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# Excitatory effect of *Clostridium perfringens* alpha toxin on the rat isolated aorta

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**1** *Clostridium perfringens* alpha toxin caused contraction of the isolated aorta of the rat in a dose-dependent manner.

**2** The contractile action caused by the toxin was inhibited or abolished by calcium antagonists such as nifedipine, verapamil and cinnarizine, or a Ca-free medium, but was not affected by phentolamine, chlorpheniramine, atropine, tetrodotoxin or a low Na medium.

**3** The toxin stimulated Ca uptake into the aorta in a dose-dependent manner.

**4** 8-N,N'-diethylaminooctyl-3,4,5-trimethoxybenzoate (TMB-8) blocked significantly both the toxin- and noradrenaline (NA)-induced contractions.

**5** Trifluoperazine (TFP) and N-(6-aminohexyl)-5-chloro-1-naphtharene sulphonamide (W-7) did not affect the contractile activity of the toxin but blocked the NA-induced contraction.

**6** The toxin also stimulated the $^{32}P$ phosphate labelling of phosphatidylinositol (PI) and phosphatidic acid (PA) in the preparation.

**7** These results indicate that the toxin-induced contraction, which is different from that induced by NA, is the result of a direct action of the toxin on the aorta and is due to an increased $Ca^{2+}$ permeability across the smooth muscle membrane. It is suggested that the contractile response to the toxin is associated with activation of phospholipid metabolism and enhanced entry of Ca into the aorta.

## Introduction

*Clostridium perfringens* alpha toxin is thought to be an important agent in gas gangrene caused by the organism (Aikat & Dible, 1957; MacLennan, 1962). The toxin is known to be lethal, dermonecrotic and haemolytic. In addition, MacFarlane & Knight (1941) reported that it possesses phospholipase C activity. The toxin can hydrolyze various phospholipids such as phosphatidylcholine in biological membranes (Tobias *et al.*, 1962; Lenard & Singer, 1968; McILwain & Rapport, 1971; Knickelbein & Rosenberg, 1980). Several workers have reported various biological properties of the crude or the partially purified toxin, especially the experimental production of lesions by administration to various tissues of the crude or partially purified alpha toxin preparation (Strunk *et al.*, 1967; Albuquerque & Thesleff, 1967; 1968; Lodge & Leach, 1973; Knickelbein & Rosenberg, 1980). On the other hand, *C. perfringens* produces other cytolytic toxins such as theta toxin, and enzymes such as collagenase and hyaluronidase, some of which are able to affect various tissues (Willis, 1969). It thus appears that contaminating proteins with tissue damaging activity interfere with the characterization of the specific biological activities of the toxin. The purification of alpha toxin produced by *C. perfringens* type A has been reported (Diner, 1970; Möllby & Wadström, 1973; Smyth & Arbuthnott, 1974). However, most of these procedures are deemed unsatisfactory. On the other hand, highly purified alpha toxin can be isolated by the procedure of Takahashi *et al.* (1974). Recently we reported that the highly purified alpha toxin possesses pressor activity as well as phospholipase C activity, and that the pressor activity is caused by increase in resistance to blood flow (Sakurai *et al.*, 1985b). It is difficult to purify large amounts of the active toxin from culture supernatant fluid of *C. perfringens* by the method of Takahashi *et al.* (1974), possibly due to instability of the toxin or differences in the strain used. In the present paper, we have investigated the effects of alpha toxin on the isolated aorta of the rat, after establishing a new purification procedure for isolating alpha toxin.

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## Methods

### *Rat isolated aorta*

Male rats, weighing 280 to 300 g, were killed by a blow on the head and exsanguination. The isolated descending aorta was cut helically at an angle of approximately 45° to the longitudinal axis into strips according to the method of Godfraind *et al.* (1982). The preparations were suspended in a 10 ml organ bath containing Tyrode solution of the following composition (mM): NaCl 137, $CaCl_2$ 1.8, KCl 2.7, $MgCl_2$ 1.8, $NaH_2PO_4$ 0.42, $NaHCO_3$ 11.9 and glucose 5.6. A low Na or Ca-free medium was prepared by replacing 68 mM NaCl with 146 mM sucrose and by omitting $CaCl_2$ in the normal Tyrode solution respectively. The tissue bath solution was maintained at 37°C and bubbled with a mixture of 95% $O_2$ and 5% $CO_2$. The upper end of the strip was connected to the lever of an isotonic transducer (Nippon Kohden Co., Tokyo, Japan). An initial resting tension of 1 g was applied to the preparation. Before the experiments started, the preparations were allowed to equilibrate for 1 h in the bath solution. During the equilibration period, the solutions were replaced every 15 min with fresh Tyrode solution.

