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Daunorubicin-induced Smooth Muscle Contraction: Involvement of Ca^{2+} Entry Mechanism

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Abstract

The mechanisms of the smooth muscle contractile action of daunorubicin were investigated using guinea-pig aortae, and the involvement of the Ca^{2+} entry mechanism was compared among different smooth-muscle preparations.

In the aorta, daunorubicin showed a concentration-dependent contractile action at concentrations of 10–200 μM. The contractile response to daunorubicin was completely dependent on extracellular Ca^{2+}, but only slightly sensitive to verapamil or nifedipine. Trifluoperazine abolished the contraction by daunorubicin, but no significant effect was noted with amiloride, phentolamine, indomethacin or staurosporine. The order of potency (sensitivity) for daunorubicin-induced smooth muscle contraction was oesophagus = gall bladder = iliac artery > bronchus = aorta, while that of maximum reactivity was iliac artery = aorta > bronchus = oesophagus = gall bladder. In the portal vein, daunorubicin showed no contractile action. Although the smooth muscle contraction induced by daunorubicin was strongly dependent on extracellular Ca^{2+} in the aorta, iliac artery, bronchus, oesophagus and gall bladder, its sensitivity to verapamil varied among the different smooth muscle preparations, with the sensitivity being iliac artery = gall bladder > bronchus = oesophagus > aorta.

These results suggest that daunorubicin has contractile action on various kinds of smooth muscle, mainly via the transplasmalemmal Ca^{2+} entry mechanism, but the degree of involvement of the voltage-dependent Ca^{2+} channel differs among the different smooth muscle preparations.

Daunorubicin, a representative anthracycline antibiotic, is reported to show vascular action in addition to its well-known cardiotoxicity. Daunomycinone, a metabolite of daunorubicin, increases coronary perfusion pressure of isolated heart (Mhatre et al 1971), and daunorubicin itself displays vasocontractile action in-vitro (Wakabayashi et al 1989). In rat aorta, daunorubicin is reported to induce contraction via activation of the voltage-dependent Ca^{2+} channel (Wakabayashi et al 1990). In vascular smooth muscle, agonist-induced contraction is dependent on transplasmalemmal Ca^{2+} entry, although the mechanisms for the Ca^{2+} entry differ for the different kinds of smooth muscle (Lodge & van Breemen 1988). In rat aorta, the contraction by agonists (e.g. noradrenaline) is inhibited in the presence of organic Ca^{2+}-channel antagonists, suggesting partial involvement of the voltage-dependent L-type Ca^{2+} channel (Godfraind & Dieu 1981). On the other hand, the agonist-stimulated Ca^{2+}-entry pathway which is distinctly different from the voltage-dependent Ca^{2+} channel was classically proposed as a receptor-operated Ca^{2+} channel (Bolton 1979; van Breemen et al 1978). Although the receptor-operated Ca^{2+} channel is not well characterized, agonist-induced contraction in guinea-pig aorta is known to be mediated by receptor stimulation, resulting in Ca^{2+} entry through Ca^{2+} channels other than the dihydropyridine-sensitive voltage-dependent Ca^{2+} channel (van Heiningen & van Zwieten 1988; Gouw et al 1990). Moreover, a voltage-dependent Ca^{2+} channel was electrophysiologically detected in guinea-pig aorta (Caffrey et al 1986). Thus, both voltage-dependent and -independent Ca^{2+}-entry pathways are present in guinea-pig aorta. This preparation was therefore suitable for elucidating whether daunorubicin activates the Ca^{2+}-entry pathway which is independent of the voltage-dependent Ca^{2+} channel. Furthermore, the potency for contractile action of daunorubicin and the involvement of the transplasmalemmal Ca^{2+}-entry mechanism were compared among different kinds of smooth-muscle preparations from guinea-pig.

