Effects of Palytoxin on Na, K and ATP Contents of Vascular Smooth Muscle of Rabbit Aorta

Hiroshi OZAKI, Hiromi NAGASE, Katsuaki ITO* and Norimoto URAKAWA
Department of Veterinary Pharmacology, Faculty of Agriculture, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan
*Department of Veterinary Pharmacology, Faculty of Agriculture, University of Miyazaki, Miyazaki 880, Japan
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Abstract—Palytoxin (PTX) at concentrations higher than 10^{-9} M increased tissue Na and decreased tissue K contents in the smooth muscle of rabbit aorta. The decrease in the tissue K content induced by PTX (10^{-8} M) was complete within 1 hr. Saponin (1 mg/ml) and Triton X-100 (0.1% wt./vol.) also rapidly decreased the tissue K content. On the other hand, a high concentration of ouabain (10^{-3} M) did not change the tissue K content within 1 hr of application, and the maximum loss of K was obtained after 6 hr. Loss of tissue Na into Na- and K-free solution from Na loaded muscle was accelerated by PTX (10^{-8} M). The PTX-induced increase in loss of Na was inhibited in proportion to the decrease in the temperature from 37 to 10°C, while the loss of Na in the absence of PTX was almost completely inhibited at 24°C. Decrease in the wet weight of the muscle induced by hyperosmotic solution was inhibited by pretreatment with PTX (10^{-8} M) or saponin (1 mg/ml) for 1 hr. PTX (10^{-9} and 10^{-8} M) had no effect on the ATP content of the muscle. However, PTX at concentrations above 10^{-7} M reduced the ATP content, and a significant amount of ATP was detected in the incubation medium. Saponin (1 mg/ml) and Triton X-100 (0.2% wt./vol.) induced a release of Na from liposomes prepared with synthesized lecithin or total lipids of rabbit red blood cells. However, no Na leak was induced by PTX (10^{-8}-10^{-6} M) in these liposomes. These results suggest that PTX at low concentrations (10^{-9}-10^{-8} M) increases the membrane permeability of vascular smooth muscle cells to Na and K ions. At higher concentrations (10^{-7}-10^{-6} M), PTX seems to form pores which are permeable to a larger molecule like ATP. The results further suggest that the mode of action of PTX is different from that of saponin or detergent.

Palytoxin (PTX) isolated from Palythoa spp. is the most potent marine toxin ever known (1, 2). Recently, the chemical structure of PTX has been fully determined by Moore et al. (3) and Cha et al. (4), independently. It is a polyalcohol with a molecular weight of 2660-2680. Several toxicological studies have suggested that the toxicity of PTX is due to its action on the cardiovascular system (5-8). Ito et al. (8) suggested that PTX exerts its toxic action through intense vasoconstriction in the whole body, particularly in the coronary and renal vascular beds, and through depression of cardiac function. PTX in an extremely low concentration induces contraction in skeletal muscle (7, 9), heart muscle (7, 10-13) and smooth muscle (14-18). These reports suggest that PTX increases the Ca permeability of the cell membrane. On the other hand, electrophysiological studies showed that PTX depolarizes the cell membrane of skeletal muscle (7), heart muscle (11, 12), smooth muscle (14) and nerve tissue (19-21) at the concentration that produces muscle contraction. These reports suggest that the membrane depolarization induced by PTX is
due to the increase in membrane Na permeability.

In the present experiments, we examined the action of PTX on transmembrane movements of Na, K and ATP in the smooth muscle of rabbit aorta. We showed that PTX increased the membrane permeability of a large molecule like ATP as well as the membrane ion permeability. In addition, we examined the effects of PTX on liposomes prepared with synthesized lecithin or a lipid extract from rabbit erythrocytes.

Materials and Methods

Preparations: The thoracic aorta was removed from male white rabbits (2.5–3.0 kg). The aorta was cut into a 3–4 mm wide spiral strip. Unless otherwise noted, the adventitial layer was separated from the media-intimal layer as described by Karaki and Urakawa (22). They were then cut into pieces of 5–10 mg. For measurement of the change in tissue wet weight, rectangular strips of 30–50 mg were prepared.