### *Purification of alpha toxin of* C. perfringens *type A*

Crude alpha toxin fraction (ammonium sulphate fraction) was prepared from the culture supernatant fluid of *C. perfringens* type A (NCTC 8237) as described previously (Sakurai *et al.*, 1985a). Purification of alpha toxin was carried out as follows. The ammonium sulphate fraction (1.6 g of protein) was applied to a Cu-chelate affinity column (1.6 × 7 cm), which was prepared by the method of Porath *et al.* (1975); the fraction had been equilibrated with 0.02 M Tris-HCl buffer (pH 7.5) containing 0.5 M NaCl. The column was first washed with 300 ml of the same buffer, and then with 500 ml of 0.02 M acetate buffer (pH 4.5) containing 0.5 M NaCl. The bulk of phospholipase C activity was eluted with 0.1 M phosphate buffer (pH 6.5) containing 0.5 M NaCl and 15 mM L-histidine. Active fractions were pooled, concentrated with PM 10 membrane (Amicon Corp., Lexington, MA), and dialyzed in 0.05 M acetate buffer (pH 5.0) containing 0.1 M boric acid (Cu-chelate fraction). The Cu-chelate fraction (86 mg) was chromatographed on DEAE-Sepharose (1.6 × 4 cm) previously equilibrated with 0.05 M acetate buffer (pH 5.0) containing 0.1 M boric acid. Phospholipase C activity was eluted with the void volume. Active fractions were pooled, concentrated with PM 10 membrane, and dialyzed in 0.02 M Tris-HCl buffer (pH 7.5) (DEAE-Sepharose fraction). The DEAE-Sepharose fraction (18 mg) was next chromatographed on Sephadex G-100 (2.2 × 50 cm) equilibrated in 0.02 M Tris-HCl buffer (pH 7.5). The fraction was separated into two peaks and the enzymatic activity was recovered with the second peak (Sephadex fraction; the purified alpha toxin). Typical data of the purification steps are summarized in Table 1. The purified preparation (50 μg) gave only one band on polyacrylamide gel electrophoresis (PAGE) or sodium dodecyl sulphate (SDS)-PAGE (Figure 1). The molecular weight of the toxin, determined by SDS-PAGE, was approximately 43,000, as described by Krug & Kent (1984). As shown in Table 2, the amino acid composition of the purified preparation (except proline and lysine) coincided with the composition of the preparation isolated by Krug & Kent (1984), which seems to be pure by several criteria. For mice injected intravenously, the specific activity of our purified toxin preparation was $1.7 \times 10^4$ $LD_{50}$ per mg protein. On the other hand, 50 μg of the purified preparation showed no activity of theta toxin, collagenase and hyaluronidase.

### *Measurement of phospholipase C activity*

Determination of phospholipase C activity was carried out by the method of Kurioka & Matsuda (1976). One unit is the amount of the purified toxin (1.8 μg) required to hydrolyze 1 nmol of *p*-nitrophenylphosphorylcholine (Sigma Chemical Company, St. Louis, MO) per min.

### *Measurement of other toxins or enzymes*

Haemolytic activity of theta toxin was determined in the presence of a reducing agent according to Roth & Pillemer (1955). Collagenase and hyaluronidase were assayed by the method of Smyth & Arbuthnott (1974). Protein was estimated by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

### *Measurement of $^{45}Ca$ uptake*

Fragments (6 mg) of rat isolated aorta were incubated in 5 ml of Tyrode solution for 1 h at 37°C. During equilibration, the solutions were replaced every 15 min with fresh media. The fragments were incubated in 2 ml of Tyrode solution containing 1 μCi $^{45}Ca$ and various concentrations of alpha toxin for 20 min at 37°C. $^{45}Ca$ uptake was measured by the method of Karaki *et al.* (1980).