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Materials and Methods

Tissue preparation

Male Hartley guinea-pigs were anaesthetized with intraperitoneal injection of sodium pentobarbital (25 mg kg^{-1}) and killed by exsanguination. The aorta, iliac artery, portal vein, oesophagus, bronchus and gall bladder were rapidly excised and placed in Krebs–Ringer bicarbonate solution (mM): NaCl (118), KCl (4·7), KH_2PO_4 (1·2), $CaCl_2$ (2·5), $MgSO_4$ (1·2), $NaHCO_3$ (25) and glucose (10). After removal of excess fat and connective tissue, helical strips of aorta (2 × 12 mm), iliac artery (2 × 15 mm) and bronchus (3 × 15 mm), and longitudinal strips of portal vein (15 mm long), oesophagus (15 mm long) and gall bladder (4 × 7 mm) were prepared for tension studies. The gall bladder, after the bile had been aspirated out, was opened along the long axis, then three or four muscle strips were obtained from each animal by cutting parallel to the long axis. In aorta and iliac artery, the endothelium was removed by gentle abrasion of the intimal surface with filter paper, since the vascular endothelium influences vascular tone through synthesis of EDRF (endothelium-derived releasing factor) (Furchgott 1983) and daunorubicin inhibits it (Wakabayashi et al 1994). Endothelial

removal was confirmed functionally by the disappearance of the 10-μM acetylcholine-induced relaxing response of the 5-μM noradrenaline-precontracted strip.

Contraction study

The strips were mounted in 10-mL organ baths containing the above solution maintained at 37°C and gassed constantly with 5% CO_2–95% O_2 (pH 7·3–7·4). Tension was recorded isometrically by means of force transducers (Nippon Kohden Kogyo Co., Tokyo, Japan) connected to a multichannel recorder. Each strip was stretched to an initial tension of 9·8 mN and allowed to equilibrate for approximately 1 h.

First, the muscle strips were contracted with 60 mM KCl and then washed with normal Krebs-Ringer bicarbonate solution. This procedure was repeated several times before each experimental protocol until a reproducible constant contractile force was obtained. In some experiments a single concentration of daunorubicin was applied to the strip and in other experiments several concentrations of daunorubicin were cumulatively applied per strip in order to obtain a concentration-response curve for daunorubicin. The contractile force was expressed in terms of the percentage of the 60 mM KCl-induced contractile force in each strip. EC50, the concentration required to induce a half-maximal contractile response, was determined graphically after calculating the linear regression of the 20–80% region of each log concentration-response curve, and the sensitivity of contractile responses to daunorubicin and noradrenaline was evaluated using pD_2, negative log EC50 (M). E_{max} was defined as the maximum contractile force of daunorubicin-induced contraction and obtained from each concentration-response curve for daunorubicin.

Drugs

The drugs used in this study were daunorubicin hydrochloride (Meiji Seika Co. Ltd, Tokyo, Japan), noradrenaline, nifedipine, phentolamine hydrochloride, amiloride hydrochloride, trifluoperazine dihydrochloride and staurosporine (Sigma Chemical Co. Ltd, St Louis, MO), verapamil hydrochloride and acetylcholine chloride (Wako Pure Chemical Co. Ltd, Osaka, Japan). Daunorubicin was dissolved in physiological saline to make up a stock solution of 10 mM and kept at 4°C. Staurosporine, amiloride and nifedipine were dissolved in dimethylsulphoxide to make up stock solutions of 0·1 mM, 10 mM and 10 mM, respectively. Indomethacin was dissolved in 1 mM Na_2CO_3 to produce a solution of 1 mM just before each use. Trifluoperazine and verapamil were dissolved in distilled water. Experiments using verapamil and nifedipine were performed in the dark. The concentration of each drug is expressed as the final concentration in the organ chamber.

Statistical analysis

Data are expressed as means with standard error. Statistical analysis of the data was performed with Student's *t*-test. *P* values less than 0·05 were considered significant.

