Contraction of the rat isolated aorta caused by *Clostridium perfringens* alpha toxin (phospholipase C): evidence for the involvement of arachidonic acid metabolism

Yoshio Fujii & ¹Jun Sakurai

Department of Microbiology, Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Yamashiro-cho, Tokushima 770, Japan

- 1 Alpha toxin produced by *Clostridium perfringens* contracted the rat isolated aorta and stimulated release of arachidonic acid in the tissue.
- 2 Quinacrine did not inhibit contraction caused by the toxin.
- 3 Indomethacin blocked contraction caused by the toxin in a dose-dependent manner and markedly increased levels of arachidonic acid released by the toxin.
- 4 The toxin-induced contraction was blocked by the thromboxane synthetase inhibitor OKY-046 and the thromboxane A₂ (TXA₂) antagonist ONO-3708.
- 5 The toxin stimulated production of TXB₂ and this was blocked by pretreatment with either indomethacin or OKY-046.
- 6 Toxin-induced contraction was diminished by pretreating aorta with collagenase or by rubbing the intimal surface to remove the endothelium.
- 7 These data suggest that the contractile response to the toxin is associated with stimulation of TXA₂ production from arachidonic acid released by the toxin in the endothelial cells of the aorta.

Introduction

Clostridium perfringens alpha toxin, which is lethal, dermonecrotic and haemolytic, has been thought to be an important toxin in gas gangrene caused by the organism (Aikat & Dible, 1957; MacLennan, 1962). The toxin is known to possess phospholipase C activity (MacFarlane & Knight, 1941) and has been shown to hydrolyze various phospholipids such as phosphatidylcholine and sphingomyelin in biological membranes (Tobias *et al.*, 1962; Lenard & Singer, 1968; McIlwain & Rapport, 1971; Knickelbein & Rosenberg, 1980). Various effects have been observed with crude preparations of alpha toxin, including increases in vascular permeability in guinea-pig skin (Elder & Miles, 1957; Stark & Duncan, 1972), increases in the ionic permeability of skeletal muscle membranes (Albuquerque & Thesleff, 1967) and a significant reduction in the acetylcholine response of smooth muscle that was partially restored by the addition of Ca²⁺ (Lodge & Leach, 1973). However, *C. perfringens* produces toxins (enterotoxin, haemolysin) and enzymes (collagenase, hyaluronidase)

which may be responsible for some of these effects (Willis, 1969) and interfere with characterization of the specific biological activities of alpha toxin.

We have recently investigated the effects of purified alpha toxin on tissues of rat and found that it potentiates the contraction of the rat isolated vas deferens elicited by noradrenaline (NA) (Sakurai *et al.*, 1985a), and causes a rise in blood pressure with a decrease in blood flow (Sakurai *et al.*, 1985b). Using alpha toxin purified on a large scale from culture supernatant fluid of *C. perfringens* type A (which produces alpha toxin, but not beta and epsilon toxins), we found that purified alpha toxin caused contraction of the rat isolated aorta (Fujii *et al.*, 1986). The present study was undertaken to define the mode of action of this contractile effect.

Methods

Rat isolated aorta

Male rats, weighing 280 to 300 g, were killed by cervical dislocation and exsanguination. The isolated

¹ Author for correspondence.

aorta was cut helically into strips (20 mm long and 2 mm wide) that were suspended in organ baths (10 ml) containing Tyrode solution of the following composition (mM): NaCl 137, CaCl₂ 1.8, KCl 2.7, MgCl₂ 1.8, NaH₂PO₄ 0.42, NaHCO₃ 11.9 and glucose 5.6. The tissue bath solution was maintained at 37°C and bubbled with a mixture of 95% O₂ and 5% CO₂. Contractions were determined isotonicity as described previously (Fujii *et al.*, 1986).

Procedures for removing endothelial cells

Two separate procedures described by Furchgott & Zawadzki (1980) were used: (a) intact aorta were cannulated and the intima exposed to 0.2% collagenase (CLS-type 1A, Worthington) for 15 min at 37°C; (b) the intimal surface was gently abraded by rubbing.

Purification of C. perfringens alpha toxin

Procedures for culture of *Clostridium perfringens* type A (strain NCTC 8237) and purification of alpha toxin have been described previously (Sakurai *et al.*, 1985a; Fujii *et al.*, 1986). This toxin preparation was free from detectable collagenase, hyaluronidase, neuraminidase and theta toxin activities.

Thromboxane B₂ assay

Rings of aorta (2 to 3 mm, weighing 20–30 mg) were equilibrated in 5 ml of Tyrode solution for 1 h at 37°C. During the equilibration period, the solutions were replaced every 15 min with fresh Tyrode solution. They were then incubated in 1 ml of Tyrode solution containing the toxin for 20 min at 37°C and thromboxane B₂ (TXB₂) in the medium was measured by radioimmunoassay (Amersham, U.K.).