### *Incorporation of $^{32}P$ into phospholipids*

Fragments (6 mg) of rat isolated aorta were incubated in 4 ml of Tyrode solution for 1 h at 37°C. During the equilibration, the solutions were replaced every 15 min with fresh media. The fragments were incubated in 4 ml of Tyrode solution containing 10 μCi $^{32}P$ for 30 min at 37°C. The toxin was added to the solution

**Table 1** Summary of purification of alpha toxin of *C. perfringens*

| *Fraction* | *Total protein* (mg) | *Total activity* ($LD_{50}$) | *Specific activity* ($LD_{50}\,mg^{-1}$) | *Recovery* (%) |
|---|---|---|---|---|
| Ammonium sulphate fraction | 1,666 | $8.3 \times 10^5$ | 500 | 100 |
| Cu-chelate fraction | 264 | $5.3 \times 10^5$ | 2,020 | 64 |
| DEAE-Sepharose fraction | 18 | $1.8 \times 10^5$ | 10,000 | 22 |
| Sephadex fraction | 9 | $1.4 \times 10^5$ | 16,667 | 17 |

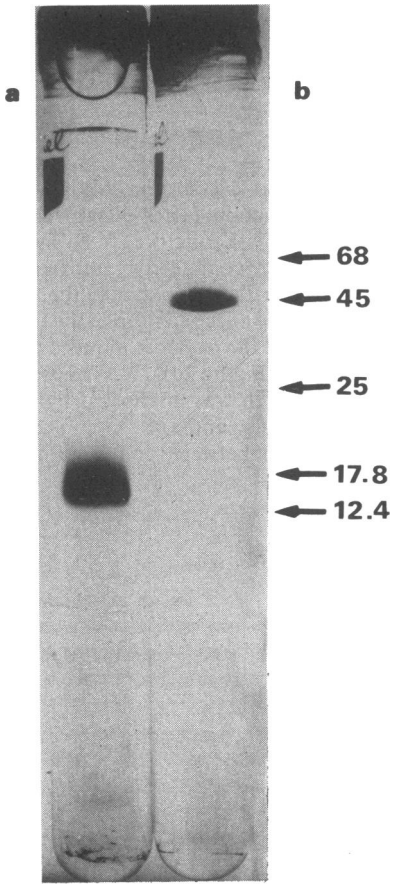


**Figure 1** Polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulphate (SDS)-PAGE of the purified toxin. (a) PAGE, (b) SDS-PAGE. PAGE was carried out according to the method of Davis (1964) and SDS-PAGE according to the method of Weber & Osborn (1969). Molecular weight standards are in thousands (arrows at the right margin).

and the incubation continued for an additional 20 min. Phospholipids in the tissues were prepared by the method of Michell (1975). Radioactive phospholipids were separated by thin-layer chromatography with chloroform-methanol-4N $NH_4OH$ (45:35:10, v/v) and identified by autoradiography, as described by Hirayama & Kato (1984).

*Statistical analysis of the data*

All mean values are shown with their calculated standard errors. Statistical analysis were performed by Student's *t* test. A *P* value of 0.05 or less was considered statistically significant.

**Table 2** Amino acid composition of alpha toxin

| *Amino acid* | *Value obtained by hydrolysis* (μmol) | *Number of residues to nearest whole number* |
|---|---|---|
| Aspartic acid | 1.50 | 72 |
| Threonine | 0.55 | 26 |
| Serine | 0.55 | 26 |
| Glutamic acid | 0.76 | 36 |
| Proline | 0.06 | 3 |
| Glycine | 0.69 | 33 |
| Alanine | 0.76 | 36 |
| Valine | 0.30 | 14 |
| Methionine | 0.17 | 8 |
| Isoleucine | 0.40 | 19 |
| Leucine | 0.40 | 19 |
| Tyrosine | 0.59 | 28 |
| Phenylalanine | 0.36 | 17 |
| Lysine | 0.59 | 28 |
| Histidine | 0.19 | 9 |
| Arginine | 0.19 | 9 |
| Cysteine | 0.04 | 2 |

The purified alpha toxin (1 mg) was hydrolyzed by the method of Simpson *et al.* (1976). Amino acid composition was determined by use of high performance liquid chromatography (LC-4A, Shimazu, Japan). Data are expressed as the number of residues per molecule of the toxin (mol. wt. 43,000).