Results

Daunorubicin-induced contraction of guinea-pig aorta and its dependence on extracellular Ca^{2+}

Daunorubicin showed a contractile effect over a range of 10 to 200 μM in a concentration-dependent manner (Fig. 1A). The contraction induced by daunorubicin was extracellular Ca^{2+}-dependent. $CaCl_2$ at concentrations from 0·1 to 1·5 mM produced a contractile response in the presence of 140 μM daunorubicin in a concentration-dependent manner (Fig. 1B). Both contractile responses to daunorubicin and KCl were abolished in the absence of extracellular Ca^{2+} (Table 1). Daunorubicin-induced contraction was only slightly inhibited by the pretreatment of the strips with verapamil (10 μM), whereas the KCl-induced contraction was markedly inhibited by it (Table 1). Nifedipine (1 μM) pretreatment also only slightly (but significantly) decreased the daunorubicin (140 μM)-induced contraction [77·4 ± 5·0% (nifedipine-pretreated) vs 87·3 ± 4·1% (control) ($P < 0·05$, n = 5)], while it strongly inhibited the KCl (60 mM-induced contraction [13·0 ± 1·7% (nifedipine-pretreated) vs 100% (control) ($P < 0·05$, n = 9)].

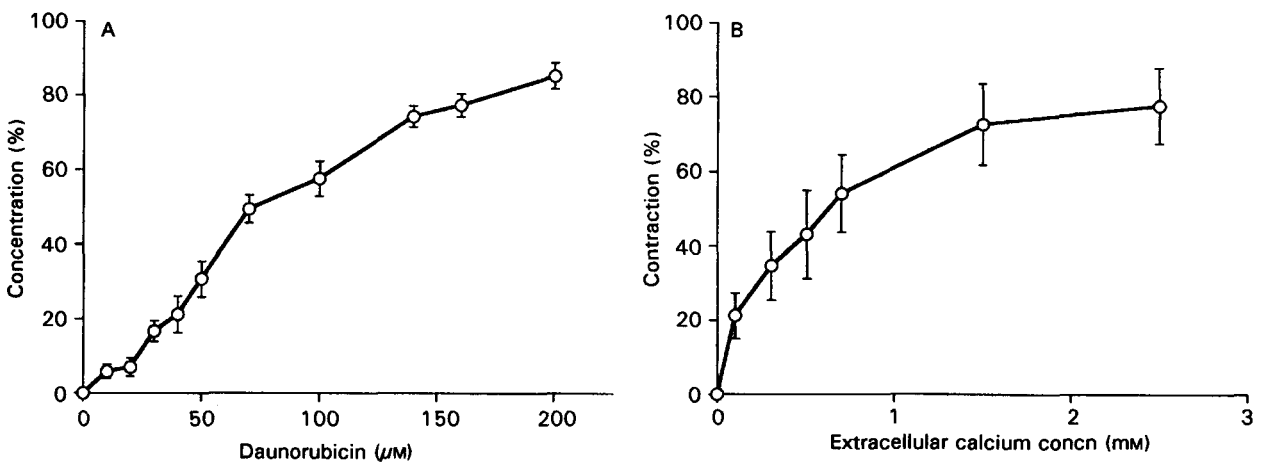

FIG. 1. Daunorubicin-induced contraction of guinea-pig aorta. A. Concentration-response relationship of daunorubicin-induced contraction. Daunorubicin was cumulatively added to the organ bath. n = 8. B. Extracellular Ca^{2+}-dependence of contractile response to daunorubicin. Aortic strips were washed three times with Ca^{2+}-free Krebs–Ringer solution containing 1 mM EGTA, and then once with Ca^{2+} and EGTA-free Krebs–Ringer solution. After stimulation with daunorubicin (140 μM), $CaCl_2$ was cumulatively added to the organ bath containing the Ca^{2+} and EGTA-free Krebs–Ringer solution. n = 7.

Table 1. Effects of extracellular Ca^{2+} removal or pretreatment with verapamil on contractile response to daunorubicin or KCl in guinea-pig aorta.

	Daunorubicin (140 μM)	KCl (60 mM)
Control	73·2 ± 2·6%	100%
Ca^{2+}-free	no response*	9·4 ± 0·7%*
Control	73·9 ± 3·0%	100%
Verapamil	63·7 ± 2·2%*	11·8 ± 2·8%

Values represent means ± s.e.m. Experiments in the absence of extracellular Ca^{2+} were performed as follows: the aortic strips were washed three times with Ca^{2+}-free Krebs–Ringer solution containing 1 mM EGTA, and then once with Ca^{2+} and EGTA-free Krebs–Ringer solution. Next, they were stimulated with daunorubicin (140 μM) or KCl (60 mM) in the Ca^{2+}- and EGTA-free Krebs–Ringer solution. In some experiments, the aortic strips were pretreated with verapamil (10 μM) or the vehicle for 60 min and then stimulated with daunorubicin (140 μM) or KCl (60 mM). *Statistically significant differences ($P < 0·05$) between the responses in the presence of verapamil or in the Ca^{2+}-free solution, and the controls (n = 5).

