THE MECHANISM OF CONTRACTILE ACTION OF PALLYTOXIN ON VASCULAR SMOOTH MUSCLE OF GUINEA-PIG AORTA

Hiroshi OZAKI, Junya TOMONO, Hiromi NAGASE and Norimoto URAKAWA
Department of Veterinary Pharmacology, Faculty of Agriculture, University of Tokyo,
1-1-1 Yayoi, Bunkyo-ku, Tokyo, 113, Japan

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Abstract—The mode of action of palytoxin (PTX) on the contractile responses and ion movements in guinea-pig aorta was investigated. PTX (10^{-8} M) induced a contraction with a latency period of 0.5–1 min, and it reached maximum after about 20 min. This contraction was not affected by phentolamine (10^{-6} M). The contraction induced by PTX was rapidly abolished by removal of external Ca. Readdition of Ca restored the contraction. Verapamil, which markedly antagonized the contraction induced by some depolarizing agents (K, Ba and tetraethylammonium), decreased the rate of rise of the PTX-induced contraction, but did not affect the sustained tension level. Removal of external Na markedly inhibited the contraction induced by PTX (10^{-10}–10^{-8} M), while it had no effect on the contraction induced by histamine (10^{-7}–10^{-5} M). PTX rapidly decreased tissue K content with a similar time course to that of the increase in tension. Ouabain (10^{-4} M) also caused contraction and decreased tissue K content in the muscle, but the rate of these changes was much slower than the PTX-induced ones. PTX increased cellular {^{45}}Ca content in normal solution, but not in Na deficient solution. These results suggest that the PTX-induced contraction in guinea-pig aorta is due to an increase in membrane Na permeability.

Palytoxin (PTX) isolated from Palythoa sp. is the most potent marine toxin known (1, 2). It is a polyalcohol whose chemical structure has recently been fully determined (3, 4). Several toxicological studies suggested that the toxicity of PTX is due to its action on the cardiovascular system (5–8). The biological action of PTX has been extensively studied, and it was suggested that PTX acts on the cell membrane of the various organs investigated such as cardiac muscle (5, 9–12), skeletal muscle (5, 13), smooth muscle (10, 14–16), nerve tissue (17–19), red blood cells (20, 21) and sperm (22).

In smooth muscles as well as in skeletal and cardiac muscles, PTX causes contractile responses. In guinea-pig taenia coli, we have reported that PTX depolarizes the cell membrane, increases spike frequency and thus induces contraction (15). In rabbit aorta, PTX also produces contraction which is due to an increased Ca influx following membrane depolarization (10). Further, PTX has been shown to induce muscle contraction in guinea-pig vas deferens by a release of endogenous catecholamine from the adrenergic nerve endings as well as by a direct action on the smooth muscle (16).

On the other hand, electrophysiological studies revealed that the depolarizing action of PTX was attenuated by lowering the external Na concentration in cardiac muscle (23), skeletal muscle (5) and nerve tissue (17–19). These results suggest that the depolarizing action of PTX is due to the increase in Na permeability of the cell membrane. However, in smooth muscle, the role of Na in the contractile actions of PTX have
not been reported.

We have previously reported that the Na accumulation induced by K-free solution causes contraction in vascular smooth muscles isolated from various animal species (24). Among the animals examined, tissues from the guinea-pig are the most sensitive to the increase in cellular Na in inducing the contractile response. Therefore, we used guinea-pig aorta in the present experiments to investigate the mode of the contractile action of PTX.

Materials and Methods

Preparations: The thoracic aorta was removed from a male guinea-pig (250–350 g) killed by a blow on the neck, placed in physiological salt solution (PSS) and cleaned of surrounding connective tissue. For tension experiments, helical strips about 10 mm long and 3 mm wide were cut. For determination of tissue K and 45Ca contents, vessels were cut open and rectangular strips weighing 5–10 mg were prepared.

Solutions: Normal PSS contained (mM): NaCl, 136.9; KCl, 5.4; CaCl2, 2.5; MgCl2, 1.0; NaHCO3, 11.9 and glucose, 5.5. High-K solution (65.4 mM) was made by adding an appropriate amount of 2 M KCl stock solution to normal PSS. The hyperosmolarity of the high K solution did not affect the contractility of this vascular smooth muscle (25). Low-Na solution was prepared by replacing NaCl with isosmolar tris-HCl or LiCl. Ca-free solution was made by omitting CaCl2 (2.5 mM) from the normal PSS and adding EGTA (0.1 mM). Solutions were aerated with 95% O2–5% CO2 and used at 37°C and pH 7.2.