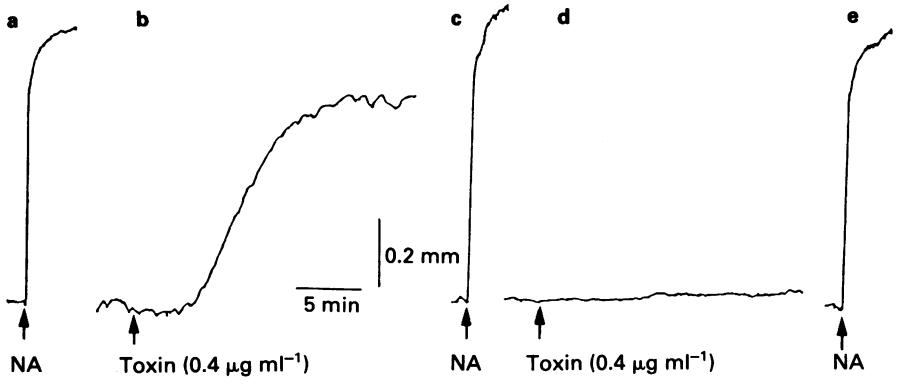


**Figure 2** Action of alpha toxin on basal and noradrenaline (NA)-induced contractility of rat aorta. A wash of 5 min followed the addition of NA ($2 \times 10^{-8}$ M), and there was a 15 min wash after the addition of alpha toxin.

*Materials*

The following drugs were used: noradrenaline (NA, Sankyo Company, Ltd., Tokyo, Japan), phentolamine methansulphonate (Regitine; Ciba-Geigy Corp., Summit, NJ), chlorpheniramine maleate (Sankyo Company), tetrodotoxin (TTX, Sankyo Company), atropine sulphate (Tokyo Kasei Company, Ltd., Tokyo, Japan), acetylcholine chloride (Daiichi Seiyaku Company, Ltd., Tokyo, Japan), histamine dihydrochloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan), nifedipine (Sigma Chemical Company), verapamil hydrochloride (Eisai Company, Ltd., Tokyo, Japan), cinnarizine (Sigma Chemical Company), 8-N,N'-diethylaminooctyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8, Sigma Chemical Company), trifluoperazine dihydrochloride (TFP, Sigma Chemical Company), N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide hydrochloride (W-7, Sigma Chemical Company). Nifedipine and cinnarizine were dissolved in acetone. Other drugs were freshly dissolved in distilled water before the experiment.

## Results

*Effect of alpha toxin on the rat isolated aorta*

A representative (8 experiments) effect of *Clostridium perfringens* alpha toxin on the aorta is shown in Figure 2. The preparation was incubated with 0.4 μg of the toxin per ml of Tyrode solution at 37°C, a contraction of the aorta began about 4 min after the application of the toxin and reached a maximum after 20 min (Figure 2b). The maximal contraction was maintained for at least 60 min. About 10 min after the maximal contraction (after 30 min exposure to the toxin), washing with toxin-free fresh Tyrode solution 5 times for 15 min completely removed the sustained contraction. On the other hand, the contractile-response induced by a second dose of the toxin reduced to less than 10% of the response induced by the first dose of the toxin (Figure 2d). NA caused a transient contraction which was unaffected by treatment with toxin (Figure 2a, c and e).