Verapamil pretreatment significantly inhibited the sensitivity of the contractile response to noradrenaline [pD_2, 6·63 ± 0·07 (verapamil-pretreated) vs 7·07 ± 0·03 (control) ($P < 0·05$, n = 7)], but did not significantly affect its maximum contractile response [152·0 ± 4·5% (verapamil-pretreated) vs 159·2 ± 3·6% (control)]. This suggests that the voltage-dependent Ca^{2+} channel is partially involved in the noradrenaline-induced contraction of guinea-pig aorta. After daunorubicin-induced contraction of aorta had reached a plateau level, removal of extracellular Ca^{2+} immediately relaxed the vessel to the basal tension, while an addition of verapamil (10 μM) only slightly relaxed it (Fig. 2).

Effects of various inhibitors on daunorubicin-induced contraction of guinea-pig aorta

The vasocontractile response to daunorubicin (140 μM) was completely abolished by trifluoperazine. No effect was noted with amiloride, phentolamine, indomethacin or staurosporine (Table 2).

Contractile action of daunorubicin, and its dependence on extracellular Ca^{2+} and sensitivity to verapamil in various kinds of smooth-muscle preparations from guinea-pig

Table 3 summarizes the effects of daunorubicin on the tone of several kinds of smooth muscles isolated from guinea-pig. The order of potency (sensitivity) for daunorubicin-induced contraction, evaluated by pD_2, in different tissues was oesophagus = gall bladder = iliac artery > bronchus = aorta, and that of reactivity, evaluated by maximum contractile force, was iliac artery = aorta > bronchus = oesophagus = gall bladder. With the portal vein, daunorubicin showed no contractile effects. With the aorta, iliac artery, bronchus, oesophagus and gall bladder, the contractile response to daunorubicin was abolished by removal of extracellular Ca^{2+}. However, the sensitivity to verapamil varied among the different kinds of preparations. With the iliac artery and gall bladder, verapamil markedly inhibited (> 50% of the control) the daunorubicin-induced contraction. The degree of the inhibitory effect was less (25–50%) in the bronchus and oesophagus, and much less (< 25%) in the aorta, than in the iliac artery and gall bladder.

Discussion

Our previous study showed that daunorubicin at 35·5–142·0 μM elicited a concentration-dependent contractile response in rat aorta (Wakabayashi et al 1989). The pD_2 values of daunorubicin-induced contraction were 4·18 ± 0·04 (Wakabayashi et al 1989) and 4·21 ± 0·04 (this study) in rat and guinea-pig aortic strips, respectively, suggesting that the potency of the vasocontractile action of daunorubicin was comparable in these vessels. In guinea-pig aorta, the contractile response to daunorubicin was completely dependent on extracellular Ca^{2+}, whereas in rat aorta it was only partially dependent (Wakabayashi et al 1989). Nifedipine inhibited the daunorubicin-induced contraction in rat aorta to a degree similar to that after removal of extracellular Ca^{2+}, suggesting that Ca^{2+} entry through the voltage-dependent Ca^{2+} channel is involved in the contractile response to daunorubicin. However, about 30% of the contractile response was resistant to pretreatment with nifedipine or removal of extracellular Ca^{2+} (Wakabayashi et al 1989). Thus, intracellular mechanism(s) not related to transplasmalemmal Ca^{2+} influx may also be involved in the contractile response to daunorubicin in rat aorta. One possible intracellular mechanism is Ca^{2+} release from intracellular Ca^{2+} pools, since it was reported that doxorubicin, another anthracycline antibiotic, at concentrations of 10–200 μM induced Ca^{2+} release and a concomitant contraction in skinned mesenteric artery from rabbit (Kanmura et al 1989). On the other hand, in the present study, the daunorubicin-induced contraction in guinea-pig aorta was only inhibited a little by verapamil or nifedipine despite the strong dependence on extracellular Ca^{2+}. Thus, a Ca^{2+} entry pathway besides the voltage-dependent Ca^{2+} channel may be involved in the daunorubicin-induced contraction of guinea-pig aorta. We also investigated the effects of several inhibitors related to intracellular signal transduction mechanisms. Trifluoperazine, a calmodulin inhibitor, abolished the daunorubicin-induced contraction. This was as expected, since calmodulin-dependent activation of myosin light-chain kinase after elevation of intracellular free Ca^{2+} triggers smooth muscle contraction (Adelstein et al 1980). However, no effect on the daunorubicin-induced contraction was noted with indomethacin, phentolamine, staurosporine or amiloride, suggesting that cyclooxygenase metabolites, activation of α-adrenergic receptors, protein kinase C-related pathway and Na^+–H^+ exchange system are not involved in this contraction. Therefore, daunorubicin may directly stimulate the Ca^{2+}-entry pathway besides the voltage-dependent Ca^{2+} channel, resulting in a tonic contraction which is dependent on Ca^{2+}-calmodulin.