Solutions: Normal physiological solution (PSS) contained (mM): NaCl, 136.9; KCl, 5.4; CaCl2, 2.5; MgCl2, 1.0; NaHCO3, 11.9; and glucose, 5.5. K-free solution was prepared by omitting KCl from the PSS. These solutions were aerated with 95% O2-5% CO2 and used at pH 7.2. Na and K-free solution contained tris(hydroxymethyl) aminomethane, 142.3; CaCl2, 2.5; MgCl2, 1.0; and glucose, 5.5 mM which was aerated with 100% O2 and adjusted to pH 7.2 with HCl. Experiments were conducted at 37°C unless otherwise stated.

Tissue Na and K contents: Tissue Na and K contents were measured by flame photometry. After incubation with the test solution, the muscles were transferred to quartz test tubes containing 0.5 ml of a mixture of equal amounts of HNO3 (61%) and HClO4 (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 standards 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 20B, Japan). In some experiments, the tissues were washed with Li-solution (LiCl, 147.4 mM; glucose, 5.5 mM; and tris-HCl, 11.9 mM) at 1°C and pH 7.2 for 15 min in order to remove extracellular and bound Na (23).

Tissue wet weight: Muscle strips were treated with various test solutions; and during the incubation period, they were removed occasionally from the organ bath. Muscles were blotted between filter paper (Toyo, No. 2) for 5 sec and weighed. It took approx. 60 sec for these procedures.

ATP content: The ATP content of the muscle or of the incubation solution was measured as described by Strehler and McElroy (24). After incubation with the test solutions, muscle strips were boiled for 5 min in 2 ml hot water and then cooled to 0°C. ATP in the boiled extract or in the incubation medium was measured with a Lumac Biometer M 1030 (Lumac B.V., The Netherlands), utilizing ATP-luciferine-luciferase luminescence.

Liposome preparation: The liposomes (multilamellar vesicles) were prepared using, 1) total lipids (TL) of red blood cells, 2) synthetic dipalmitoyllecithin (DPPC), cholesterol (CHOL) and dicetylphosphate (DP) in a molar ratio of 2 : 1.5 (or 0) : 0.11 and 3) combination of 1) and 2). The lipids were extracted from rabbit red blood cells as described by Folch et al. (25). The dried films were dispersed by agitation with a Vortex mixer in a sufficient volume of Na-solution (NaNO3, 214.2 mM; tris-HCl, 23.8 mM, pH 7.2). Untrapped marker ions were removed by centrifugation (12,000 rpm) for 10 min each at 3°C with three changes of Na-free solution (238 mM tris-HCl, pH 7.2).

Release of Na from liposomes: The loss of Na into the Na-free solution was measured by "thin-layer potentiometric analysis" (26, 27), using a Na selective electrode (Type IS561 Na, Philips Japan, Japan). Measurements were performed at room temperature (19±1°C). Comparative studies were always done from one stock liposome solution.

Drugs: PTX isolated from Palythoa tuberculosa was kindly donated by the late Prof. Y. Hashimoto of The University of Tokyo and Prof. Y. Hirata of Meijo University. PTX from Prof. Hirata was used in the experiments on the ATP movements and liposomes. The
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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, F.R.G.), saponin (Sigma, St. Louis, U.S.A.) and Triton X-100 (Sigma, St. Louis, U.S.A.). Dipalmitoyl L-α-phosphatidyl choline (DPPC), dicetyl phosphate (DP) and cholesterol (CHOL) were purchased from Sigma (St. Louis, U.S.A.). Luciferin-luciferase mixture was purchased from Boehringer Mannheim (Mannheim, F.R.G.).

**Results**

**Effects of PTX on Na and K contents:** As shown in Fig. 1, PTX (10^{-8} M) rapidly decreased the tissue K content of the media-intimal layer of rabbit aorta. Saponin (1 mg/ml), a plant glycoside, and Triton X-100 (0.1% wt./vol.), a nonionic detergent, also decreased tissue K content. A high concentration of ouabain (10^{-3} M) also decreased tissue K content, although a significant decrease was only obtained after 3 hr treatment with ouabain, and the plateau was reached after about 6 hr. A concentration-response relationship for the effects of PTX on tissue Na and K contents is shown in Table 1.