Measurement of tension: Muscle strips were suspended in an organ bath (20 ml), and contractions were recorded isometrically with an force transducer (Nihon Kohden). The resting tension applied to the muscle strips was 1 g. The muscles were allowed to equilibrate in normal PSS for at least 3 hr until the response to high-K (65.4 mM) solution became stable. The magnitude of the contraction induced by a 30 min application of high-K (65.4 mM) solution, averaging 1.35±0.14 g (n=10), was considered as the reference response (100%).

Measurement of tissue K content: Tissue K content was measured by flame photometry. After incubation with test solution, the muscles were transferred to quartz test tubes containing 0.5 ml of a mixture of equal amount of HNO3 (61%) and HCIO4 (60%), and heated overnight at 180–240°C. Immediately before determination, the dried samples were dissolved in 0.01 N HCl. CsCl (1 g/l) was added to the standard and diluted solutions to inhibit mutual interference of Na and K. Ion concentration of the diluted samples was measured by a flame photometer (Hitachi, Type 208).

Measurement of cellular 45Ca content: Cellular 45Ca content was measured by a modified “La method” which was developed by Karaki and Weiss (26) for rabbit aorta. After incubation with various test solutions labelled with 45Ca (0.5 μCi/ml, New England Nuclear) the muscle were exposed to a high-La solution (LaCl3, 73.8 mM; glucose, 5.5 mM; and tris-HCl, 11.9 mM) at 1°C and pH 7.2 for 1 hr in order to remove extracellularly bound 45Ca. The muscles were then placed in scintillation vials containing 0.5 ml of tissue solubilizer (Soluene-350, Packard) and were digested overnight at 50–60°C. The solubilized samples were then mixed with 5 ml of scintillator (Insta-Gel, Packard), and radioactivity was determined with a liquid scintillation spectrometer (Tri-Carb 3380, Packard).

Reserpine pretreatment: Reserpine (1 mg/kg, i.p.) was given daily to guinea-pigs for 2 days. The experiments were performed 24 hr after the last injection.

Drugs: PTX isolated from Palythoa tuberculosa was kindly donated by the late
Prof. Y. Hashimoto of the University of Tokyo. The toxin was dissolved in distilled water at a concentration of $10^{-4}$ M and kept frozen as a stock solution. Other drugs used were ouabain (Merck, Darmstadt, FRG), histamine dihydrochloride (Wakoh Junyaku, Tokyo, Japan), tetraethylammonium (TEA) (Nakarai, Tokyo, Japan), phentolamine mesylate (Ciba Geigy, Basel, Switzerland), tetrodotoxin (TTX) (Sankyo, Tokyo, Japan), verapamil (Eisai, Tokyo, Japan), diltiazem (Tanabe, Tokyo, Japan), nifedipine (Bayer, Leverkusen, FRG) and reserpine (Daiichi, Tokyo, Japan). Nifedipine was dissolved in dimethylformamide and diluted with PSS to give a final concentration of 0.01%.

**Results**

**Effects of PTX on the contractile responses:**

In guinea-pig aorta, PTX ($10^{-8}$ M) produced a contraction after a latency of 0.5–1.0 min and then gradually decreased to some level (Fig. 1A). The maximum tension ($1.29 \pm 0.15$ g, n=10) was reached after about 20 min. The threshold concentration of PTX was $10^{-9}$ M, and the maximum was obtained at $10^{-7}$ M. Phentolamine ($10^{-6}$ M) had no effect on the PTX-induced contraction. In the preparations isolated from reserpine-treated animals, PTX produced a similar magnitude of contraction as was observed in untreated preparations. Removal of PTX from the medium did not reduce the developed contraction for at least 3 hr.

PTX did not produce any contraction in Ca-free medium (containing 0.1 mM EGTA). Contraction induced in the presence of Ca was rapidly and completely inhibited after Ca removal (Fig. 1A). Readdition of Ca (2.5 mM) to the Ca-free solution restored the contraction.

It has been reported that ouabain or Na removal produced a contraction by a release of endogenous catecholamine in vascular smooth muscles (27). Therefore, in the following experiments, phentolamine ($10^{-6}$ M) was added to all solutions in order to inhibit the vasoactive effects of endogenous catecholamines. This concentration of phentolamine completely inhibited the contraction induced by low-Na solution or exogenous application of norepinephrine ($10^{-5}$ M).

Ouabain ($10^{-4}$ M), an inhibitor of the Na pump, produced contraction, but the rate was much slower than that of the PTX-induced one (Fig. 1B). The latent period was about 10 min, and the maximum was reached after about 120 min. The contraction was highly dependent on the presence of external Ca and Na, as reported previously (28).