A correlation between contractions of the aorta

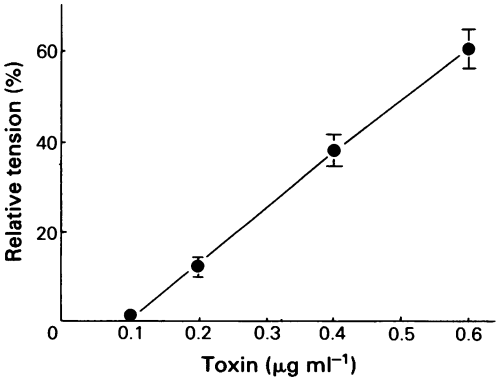


**Figure 3** Concentration-dependence of the contractile response of the isolated aorta of rat to alpha toxin. Relative tension was expressed as a percentage of the reference contraction induced by noradrenaline ($1 \times 10^{-7}$ M). Each point is the mean value of nine or ten determinations with s.e. shown by vertical lines.

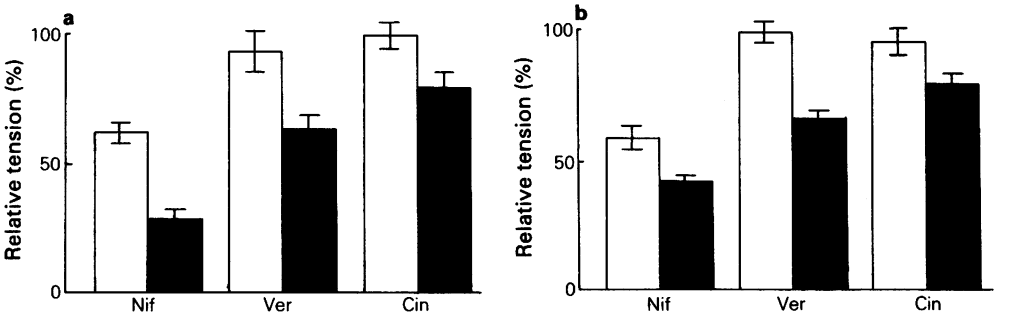


**Figure 4** Effect of nifedipine, verapamil and cinnarizine on contractions of rat aorta induced by alpha toxin and noradrenaline (NA). Alpha toxin (a) or NA (b) was applied 15 min after administration of nifedipine (Nif), or verapamil (Ver), and 30 min after administration of cinnarizine (Cin). Open column $1 \times 10^{-7}$ M; closed column $3.5 \times 10^{-6}$ M. Vertical lines indicate s.e.mean ($n = 6$). Differences between contractions of untreated aorta and nifedipine, verpamil and cinnarizine-treated aorta induced by the toxin or NA were significant.

caused by the toxin and doses of the toxin was investigated. The contraction caused by the toxin increased with increasing toxin concentrations in the range of 0.2 μg ml$^{-1}$ to 0.6 μg ml$^{-1}$ in a dose-dependent manner (Figure 3). Concentrations of less than 0.1 μg ml$^{-1}$ of the toxin caused no contraction of the aorta.

We next determined interactions between the toxin and chemical mediators that can induce a contraction in blood vessels. The toxin-evoked contraction was unaffected by a concentration of phentolamine ($2 \times 10^{-6}$ M) sufficient to prevent a submaximal response to NA ($1 \times 10^{-7}$ M). Likewise, the toxin-induced contraction was not inhibited by pretreatment with atropine ($5 \times 10^{-4}$ M) or chlorpheniramine ($1 \times 10^{-6}$ M).

*Effect of Ca on the toxin-evoked contraction*

The relationships between the toxin-evoked contraction and Ca were investigated. Figure 4 shows the effects of nifedipine, verapamil and cinnarizine on the contraction caused by the toxin (0.5 μg ml$^{-1}$) or NA ($1 \times 10^{-7}$ M). These agents inhibited the toxin-induced contraction. However, there was a wide variation among the inhibitory effects of the agents. The effects were in the following order; nifedipine > verapamil > cinnarizine (Figure 4). This order tended to be consistent with that for NA-induced contraction. In addition, the contraction induced by the toxin was completely abolished in Ca-free medium, and completely restored by the addition of Ca (Figure 5).

