Palytoxin-Induced K^+ Efflux from Ileal Longitudinal Smooth Muscle of the Guinea-Pig

Masatoshi HORI, Kazumasa SHIMIZU, Shinjiro NAKAJYO
and Norimoto URAKAWA

Department of Veterinary Pharmacology, Nippon Veterinary and Zootechnical College,
Musashino, Tokyo 180, Japan

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Abstract—In guinea-pig ileal longitudinal smooth muscle, both palytoxin (PTX) and carbachol (CCh) increased K^+ efflux with an EC_{50} of 1.8×10^{-10} M and 4.1×10^{-7} M, respectively. Atropine (10^{-6} M) did not inhibit the K^+ efflux due to PTX (3×10^{-9} M), but completely inhibited the efflux due to CCh (10^{-5} M). External Ca^{2+} removal and verapamil (10^{-5} M) did not change the PTX-induced K^+ efflux, although the CCh-induced K^+ efflux was inhibited about 77% and 71%, respectively. PTX-induced K^+ efflux was reduced to 31% by a depletion of intracellular Ca^{2+} . Tetraethylammonium (15 mM) inhibited the K^+ efflux due to PTX or CCh to 61% or 75%, respectively. The PTX-induced K^+ efflux was also inhibited by cymarin (3×10^{-8} M), ouabain (10^{-5} M) and digitoxin (10^{-5} M). These results suggest that the PTX-induced K^+ efflux is less dependent on Ca^{2+} influx than that due to CCh. Furthermore, the binding sites for PTX in the ileal muscle of guinea-pig may be Na^+, K^+ -ATPase, as has been suggested in other types of cells.

Palytoxin (PTX; $C_{129}H_{223}N_3O_{54}$, MW 2677) isolated from Zoanthid, *Palythoa tuberculosa*, is known to be the most potent animal toxin (1), and its toxicity is due to action on the cardiovascular system (2–4). The chemical structure of PTX is characterized as a polyalcohol consisting of a very long, partially unsaturated aliphatic chain interspaced with five sugar moieties, which starts with an OH group and ends with an NH_2 group (5, 6). PTX at an extremely low concentration increased the Ca^{2+} permeability of cell membranes and induced a contraction in skeletal muscle (3), heart muscle (3, 7, 8) and intestinal and vascular smooth muscles (9–12). Electrophysiological studies showed that PTX, at the concentration that produces muscle contraction, depolarized the cell membranes of skeletal muscle (3), heart muscle (8), intestinal and vascular smooth muscles (9) and nerve tissue (13). PTX also caused K^+ efflux from the erythrocytes of man, sheep, dog, rat and rabbit (14–17). Since PTX decreased tissue K^+ content in intestinal and

vascular smooth muscle (11, 12), it is possible that PTX increases K^+ efflux in these tissues, and the K^+ efflux and the decrease in tissue K^+ content by PTX were inhibited by ouabain (12, 15, 18, 19). However, mechanisms of the PTX-induced K^+ efflux are not clear.

In guinea-pig ileal longitudinal smooth muscle, carbachol (CCh) also produced an increase in K^+ efflux, membrane depolarization and a contraction (20). In this experiment, we compared the properties of K^+ efflux induced by PTX and CCh in guinea-pig ileal longitudinal smooth muscle. K^+ efflux was continuously monitored with a K^+ -selective electrode. Results suggest that the PTX-induced K^+ efflux is less dependent on Ca^{2+} influx than that due to CCh.

Materials and Methods

Preparations: Guinea-pigs (300–400 g, male) were sacrificed by a blow on the neck. After exsanguination, the abdomen was opened, and the lower part of the ileum (10–

15 cm long) was removed. The ileal longitudinal muscle preparation, about 4 cm long and weighing 40–50 mg, was prepared as described by Paton and Aboo Zar (21).