Verapamil ($2 \times 10^{-6}$ M), added to the medium after the PTX ($10^{-8}$ M)-induced contraction had reached its maximum, produced a slight decline of the tension (less than 10%). On the other hand, pretreatment of the muscle with verapamil decreased the rate of rise of the PTX-induced contraction, but it had no effect on the plateau phase of the contraction (Fig. 2). Other types of organic Ca antagonists such as diltiazem ($2 \times 10^{-6}$ M) and nifedipine ($10^{-7}$ M) also inhibited the early phase of the PTX-induced contraction.

**Fig. 1.** Contractile effects of PTX ($10^{-8}$ M) (A) and ouabain ($10^{-4}$ M) (B) on isolated guinea-pig aorta. A reference contraction was produced by high-K (85.4 mM) solution. In the upper trace, after the contraction had reached maximum, the external medium was changed to Ca-free solution (0.1 mM, EGTA), and then Ca (2.5 mM) was readded.
Fig. 2. Effect of verapamil on PTX-induced contraction. The muscles were pretreated with verapamil (2 x 10^{-6} M) for 5 min before they were contracted by PTX (10^{-8} M). Ordinate: relative tension (%). Abscissa: time (min). Mean values±S.E.M. (n=6).

contraction. These agents completely inhibited the contractions induced by the depolarizing agents such as KCl (45.4 mM), BaCl_2 (40 mM) and tetroethylammonium (15 mM). On the other hand, Na channel blocker TTX (3.1 x 10^{-7} M) had no effect on the time course of the contraction induced by PTX (10^{-8} M).

The effect of NaCl (136.9 mM) removal on the contractile action of PTX was examined. Since sucrose- and choline-substituted solutions induce a rise in the resting tension, we employed tris and Li as Na substitutes. Figure 3 shows the dose-response relationship of the PTX-induced contraction in normal- and low-Na solutions. Both in tris- and Li-substituted solutions, the contraction induced by PTX was markedly inhibited. The rate of rise of the PTX-induced contraction was also slowed in the low-Na solutions. On the other hand, histamine (10^{-7}–10^{-5} M) produced the same magnitude of contraction either in normal-Na or low-Na medium.

Effects of PTX on tissue K content: In the previous reports (24, 29–31), we measured cellular Na content after washing the tissue with cold Li-substituted solution for several minutes to remove extracellular Na ("Li method") (32). However, we found in guinea-pig aorta that the total Na+K content of the muscle pretreated with PTX was greatly reduced during the cold Li-wash. This finding suggests that cold Li-solution could not completely inhibit the transmembrane Na and K leak in PTX-treated tissue.

In the present experiments, we again attempted to measure the change in tissue Na content due to PTX. However, the values were so variable that we could not find any significance between the values. This may be due to the fact that the bulk of tissue Na is located extracellularly, and it is affected substantially by the change in the volume of extracellular fluid that occurs during the contraction of the vascular wall. It is generally known that the change of cellular K for extracellular Na induced by the inhibition of the Na pump is on an almost equimolar basis. Therefore, we estimated the changes in the intracellular Na indirectly by measurement of tissue K.

The tissue K content in normal PSS was...
24.6±1.7 mmole/kg wet weight (n=6). As demonstrated in Fig. 4, PTX (10⁻⁸ M) rapidly decreased tissue K content. A plateau phase was reached after about 40 min. Ouabain (10⁻⁴ M) also reduced the K content. However, the rate of the ouabain-induced loss of K was much slower than that induced by PTX.

**Effects of PTX on cellular ⁴⁵Ca content:**
We have reported that, in guinea-pig aorta, the resting ⁴⁸Ca uptake reached a steady state within 30 min (24). Thus, the effect of PTX on the cellular ⁴⁵Ca content was investigated using a 30 min incubation period. As shown in Table 1, PTX (2×10⁻⁸ M) significantly (P<0.05) increased the cellular ⁴⁵Ca content in normal solution. In contrast, PTX did not significantly increase the ⁴⁵Ca content in low-Na solution.

**Discussion**
In the present experiments, we investigated the mode of contraction induced by PTX in isolated guinea-pig aorta. The major findings were as follows: (a) PTX at concentration above 10⁻⁹ M causes contraction which is not affected by the pretreatment with phentolamine or by TTX. Further, PTX causes contraction in reserpine treated muscle. (b) The contractile effects of PTX are dependent on the presence of both external Ca and Na. (c) Organic Ca antagonists such as verapamil, diltiazem and nifedipine only inhibit the early phase of the PTX-induced contraction. (d) PTX decreases cellular K with a similar time course to the increase in the contractile responses. (e) PTX is able to increase the cellular ⁴⁵Ca content only in the presence of external Na ions.