When the aorta was pretreated with $1 \times 10^{-5}$ M

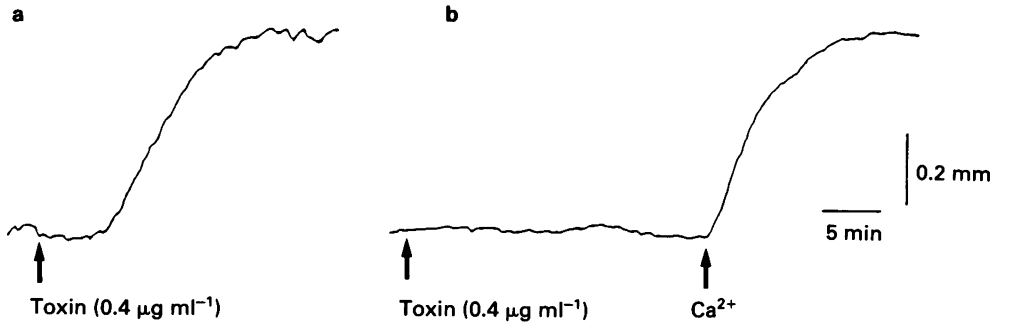


**Figure 5** Action of alpha toxin on the rat aorta in Ca-free medium. $CaCl_2$ solution (1.8 mM) was added 25 min after the application of the toxin (0.4 μg ml$^{-1}$). (a) Control; (b) Ca-free medium. This addition of calcium did not cause vasoconstriction in the absence of alpha toxin.

TMB-8 (the putative intracellular Ca antagonist) for 15 min at 37°C, the contractions induced by the toxin and NA were reduced by 60 and 40%, respectively (data not shown). Treatment with TFP ($1 \times 10^{-5}$ M) for 15 min completely inhibited the contractile activity of NA, but did not affect the contraction elicited by the toxin (Figure 6). In addition, treatment with the calmodulin inhibitor W-7 ($1 \times 10^{-5}$ M) for 15 min markedly decreased the activity of NA (to about 55% of control), but did not alter the contractile response to the toxin (Figure 6). By contrast, pretreatment with tetrodotoxin ($1.5 \times 10^{-6}$ M) had no effect on the toxin-evoked contraction (data not shown). Furthermore, the contraction induced by the toxin was not inhibited after a 15 min incubation in low Na medium (data not shown).

*Effects of the toxin on Ca uptake*

The toxin caused a dose-dependent increase in $^{45}Ca$ uptake into the aorta (Table 3). After treatment with 0.5 μg or 2.0 μg ml$^{-1}$ of the toxin, Ca uptake was increased from 6.4 to 10.2 or 13.8 nmol g$^{-1}$ wet weight, respectively. While verapamil ($3.5 \times 10^{-6}$ M) significantly inhibited the contractile response to the toxin (Figure 4), pretreatment with verapamil ($3.5 \times 10^{-6}$ M) for 15 min reduced Ca uptake induced by 0.5 μg or 2.0 μg ml$^{-1}$ of the toxin to approximately 68% or 64% of uptake induced by the toxin in the absence of verapamil, respectively (Table 3).

*Effect of alpha toxin on phospholipid metabolism*

Michell (1982) has reported that the entry of Ca into the cells is associated with the metabolism of phospholipids in biological membranes. On the other hand, it has been reported that alpha toxin, which possesses phospholipase C activity, can hydrolyze phospholipids in various biological membranes (Allan *et al.*, 1978; Hofmann *et al.*, 1982; Honeyman *et al.*, 1983; Philipson *et al.*, 1983). We thus determined whether the phospholipid metabolism in the rat aorta is activated by exposure to alpha toxin. As shown in Table 4, incorporation of $^{32}P$ into PI and PA was determined by counting the radioactivity of the bands after autoradiography of phospholipids in the tissue treated with the toxin. Exposure to the toxin increased significantly incorporation of the radioactivity into PI and PA by approximately 170% and 190%, respectively. A stimulatory effect of the toxin on $^{32}P$ phosphate incorporation was manifest in PI and PA. However, the toxin did not enhance $^{32}P$ incorporation into phosphatidylethanolamine (PE).