In previous reports (28–30), we measured cellular Na and K after washing the tissue with cold Li-substituted solution for several minutes to remove extracellular and bound...
Na ("Li-method") (23). In the present study, we examined the effects of PTX on Na and K contents using this technique. After incubation with various concentrations of PTX for 1 hr, the muscles were exposed to Li-solution at 0.5°C for 15 min. As shown in Fig. 2, the concentration-response diagram for the K content was sigmoidal. The maximum effect was obtained at 10^{-8} M. On the other hand, the curve for the Na content was bell shaped. The total Na+K content was decreased when the PTX concentration was more than 10^{-8} M. These results suggest that the "Li-method" can be applied to the muscles pretreated with PTX at concentrations lower than 10^{-9} M. At 10^{-8} M, the cellular Na and K contents would be underestimated by about 15%.

In the next series of experiments, the effects of PTX on the loss of Na into Na- and K-free solution (tris-substituted) were investigated (intact aorta) (Fig. 3). When the muscles were treated with K-free solution for 10 hr, tissue Na content increased from 68.8±2.2 to 134.3±6.7 mmole/kg (n=6 each). The Na loaded tissues were then transferred to the Na- and K-free solution. Tissue Na decreased gradually for more than 3 hr. Other muscle strips were preloaded with Na by incubating with PTX (10^{-8} M) in normal PSS for 1 hr (Na: 89.2±3.9 mmole/kg, n=6), and they were then transferred to Na- and K-free solution. The rate of loss of tissue Na in PTX-treated aorta was much faster than in the K-free treated one. Figure 3 also shows the effects of lowering the temperature on the loss of tissue Na. The decrease in Na content induced by PTX was proportionally inhibited by lowering the temperature from 37 to 10°C. On the other hand, the loss of Na in the absence of PTX was almost completely inhibited at 24°C after the initial washout of extracellular Na.

Effects of PTX on the change in tissue wet weight: The effects of PTX and saponin were examined on the change in tissue wet weight induced by a hyperosmotic solution (PSS plus 200 mM NaCl) (intact aorta). When the muscles were exposed to the hyperosmotic solution, muscle wet weight decreased by about 15% (Fig. 4). After the muscles had been pretreated with PTX (10^{-8} M) or saponin (1 mg/ml) for 1 hr, the decrease in tissue wet weight was greatly inhibited.

Effects of PTX on ATP content: The changes in muscle ATP content are summarized in Table 2. In this series of experiments, we compared the action of PTX supplied from Prof. Hashimoto (group A) and Prof. Hirata (group B). The ATP content in normal solution was 0.29±0.06 (n=6) and 0.41±0.08 (n=8) mmole/kg tissue in group A and group B, respectively. Both in groups A and B, PTX (10^{-9} and 10^{-8} M) did not affect the ATP content, while PTX (10^{-7} and 10^{-6} M) significantly decreased it to a similar extent. Figure 5 shows the time course of the efflux of ATP induced by PTX (10^{-6} M) into the incubation medium. The maximum loss of ATP was obtained at 15 min after the addition of PTX (10^{-6} M). The resting loss of tissue ATP was negligibly small.
Saponin and Triton X-100 are known to destroy the cell membrane and to make it permeable to large molecules. Therefore, these agents are frequently used for chemical "skinning" in various tissues. In this experiment, we examined the effects of these agents on ATP content of the vascular smooth muscle. As shown in Table 2, saponin (1 mg/ml) and Triton X-100 (0.2% wt./vol.) significantly decreased the ATP content to 0.05 mmole/kg tissue and to an undetectable level, respectively. Treatment of the muscles with buffered water (containing only 11.9 mM NaHCO₃) also depleted the tissue ATP content. When the muscle was incubated with water, ATP was rapidly released as shown in Fig. 5. Although the time course of the ATP leak was different from tissues treated with PTX and water, the total amount of ATP released was almost the same.

### Effects of PTX on liposomes

Effects of PTX, saponin and Triton X-100 on the Na release from lecithin liposomes with or
without cholesterol (DPPC-CHOL-DP or DPPC-DP) were investigated. As shown in Fig. 6, saponin (1 mg/ml) and Triton X-100 (0.2% wt./vol.) induced a strong Na leak from the liposomes. However, no release was observed from the liposomes in the presence of PTX (10^{-8}-10^{-6} M).

In the next experiments, we extracted total lipids (TL) from rabbit red blood cells and prepared liposomes. Further, we prepared liposomes with TL, DPPC, CHOL and DP (16:37:44:3, dry weight ratio). As was the case with lecithin liposomes, no observable Na leak was induced by PTX (10^{-6} M) in these liposomes (Fig. 7).