Solution: Physiological salt solution (PSS) has the following composition (mM): NaCl, 136.8; KCl, 5.4; CaCl_2 , 2.5; MgCl_2 , 1.0; HEPES, 11.9 and glucose, 5.5; and pH was adjusted to 7.2 by 1N NaOH. KCl was omitted from PSS when K^+ efflux was measured. Ca^{2+} -deficient solution was prepared by simply omitting CaCl_2 from the PSS.

Measurement of K^+ efflux: The PSS (15 ml) in a 30 ml volume-organ bath was aerated with 100% O_2 at 37°C. The solution was usually stirred by a stirrer. After the pre-incubation for 60 min in the PSS, the mus-

cle was transferred to K^+ -deficient solution, and changes in external K^+ concentration were measured by a K^+ -selective electrode (Philips IS561K) and ion meter (Beckman Serection 500). The maximum release of K^+ was measured after adding saponin (10 $\mu\text{g}/\text{ml}$) for 30 min. PTX or CCh was applied to the medium either at the start of the K^+ -free wash (Fig. 1A) or 20 min or 25 min after beginning the wash (Fig. 1B). Residual K^+ contents in the muscle were calculated by subtracting K^+ concentrations in the medium (measured every two minutes) from the total K^+ concentration released by saponin. The total K^+ content was taken as 100%, and the results of the experiments are shown by either relative K^+ content (%) or rate of K^+

Fig. 1. Effect of PTX or CCh on K^+ efflux of guinea-pig ileal longitudinal muscle in the absence (A) or presence (B) of atropine. Each line was obtained from the mean of 4–6 experiments. A: K^+ efflux induced by various concentrations of PTX or CCh. B: Effect of atropine (10^{-6}M) on K^+ efflux due to PTX ($3 \times 10^{-9}\text{M}$) or CCh (10^{-5}M). Ordinate shows relative K^+ content in the muscle (%) taking maximum K^+ content as 100%. The maximum K^+ content was obtained by adding saponin (10 $\mu\text{g}/\text{ml}$) for 30 min. Abscissa shows time (min).

efflux.

Drugs: PTX isolated from *Palythoa tuberculosa* was kindly donated by Dr. Hirata (Meijo University, Nagoya). The toxin was dissolved in distilled water containing 0.1% bovine albumin and 10 mM HEPES at pH 7.4 to prepare a 10^{-4} M stock solution, which was kept frozen at -20°C . Other drugs used were CCh (Sigma), atropine (Sigma), verapamil (Eisai), TEA (Sigma), cymarin (Sigma), ouabain (Merck), digitoxin (Aldrich) and saponin (ICN).

Results

PTX or CCh-induced K⁺ efflux: In guinea-pig ileal longitudinal smooth muscle, tissue K⁺ was gradually lost in a K⁺-free solution with a half time ($T_{1/2}$) of 27.9 min. PTX (10^{-11} M– 10^{-8} M) or CCh (10^{-7} M– 10^{-4} M) increased the K⁺ efflux in a concentration-dependent manner. The EC₅₀ values of PTX- or CCh-induced K⁺ efflux was 1.8×10^{-10} M

or 4.1×10^{-7} M, respectively. The $T_{1/2}$ of the K⁺ efflux due to PTX (10^{-9} M) or CCh (10^{-6} M) was 14.7 min or 16.2 min, respectively (Fig. 1A).

Effect of atropine: Atropine (10^{-6} M) was applied 15 min after transferring the muscle to K⁺-free solution, and PTX (3×10^{-9} M) or CCh (10^{-5} M) was applied 10 min after the application of atropine (Fig. 1B). Atropine affected neither the resting K⁺ efflux nor the PTX (3×10^{-9} M)-induced K⁺ efflux. However, the CCh-induced K⁺ efflux was completely inhibited by atropine (Fig. 2).