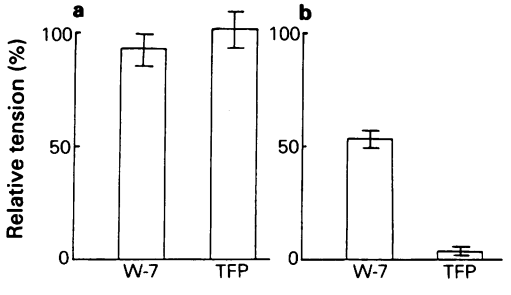


**Figure 6** Effect of W-7 and TFP on contractions induced by alpha toxin and noradrenaline (NA). Alpha toxin (0.5 μg ml$^{-1}$) (a) or NA ($1 \times 10^{-7}$ M) (b) was applied 15 min after administration of W-7 or TFP. Vertical lines indicate s.e.mean ($n = 6$). Differences between contractions of untreated aorta and W-7 or TFP-treated aorta induced by NA were significant ($P < 0.001$).

## Discussion

The data presented here show that alpha toxin induces a dose-dependent contraction of the rat isolated aorta within the dose range tested (0.2 μg ml$^{-1}$ to 0.6 μg ml$^{-1}$). The toxin-induced contraction was abolished by repeated washing, suggesting that binding of the toxin on the surface of the aorta is essential for contraction. The response to repeated doses of the

**Table 3** Effect of alpha toxin on $Ca^{2+}$ uptake into the isolated aorta of rat

| *Treatment* | *$^{45}Ca$ uptake*[a] |
|---|---|
| | (nmol g$^{-1}$ wet wt.) |
| None | 6.4 ± 1.2 |
| Alpha toxin (0.5 μg ml$^{-1}$) | 10.2 ± 0.5* |
| Alpha toxin (2.0 μg ml$^{-1}$) | 13.8 ± 0.5** |
| None | 6.5 ± 1.4 |
| Verapamil alone ($3.5 \times 10^{-6}$ M) | 6.5 ± 0.9 |
| Alpha toxin (0.5 μg ml$^{-1}$) | 13.0 ± 0.6 |
| + verapamil ($3.5 \times 10^{-6}$ M)[b] | 8.8 ± 0.8*** |
| Alpha toxin (2.0 μg ml$^{-1}$) | 15.1 ± 0.9 |
| + verapamil ($3.5 \times 10^{-6}$ M)[b] | 9.7 ± 0.7**** |

[a]Mean ± s.e. ($n = 7$).
[b]Verapamil was added 15 min before the application of the toxin.
*Significantly different from untreated (None) ($P < 0.02$).
**Significantly different from alpha toxin (0.5 μg ml$^{-1}$) ($P < 0.001$).
***Significantly different from alpha toxin (0.5 μg ml$^{-1}$) alone ($P < 0.002$).
****Significantly different from alpha toxin (2.0 μg ml$^{-1}$) alone ($P < 0.001$).

**Table 4** Distribution of radioactivity between phospholipids in the aorta

| *Phospholipid* | *Radioactivity (c.p.m.)* *Control* | *Toxin* |
|---|---|---|
| PI | 3843 ± 303 | 6687 ± 1130* |
| PA | 1064 ± 220 | 2028 ± 192** |
| PE | 6595 ± 319 | 6245 ± 507 |

Fragments of rat isolated aorta were incubated in the absence (Control) or the presence (Toxin) of the toxin (0.5 μg ml$^{-1}$) as shown in the text.
The phospholipids analyzed by thin-layer chromatography as shown in the text were scraped into vials, and the radioactivity was measured by liquid scintillation counting. Each value is the mean ± s.e. of 6 experiments.
*Significantly different from control ($P < 0.05$).
**Significantly different from control ($P < 0.002$).

toxin was found to be tachyphylactic. However, while the mechanism of this tachyphylaxis is unclear, the toxin did not affect the response to NA. It is thus unlikely that in our experiments the purified toxin causes tissue destruction. On the other hand, Lodge & Leach (1973) reported that the crude alpha toxin destroyed the longitudinal muscle preparation of the guinea-pig ileum. The crude or partially purified preparation contains other toxins and hydrolytic enzymes such as theta toxin (haemolysin), kappa toxin (collagenase) and mu toxin (hyaluronidase), which can destroy various tissues or cause synergistic effects of certain combinations of these toxins observable at biological actions. It is imperative to probe membrane structure and function only with a purified toxin preparation.