It is known that removal of external K or addition of cardiac glycosides induces contraction in various types of vascular smooth muscles (27). In guinea-pig aorta, we have reported that an increase in intracellular Na concentration, resulting from inhibition of the Na pump, causes contraction by an increase in Ca influx through a Na-Ca exchange mechanism (28). Further, this process was not influenced by organic Ca antagonist like verapamil (29, 30). The present results demonstrate that the major part of the PTX-induced contraction is resistant to verapamil. In addition, the PTX-induced contraction and ⁴⁵Ca uptake are greatly dependent on the presence of external Na ions. These results suggest a similarity between the contractions

![Figure 4](image-url)

**Fig. 4.** Effect of PTX (10⁻⁸ M) (○) and ouabain (10⁻⁴ M) (●) on tissue K content. Ordinate: tissue K content (%). Abscissa: time (hr). Mean values ±S.E.M. (n=6).

**Table 1.** Effects of PTX (2×10⁻⁸ M) on cellular ⁴⁵Ca content measured by the modified "La method"

|            | Na: 148.7 mM | Na: 11.9 mM (tris substituted) |
|------------|--------------|---------------------------------|
| Control    | 204.5±16.1   | 194.7±9.0 (11)                  |
| PTX (2×10⁻⁸ M) | 269.6±22.6*  | 213.7±8.7 (13)                  |

Values are means±S.E.M. (μmole/kg wet weight). Parentheses indicate the number of experiments. *Significantly different from the control (P<0.05).
induced by PTX and inhibition of the Na pump. It remains possible that the increased cellular Na inhibits the reuptake of Ca into some sequester sites and thus increases the cellular free Ca concentration. However, at present, such a possibility has not been elucidated in vascular smooth muscles. The results also suggest that the early phase of the contraction, which is sensitive to the organic Ca antagonists, is due to membrane depolarization.

Recently, it has been suggested that fast Na channels exist in the smooth muscle membrane of guinea-pig vas deferens (33). This assumption comes from the observation that ciguatoxin induces supersensitivity in the smooth muscle cells by an increase in Na permeability which is blocked by TTX. However, such fast Na channels are not involved in the PTX-induced contractions of guinea-pig aorta since the contractions are not affected by pretreatment with TTX.

The present experiments indicate that PTX dissipates the Na and K gradients of the vascular smooth muscle. This dissipation was obtained at a much faster rate than that induced by maximal inhibition of the Na pump with $10^{-4}$ M ouabain. Further, PTX markedly increases loss of Na from Na-loaded vascular smooth muscle into low-Na solution (34). It has been reported that PTX does not influence the Na,K-ATPase activity of erythrocyte (21) and heart muscle (35) in concentrations up to $10^{-7}$ M. Based on these observations, it is suggested that PTX acts on the cell membrane and increases the Na permeability.

A Na ionophore monensin has been shown to increase the cellular Na content in rabbit aorta (31). However, such an increase can be seen only when the Na pump is inhibited by ouabain. This suggests that monensin-induced elevation of cellular Na, resulting from the increase in Na permeability, is cancelled by activation of the Na pump in the absence of ouabain. In contrast to the effects of monensin, PTX is able to increase cellular Na when the Na pump is intact. Thus, it is likely that PTX is a much more potent agent than monensin in its ability to increase membrane Na permeability. At present, it remains unknown whether PTX increases the membrane permeability by acting as an ionophore or by acting on the ion-transport mechanism present in the membrane of smooth muscle cells.

In our previous report with rabbit aorta (10), it was shown that the properties of PTX-induced contraction were similar to those of high-K induced contraction, and we concluded that PTX induces contraction by an increase in Ca influx through voltage sensitive Ca channels (verapamil sensitive). In contrast to the above conclusion in rabbit aorta, the present experiments with guinea-pig aorta show that the contraction induced by PTX is only partly sensitive to verapamil. These results suggest that the mechanism of the PTX-induced contraction is different in these two animal species. Such a species difference is seen in the contractile responses to monensin; the verapamil-sensitive component of the monensin-induced contraction is greater in rabbit aorta than in guinea-pig aorta (under preparation).

In conclusion, it is suggested that the major mechanism of the PTX-induced contraction in guinea-pig aorta is explained by an increase in the Na permeability of the cell membrane.

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