Arachidonic acid assay

Rings of aorta (2 to 3 mm, weighing 15–20 mg) were equilibrated in 5 ml of Tyrode solution for 1 h at 37°C. During the equilibration period, the solutions were replaced every 15 min with fresh Tyrode solution. Next, the tissues were incubated in 2 ml of Tyrode solution containing the toxin for 20 min at 37°C, and homogenized in a fluid volume of 4 ml of Tyrode solution. The homogenates (2 ml) were vigorously mixed with a mixture containing 4 ml of isopropyl alcohol-hexane (7:3), 1 ml of 2N H₂SO₄, and nonadecanoic acid (C_{19:0}) as the internal standard for 30 s and allowed to separate on standing. The hexane phase was pipetted into a vial containing 60 mg of silicic acid. The vial was rotated, allowed to stand for 10 min at room temperature and dried in a

rotary evaporator. The residue was esterified with excess ethereal diazomethane, and excess reagent was evaporated under a nitrogen stream. Esterified fatty acids were analysed by gas chromatography using a flame ionization detector (Shimadzu GC-7AG, Kyoto, Japan). The silanized, coiled glass column (2.1 m × 3 mm i.d.) used was packed with either 2% (w/w) OV-17 on Chromosorb W (AW-DMCS), 80–100 mesh (Shimadzu) or 3% (w/w) FFAP on Chromosorb W (AW-DMCS), 80–100 mesh (Shimadzu).

Materials

The following drugs were used: indomethacin (Sigma Chemical Company, St. Louis, MO, U.S.A.), aspirin (Sigma Chemical Company), arachidonic acid (Sigma Chemical Company), quinacrine (Sigma Chemical Company), noradrenaline (NA) (Sankyo Company, Ltd., Tokyo, Japan), nonadecanoic acid (Serdary Research Lab., London, Ontario, Canada), collagenase type 1A (Sigma Chemical Company). OKY-46 ((E)-3-[4-(imidazolylmethyl)phenyl]-2-propenoic-acid hydrochloride-monohydrate) and ONO-3708 ((9,11)(11,12)-dideoxa-9 α ,11 α -dimethylmethano-11,12-methano-13,14-dihydro-13-aza-14-oxo-15-cyclopentyl-16,17,18,19,20-pentanor-15-epi-thromboxane-A₂) were kindly provided by Ono Pharmaceutical Company (Osaka, Japan).

Results

Effect of alpha toxin on arachidonic acid release in the rat isolated aorta

The relationships between doses of the toxin and release of arachidonic acid were investigated (Figure 1). After treatment with 0.54 $\mu\text{g ml}^{-1}$ of the toxin, release of arachidonic acid was increased from 22.0 ± 3.8 to $42.1 \pm 5.6 \text{ ng mg}^{-1}$ wet weight, suggesting that the toxin stimulates release of arachidonic acid. On the other hand, when the tissue was treated with the toxin in the presence of the cyclo-oxygenase inhibitor indomethacin, arachidonic acid release was $61.0 \pm 3.0 \text{ ng mg}^{-1}$ wet weight (Figure 1). The data shows that in the presence of indomethacin arachidonic acid released by the toxin accumulates at a significantly higher level.

To test if the action of the toxin was dependent on phospholipase A₂, the effect of the phospholipase A₂ inhibitor quinacrine (Frye & Holz, 1983) was evaluated. Treatment with quinacrine ($5 \times 10^{-5} \text{ M}$) for 15 min, which was sufficient to prevent a submaximal response to NA ($1 \times 10^{-7} \text{ M}$), did not affect either

Figure 1 Effect of indomethacin on arachidonic acid release induced by alpha toxin in the rat isolated aorta. Tissues were incubated for 15 min in the absence (A) or the presence (B) of indomethacin (1×10^{-5} M). The toxin ($0.54 \mu\text{g ml}^{-1}$) was added after 15 min of incubation in the absence (C) or the presence (D) of indomethacin (1×10^{-5} M). These treated tissues were incubated at 37°C for 1 h in Tyrode solution, and the arachidonic acid assay was carried out as described in the text. *Significantly different from (A) or (B) ($P < 0.01$). **Significantly different from (C) ($P < 0.02$). Vertical lines indicate s.e.mean ($n = 4$).

the contraction or the release of arachidonic acid caused by the toxin (data not shown).

The relationship between arachidonic acid metabolism and contraction induced by the toxin

Figure 2 shows that the cyclo-oxygenase inhibitors indomethacin and aspirin both decreased contraction caused by the toxin.