The toxin-induced contraction was not inhibited by an α-adrenoceptor blocking agent (phentolamine), a histamine receptor blocking agent (chlorpheniramine), or a muscarinic receptor blocking agent (atropine). It thus appears that the contractile action of the toxin was not due to activation of amine receptors and/or the release of these chemical mediators. These observations suggest that the response to the toxin seems to be the result of a direct action on the aorta.

Ca entry blockers such as verapamil and nifedipine antagonize Ca channel function in smooth muscles (Nueten & Vanhoutte, 1981). The toxin-induced contraction was inhibited by nifedipine, verapamil and cinnarizine. Furthermore, the toxin-induced contraction was not observed in Ca-free medium, and was immediately restored by the addition of Ca. The toxin-induced contraction and $^{45}Ca$ uptake into the tissue increased with increasing doses of the toxin. Moreover, Ca influx induced by the toxin was significantly inhibited by verapamil. Therefore, the data indicate that the direct action of the toxin is mainly due to an increase in Ca permeability across the smooth muscle cell membrane (Ca channel). By contrast, the response to the toxin was not blocked or affected by the Na channel blocker, tetrodotoxin, or by sodium deprivation, indicating that an increase in the membrane permeability to Na is not associated with its mode of action.

TMB-8, an antagonist of intracellular Ca transport and Ca release (Chiou & Malagodi, 1975), inhibited contractions induced by the toxin and NA. Since NA-induced contraction depends upon Ca uptake and intracellular Ca transport and release (Godfraind *et al.*, 1982), it appears that intracellular Ca transport and Ca release are also essential for contraction induced by the toxin.

The inhibition of the toxin-induced contraction by Ca entry blockers and by TMB-8 paralleled the inhibition of NA-induced contraction by these agents. However, calmodulin inhibitors such as TFP (Zavecz *et al.*, 1982) and W-7 (Tanaka *et al.*, 1982a,b) inhibited contraction induced by NA, but not by toxin. The data show that the mechanism of the toxin-induced contraction is different from that of the NA-induced contraction.

TFP has been reported to be a Ca entry blocker. However, our data indicate that nifedipine, verapamil and cinnarizine blocked contraction caused by the toxin, but TFP did not. TFP is classed with cinnarizine, on the basis of structure-activity relationships and lipophilicity (Spedding, 1985). Under our experimental conditions, cinnarizine was less efficient as a blocker than nifedipine and verapamil (Figure 4). Therefore, our interpretation of the mechanism based on Ca entry does not seem unreasonable.

Alpha toxin (phospholipase C) can hydrolyze various phospholipids, especially PI and phosphatidylcholine, to diacylglyceride in biological membranes (MacFarlane & Knight, 1941; Stahl, 1973; Krug & Kent, 1984). We have reported that the pressor activity of the toxin may be associated with both vasocontraction caused by the toxin and phospholipase C activity of the toxin (Sakurai *et al.*, 1985b). In the present study, labelling by $^{32}P$ phosphate of PI and PA in fragments of the aorta was enhanced by alpha toxin. Michell (1975, 1982) has proposed that PI breakdown is associated with a functional role in the activation of Ca gating. Tyson *et al.* (1976) and Serhan *et al.* (1981) reported that PA can act as a calcium ionophore. Therefore, Ca influx caused by the toxin is thought to be associated with the stimulation of phospholipid metabolism. On the other hand, it is possible that the increased turnover of phospholipids caused by the toxin induces a stimulation of PI breakdown to diacylglycerol and inositolphosphates. Suematsu *et al.* (1984) suggested that

inositol 1,4,5-triphosphate (Ins-$P_3$) plays the role of messenger in increasing the cytosolic Ca, and producing a contraction. Ca release from intracellular store sites and intracellular Ca transport, which seem to be important for contraction caused by the toxin, may be associated with the breakdown of phosphoinositides.

It is concluded that in rat aorta, alpha toxin hydrolyzes phospholipids in biological membranes, so that PI metabolism is activated, which in turn promotes Ca availability and thereby elicits contraction.

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