Effects of Ca²⁺_o removal and verapamil: The PTX-induced K⁺ efflux was not inhibited by Ca²⁺_o removal, whereas the CCh-induced K⁺ efflux was inhibited by 77% (Fig. 3). Although the PTX-induced K⁺ efflux was not inhibited by verapamil, an organic Ca²⁺ antagonist, K⁺ efflux induced by CCh (10^{-5} M) was inhibited by 71% (Fig. 4).

Effect of Ca²⁺_i depletion: A Ca²⁺_i depleted

Fig. 2. The effect of atropine (10^{-6} M) on the PTX- or CCh-induced K⁺ efflux in the ileal muscle. Ordinate: The rate of K⁺ efflux shown by 100 times the slope of the regression coefficient for decrease in tissue K⁺ content. Tissue K⁺ content was obtained by K⁺ subtracting K⁺ concentration in the medium measured every two minutes from the K⁺ concentration after the saponin-treatment. Mean $\pm$ S.E. of 4–8 experiments is shown. **: significantly different from the value of PTX (3×10^{-9} M) or CCh (10^{-5} M) ($P < 0.01$).

Fig. 3. The effect of Ca²⁺_o removal on the PTX- or CCh-induced K⁺ efflux in the ileal muscle. Ordinate: Mean $\pm$ S.E. of 4–8 experiments is shown. **: significantly different from the value of PTX (10⁻⁸ M) or CCh (10⁻⁵ M) ($P < 0.01$). *: significantly different from the value of Ca²⁺ (-) + CCh (10⁻⁵ M) ($P < 0.05$). Ca²⁺_o removal had no effect on the basal K⁺ efflux from the muscle.

muscle was made by the one min-application of CCh (10⁻⁵ M) twice in a 40 min incubation period, in a Ca²⁺_o-free solution containing 5.4 mM KCl. Muscle was then transferred to a Ca²⁺_o- and K⁺-free solution with or without PTX. Ca²⁺_i depletion inhibited the PTX (3 $\times$ 10⁻⁹ M)-induced K⁺ efflux by 31% (Fig. 5).

Effect of TEA: Pretreatment with 15 mM TEA, a specific K⁺ channel blocker (16), inhibited the PTX- or CCh-induced K⁺ efflux by 65% or 75%, respectively (Fig. 6).

Effects of cardiac glycosides: The rate of K⁺ efflux induced by PTX was markedly decreased by cardiac glycosides, cymarin, ouabain and digitoxin. The threshold concentrations of cymarin, ouabain and digitoxin were about 3 $\times$ 10⁻⁸ M, 10⁻⁵ M and 10⁻⁵ M, respectively (Fig. 7). In contrast to this, the CCh-induced K⁺ efflux was not inhibited by cymarin at the concentration (10⁻⁶ M) that completely inhibited the PTX-induced K⁺ efflux.

Discussion

In the present experiment, K⁺ efflux was continuously measured by a K⁺-selective electrode. In this experiment, K⁺ concentration in the bath gradually increased reaching to 0.12–0.17 mM at the end of the measurements. However, the rate of K⁺ efflux did not change during the measurements, suggesting that such a small change in external K⁺ concentration does not modify the Na⁺, K⁺-ATPase activity. PTX increased K⁺ efflux from guinea-pig ileal longitudinal smooth muscle in a dose-dependent manner, and the EC₅₀ was 1.8 $\times$ 10⁻¹⁰ M. A similar value (5.0 $\times$ 10⁻¹⁰ M) was reported with rabbit erythrocytes. In rabbit erythrocytes, almost all the cellular K⁺ was lost in about 10–15 min (17). However, it took about 30–40 min in the ileal muscle to lose 80–90% of the K⁺. Because the ileal muscle tissue consists of smooth muscle cells, connective tissue, capillaries and nerves, diffusion of ions and PTX in this

Fig. 4. The effect of verapamil (10^{-5} M) on the PTX- or CCh-induced K⁺ efflux in the ileal muscle. Mean $\pm$ S.E. of 4–8 experiments is shown. **: significantly different from the value of PTX (3×10^{-9} M) or CCh (10^{-5} M) ($P < 0.01$). *, **: significantly different from the value of verapamil (10^{-5} M) + PTX (3×10^{-9} M) or CCh (10^{-5} M) ($P < 0.05$, $P < 0.01$). Verapamil (10^{-5} M) had no effect on the basal K⁺ efflux from the muscle.