In order to investigate further the role of cyclo-oxygenase products in toxin-evoked contractions, the effects of the TXA_2 synthetase inhibitor OKY-46 (Naito *et al.*, 1983; Cerletti *et al.*, 1986) and TXA_2 antagonist ONO-3708 (Fujioka *et al.*, 1986; Toda *et al.*, 1986) were investigated and compared with their actions on NA-induced contractions (Table 1). When the tissue pretreated with 10^{-5} M and 10^{-4} M OKY-046, the toxin-induced contraction was decreased to approximately 36% and 14% of control, respectively. ONO-3708 also inhibited the toxin-induced contraction in a dose-dependent manner. The NA-induced contraction was not inhibited by either OKY-046 or ONO-3708 at 10^{-4} M. These data suggest that TXA_2 is an important factor in the contraction induced by the toxin, but not by NA. Consistent with this, the stable metabolite of TXA_2 , TXB_2 , was significantly increased from 18.0 (basal release) to 40.2 pg mg^{-1} wet weight by the treatment with the toxin (Table 2). This toxin-induced increase in TXB_2 production was blocked by indomethacin or OKY-046.

Figure 2 Effect of indomethacin or aspirin on alpha toxin-induced contraction. The tension (%) was expressed as a percentage of control contractions induced by alpha toxin alone (A). The toxin ($0.54 \mu\text{g ml}^{-1}$) was applied 15 min after administration of indomethacin 1×10^{-6} M (B) or 3×10^{-6} M (C) or aspirin 5×10^{-5} M (D). * Significantly different from (A) ($P < 0.002$). ** Significantly different from (A) ($P < 0.001$) and (B) ($P < 0.01$). *** Significantly different from (A) ($P < 0.001$). Vertical lines indicate s.e.mean ($n = 5$).

Effect of the toxin on the isolated aorta without endothelium

The effect of the toxin or NA on the untreated and the collagenase-treated tissue was investigated. Collagenase treatment resulted in no loss of the contractile response to NA (data not shown), as observed by

Table 1 Effect of OKY-046 and ONO-3708 on alpha toxin- and noradrenaline (NA)-induced contractions

Inducer	Agent	Tension (%)
Toxin	None	100
Toxin	OKY-046, 1×10^{-5} M	$36.0 \pm 4.6^*$
Toxin	OKY-046, 1×10^{-4} M	$14.5 \pm 4.8^{***}$
Toxin	ONO-3708, 1×10^{-5} M	$18.0 \pm 10.3^{***}$
Toxin	ONO-3708, 1×10^{-4} M	$11.3 \pm 5.6^{***}$
NA	None	100
NA	OKY-046, 1×10^{-4} M	98.2 ± 10.3
NA	ONO-3708, 1×10^{-4} M	94.0 ± 8.0

The toxin ($0.54 \mu\text{g ml}^{-1}$) or NA (1×10^{-5} M) was applied 15 min after administration of OKY-046 or ONO-3708. The tensions (%) were expressed as a percentage of contraction induced by alpha toxin ($0.54 \mu\text{g ml}^{-1}$) or NA (1×10^{-5} M). The results are the mean $\pm$ s.e.mean of 4 experiments.

* Significantly different from control ($P < 0.001$).

** Significantly different from treatment with 1×10^{-5} M OKY-046 ($P < 0.05$).

*** Significantly different from control ($P < 0.005$).

Table 2 Effect of indomethacin and OKY-046 on thromboxane B₂ generated by alpha toxin in rings of rat isolated aorta

Toxin	Agent	Thromboxane B ₂ (pg mg ⁻¹ wet wt.)
-	None	18.0 ± 2.3
+	None	40.2 ± 3.9*
-	Indomethacin	20.0 ± 2.0
+	Indomethacin	21.0 ± 1.4**
+	OKY-046	24.0 ± 3.4***
-	OKY-046	25.5 ± 2.1

Rings of isolated aorta were incubated for 15 min in the absence or presence of indomethacin (1×10^{-6} M) or OKY-046 (1×10^{-5} M), where indicated. (+) Toxin ($0.54 \mu\text{g ml}^{-1}$) was added after this incubation. The tissues were incubated in Tyrode solution at 37°C for 1 h, and the thromboxane B₂ assay was carried out as described in the text. The results are the mean ± s.e.mean of 6 experiments.

* Significantly different from the untreated tissues ($P < 0.005$).

** Significantly different from the toxin-treated tissues ($P < 0.01$).

*** Significantly different from the toxin-treated tissues ($P < 0.05$).

Furchgott & Zawadzki (1980), suggesting that aortic smooth muscle was not damaged by collagenase. However, when endothelial cells had been removed by preincubation with collagenase (Furchgott & Zawadzki, 1980), the toxin-induced contraction was reduced by about 75%, compared to the toxin-induced contraction of the untreated tissue (Figure 3a). To obtain further evidence for the relationship between the presence of endothelium and contraction induced by the toxin, endothelial cells were removed by rubbing the intimal surface (Furchgott & Zawadzki, 1980). Contractions evoked by the toxin were decreased to approximately 35% of control (Figure 3b).