We also investigated the effects of daunorubicin on the vascular tonus of other blood vessels in guinea-pig. In the iliac artery, daunorubicin induced contraction which was dependent on extracellular Ca^{2+} and markedly inhibited by verapamil, suggesting a mechanism involving activation of the voltage-dependent Ca^{2+} channel. On the other hand, in the portal vein, daunorubicin had no contractile action. Thus, even in the same species, the vascular effects of daunorubicin differ at different

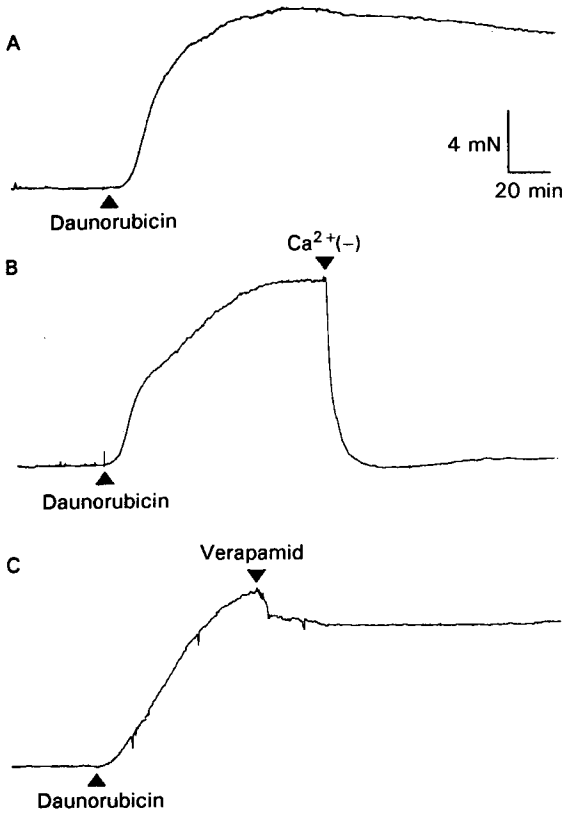

FIG. 2. Representative tension recordings of guinea-pig aortas. A. Daunorubicin induced a contractile response. B. Replacement of the normal bath solution with the Ca^{2+}-free one immediately relaxed the daunorubicin-induced contraction to the basal tension. C. Verapamil addition slightly relaxed the daunorubicin-induced contraction. In case of removal of extracellular Ca^{2+} [Ca^{2+}(−)], the bath solution was immediately changed to Ca^{2+}-free Krebs–Ringer solution containing 140 μM daunorubicin. Daunorubicin (140 μM); verapamil (10 μM).

loci of the blood vessels. We also evaluated the effects of daunorubicin on the tonus of non-vascular smooth muscle preparations. To our knowledge, there are no previous reports describing the effects of daunorubicin on the tonus of non-vascular smooth muscles. In the bronchus, oesophagus and gall bladder, daunorubicin elicited a contractile response which was strongly dependent on extracellular Ca^{2+}. However, the reactivity of daunorubicin-induced contraction, evaluated by

Table 2. Effects of several inhibitors on daunorubicin-induced contraction in guinea-pig aorta.