**Discussion**

In the present experiment, PTX increased tissue Na and decreased tissue K contents of the rabbit aorta. PTX also increased Na and K contents of a fraction not removable by washing the tissue with cold Li-solution for 15 min. Friedman and his co-workers have suggested that the residual Na after a wash in cold Li-solution is cellular in vascular smooth muscle (23, 32, 33). These results suggest that PTX dissipates both the transmembrane Na- and K-gradients of the vascular smooth muscle.

The dissipation of the Na- and K-gradients induced by PTX was observed to occur at a much faster rate than that induced by a high concentration of ouabain (present experiment) or by K-free solution (29). It has been reported that PTX at concentrations below 10^{-7} M does not affect the activity of the Na,K-ATPase prepared from red blood cells (34) and heart muscle (35). Furthermore, the present results showed that PTX markedly increased the loss of cellular Na into Na-free solution. Based on these observations, it is suggested that PTX acts directly on the cell membrane and increases Na and K permeability.

It is known that ionophores increase permeability in the lipid bilayer membrane by two different mechanisms. Pore type ionophores (amphotericin B, nystatin, filipin, etc.)
form stationary structures that allow the passage of ions. Carrier type ionophores (monensin, nigericin, X-537A, A23187, etc.), in contrast, move through the membrane in association with individual (specific) ions. Thus, the change in the membrane fluidity obtained by lowering the temperature affects the two permeation mechanisms differently; low temperature more strongly inhibits ion movements due to carrier type ionophores than those due to pore type ionophores. In vascular smooth muscle, we have reported that the monensin-induced increase in Na permeability is completely inhibited by lowering the temperature to 0.5°C (36). The present results demonstrated that the Na loss into Na-free solution induced by PTX (10^{-8} M) was proportionally reduced by lowering the temperature from 37 to 10°C (Fig. 3), but not completely inhibited at 0.5°C (Fig. 2). Furthermore, in PTX-treated muscle, the tissue wet weight (cell volume) was not reduced by the increase in the osmolarity of the external solution. These findings eliminate the possibility that PTX forms complexes with cations and diffuse through the membrane and transports cations like the carrier type of ionophores. The above concept would be further supported by the fact that PTX is only soluble in water and entirely insoluble in any organic solvents.

In the present experiments, we attempted to measure the change in cellular Na and K contents by washing the tissue with cold Li-solution for 15 min at 0.5°C (“Li-method”) (23). This method is based on the observation that cold Li-substituted solution displaces Na and K from extracellular sites but preserves the cellular compartment. At concentrations below 10^{-8} M, PTX increased tissue Na of a fraction not removable by the cold Li-solution, while at higher concentration, the Na content significantly decreased. On the other hand, the K content was decreased by PTX in proportion to the PTX-concentration. These results suggest that cellular Na and K were lost during the cold Li-wash (0.5°C) when the PTX concentration was higher than
10^{-7} M. The present study further showed that at the higher concentration ranges (above 10^{-7} M), PTX released cellular ATP into the incubation solution.

In the present experiments, we examined the effects of PTX on the Na permeability of artificial phospholipid bilayer membranes (liposome). Since the presence of cholesterol in phospholipid membranes is a requirement for the sensitivity of polyene antibiotic ionophores such as amphotericin B and nystatin (37–39), we prepared liposomes with or without cholesterol. The present results clearly demonstrated that PTX (10^{-8}–10^{-6} M) had no effect on these liposomes, while saponin and Triton X-100 strongly induced Na release. Furthermore, PTX had no effect on liposomes prepared with total lipids extracted from rabbit red blood cells. On the other hand, it has been reported that PTX causes a cytolysis of the red blood cells (34, 40, 41). These results suggest that PTX cannot interact with some lipids.

In summary, the results of the present study show that PTX increased membrane permeability to Na and K ions, and this was relatively resistant to lowering the temperature. In addition, at higher concentrations (above 10^{-7} M), PTX increased membrane permeability to a larger molecule like ATP. Further, PTX abolished the osmotic resistance. These results suggest that PTX directly acts on the cell membrane and forms pores. The size of the pores seems to be determined by the concentration of the toxin. The action of PTX does not seem to be mediated by an interaction with membrane lipids.

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