Discussion

Alpha toxin purified from culture supernatant fluid of *Clostridium perfringens* alpha toxin has been shown to possess phospholipase C activity (Krug & Kent, 1984), and we have recently demonstrated that toxin-induced contraction of rat isolated aorta was associated with *de novo* production of phosphatidylinositol and phosphatidic acid (Fujii *et al.*, 1986). In the present study, we found that the toxin-induced contraction was significantly inhibited by cyclo-oxygenase inhibitors such as indomethacin and

Figure 3 Effect of alpha toxin on the isolated aorta without endothelium. (a) Treatment of the tissue with collagenase. (A) The untreated tissue (control), (B) the treated tissue. (b) Rubbing of the intimal surface of the tissue. (C) The untreated tissue (control), (D) the treated tissue. The toxin ($0.54 \mu\text{g ml}^{-1}$) was added 15 min after incubation of the treated tissue in Tyrode solution. The tension (%) was expressed as a percentage of the reference contraction induced by noradrenaline (1×10^{-5} M). * Significantly different from control (A) ($P < 0.01$). ** Significantly different from control (C) ($P < 0.01$). Vertical lines indicate s.e.mean ($n = 5$).

aspirin. Furthermore, the toxin was found to stimulate release of arachidonic acid in the tissue. Therefore, stimulation of arachidonic acid release may be related to activation of phospholipid metabolism by the toxin. Arachidonic acid has generally been thought to be metabolized to cyclo-oxygenase products (Hamberg & Samuelsson, 1973) and lipoxygenase products (Murphy *et al.*, 1979). The release of arachidonic acid caused by the toxin in the presence of the cyclo-oxygenase inhibitor indomethacin was approximately 1.5 times higher than that in the absence of the inhibitor, suggesting that arachidonic acid is mainly metabolized to cyclo-oxygenase products under our experimental conditions. These data support the view that toxin-induced contractions are associated with cyclo-oxygenase product(s) metabolized from arachidonic acid.

The toxin-evoked contractions were unaffected by quinacrine at concentrations which completely inhibited NA-induced contractions. Furthermore, the release of arachidonic acid caused by the toxin was not inhibited by quinacrine. In these experiments, quinacrine was used to inhibit phospholipase A₂ (Lapetina, 1982; Frye & Holz, 1983). However, arachidonic acid can be released not only from phosphatidic acid by phospholipase A₂ (Lapetina, 1982) but also from monoacylglycerol by monoacylglyceride lipase (Chau & Tai, 1981). Therefore, since quinacrine was inactive, the release of arachidonic acid seems to be due to hydrolysis of monoacylglycerol or diacylglycerol by monoacylglyceride or diacylglyceride lipase. This is consistent with the

toxin causing hydrolysis of membrane phospholipids resulting in a rise in the level of diacylglycerol.

To investigate further the role of cyclo-oxygenase products, OKY-046 was used as an inhibitor of TXA₂ synthetase (Naito *et al.*, 1983; Cerletti *et al.*, 1986), and ONO-3708 as an antagonist of TXA₂ (Fujioka *et al.*, 1986; Toda *et al.*, 1986). OKY-046 and ONO-3708 were found to inhibit contractions caused by the toxin in a dose-dependent manner. Furthermore, the production of TXB₂, the stable metabolite of TXA₂, was stimulated by the toxin and this effect was also blocked by indomethacin and OKY-046. Although these agents completely inhibited the production of TXA₂ by the toxin, they did not completely inhibit toxin-induced contractions. In contrast, indomethacin 3×10^{-6} M almost abolished toxin-induced contractions. Thus, it is possible that other cyclo-oxygenase products apart from TXA₂ contribute to the contraction.

Several workers have shown that TXA₂ is produced enzymatically by endothelial cells of blood vessels from rabbit (Ingberman-Wojenski *et al.*, 1981b),

bovine (Ingberman-Wojenski *et al.*, 1981a), and human umbilical blood vessels (Tuvemo *et al.*, 1976). Thus, it was possible that toxin-induced contractions of rat isolated aorta associated with the production of TXA₂ required intact endothelium. Consistent with this, the toxin did not induce contraction after treatment of the isolated aorta with collagenase or rubbing of the tissue to remove endothelial cells from the intimal surface of the tissue (Furchgott & Zawadzki, 1980). Since collagenase treatment and rubbing resulted in no effect on the sensitivity of the tissues to NA, we conclude that aortic smooth muscle was not damaged by these treatments.

From these observations it appears that in endothelial cells of rat aorta, alpha toxin stimulates arachidonic acid release, leading to the production of TXA₂ which then elicits contraction of adjacent aortic smooth muscle.

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