	Inhibitor (+)	Control
Indomethacin	79·4 ± 0·7	80·6 ± 2·0
Phentolamine	88·6 ± 3·1	91·4 ± 4·8
Amiloride	84·9 ± 4·3	90·3 ± 2·8
Trifluoperazine	0·8 ± 0·4*	87·8 ± 4·0
Staurosporine	85·9 ± 3·7	86·0 ± 5·2

Values represent means ± s.e.m. The aortic strips were pretreated with indomethacin (10 μM), phentolamine (10 μM), amiloride (1 μM), trifluoperazine (100 μM) or staurosporine (50 μM) for 60 min, and then stimulated with daunorubicin (140 μM). The controls were strips pretreated with each vehicle for 60 min before daunorubicin stimulation. *Statistically significant difference ($P < 0·05$) from the control (n = 5).

maximum contractile force, was less in non-vascular smooth muscles compared with that in the aorta and iliac artery. The sensitivity of daunorubicin-induced contraction to verapamil also differed among the different kinds of tissues, with the order being gall bladder > bronchus = oesophagus. This might be due to the differences in distribution of L-type Ca^{2+} channel in smooth muscle or that in the signal transduction mechanisms coupled to L-type Ca^{2+} channel among different kinds of smooth muscle in guinea-pig.

Anthracycline antibiotics are injected intravenously for general administration or selectively intra-arterially using angiographical techniques for local administration in malignant diseases. Thus, the effects of daunorubicin on blood vessels are clinically more important compared with those on the other smooth muscles. During chemotherapy, the concentration of daunorubicin in peripheral blood at 5 min after intravenous injection and its calculated maximum concentration in body fluid after non-excretory regular distribution were reported to be 0·5 μM and 8 μM, respectively (Burns & Dow 1980; Ogawa et al 1987). These concentrations were lower than those eliciting vasoconstriction in this study. However, local concentrations of daunorubicin can be much higher just after injection, and long exposure of the blood vessels to lower concentrations might induce some vascular response. The threshold concentration which induces acute myocardial disturbance in perfused rat hearts was reported to be 19 μM (Burns

Table 3. Daunorubicin-induced contraction and its sensitivity to removal of extracellular Ca^{2+} or treatment with verapamil in smooth-muscle preparations isolated from guinea-pig.

Preparation	Contraction pD_2	E_{max}	Sensitivity to extracellular Ca^{2+} removal	Sensitivity to verapamil treatment
Aorta	4·21 ± 0·04	85·3 ± 3·5	+ +	±
Iliac artery	4·35 ± 0·06	96·2 ± 3·6	+ +	+ +
Portal vein	no response	no response	n.d.	n.d.
Bronchus	4·23 ± 0·04	51·7 ± 1·5	+ +	+
Oesophagus	4·47 ± 0·05	45·5 ± 3·2	+ +	+
Gall bladder	4·36 ± 0·02	38·2 ± 1·8	+ +	+ +

Values represent means ± s.e.m. The sensitivity and reactivity of the contraction were evaluated by pD_2 and E_{max}, respectively (n = 5). Removal of extracellular Ca^{2+} and treatment with verapamil were carried out as explained in Table 1, and then the strips were stimulated with daunorubicin (140 μM). The sensitivity of daunorubicin-induced contraction to extracellular Ca^{2+} removal or verapamil (10 μM) was evaluated by the degree of decreased contractile force by extracellular Ca^{2+} removal or verapamil treatment, and expressed as % decrease of maximal contractile force of the control (±, < 25%; +, 25–50%; + +, > 50%). n.d., not determined.

& Dow 1980), which is similar to that eliciting vasoconstriction in this study. Further studies are necessary to elucidate whether the vasoconstrictive action of daunorubicin is involved in daunorubicin-induced side effects, including cardiotoxicity during chemotherapy.

In conclusion, daunorubicin has extracellular Ca^{2+}-dependent contractile action in various kinds of smooth muscle preparations and the degree of involvement of the voltage-dependent Ca^{2+} channel in the contraction differs with the type of tissue.

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