Fig. 5. The effect of Ca²⁺_i depletion on K⁺ efflux due to PTX (3×10^{-9} M) in the ileal muscle. Ca²⁺_i depleted muscle was made by the applications of CCh (10^{-5} M) in Ca²⁺_o removal solution. Mean $\pm$ S.E. of 4–8 experiments is shown. *: significantly different from the value of PTX (3×10^{-9} M) ($P < 0.05$). *: significantly different from the value of Ca²⁺(-) + PTX (3×10^{-9} M) ($P < 0.05$).

Fig. 6. The effect of TEA (15 mM) on the PTX- or CCh-induced K⁺ efflux. Mean $\pm$ S.E. of 4–8 experiments is shown. **: significantly different from the value of PTX (3×10^{-9} M) or CCh (10^{-5} M) ($P < 0.01$). *: significantly different from the value of TEA (15 mM) + PTX (3×10^{-9} M).

Fig. 7. The effect of cardiac glycosides on K⁺ efflux due to PTX (3×10^{-9} M) in the ileal muscle. Ordinate: The relative rate of K⁺ efflux. The rate of K⁺ efflux due to PTX (3×10^{-9} M) was taken as to 100%. (●): cymarin, (○): ouabain (▲): digitoxin. Abscissa: Concentration ($-\log$ M) of cardiac glycosides. Mean $\pm$ S.E. of 4–8 experiments is shown.

tissue may be slower than in erythrocytes.

Atropine did not affect the PTX-induced K⁺ efflux in the ileal muscle, suggesting that the effect of PTX is not mediated by endogenous acetylcholine. However, since PTX has been shown to increase the metabolites of arachidonic acid (22, 23), the present results do not exclude the possibility that the effect of PTX is mediated by an endogenous substance.

The PTX-induced K⁺ efflux was inhibited partially by Ca²⁺_o removal in rabbit erythrocytes (24). However, the effect of PTX in the ileal muscle of guinea-pig was not affected by Ca²⁺_o removal and verapamil (10⁻⁵ M). Since high concentration of CCh releases Ca²⁺_i in guinea-pig taenia coli (25), we attempted to deplete Ca²⁺_i in the muscle preparation by two applications of CCh (10⁻⁶ M) in Ca²⁺_o-free solution. In this Ca²⁺_i-depleted muscle, the PTX-induced K⁺ efflux was partially inhibited, supporting the possibility that PTX releases Ca²⁺_i to increase the K⁺ efflux. As TEA, a specific K⁺ channel blocker, markedly inhibited the PTX-induced K⁺ efflux, the larger portion of the K⁺ efflux may be a function of the K⁺ channel. However, the PTX-induced K⁺ efflux in rabbit erythrocytes was not affected by TEA (10 mM) (17).

CCh induces contraction and increases in K⁺ efflux in the guinea-pig ileal longitudinal muscle (20). As the CCh-induced K⁺ efflux was markedly inhibited by atropine (10⁻⁶ M), Ca²⁺_o removal and verapamil (10⁻⁵ M), the CCh-induced K⁺ efflux seems to be due to receptor-linked Ca²⁺ influx. Furthermore, the CCh-induced K⁺ efflux was inhibited by a K⁺ channel blocker, TEA (15 mM). It has been reported that acetylcholine opens the Ca²⁺_i-activated K⁺ channel in rabbit jejunum (26), and that there were at least two K⁺ currents (Ca²⁺_o-dependent K⁺ current and delayed K⁺ current) in rabbit intestinal smooth muscle cells (27). From these and our results, the K⁺ efflux due to CCh in guinea-pig ileum may be produced by Ca²⁺_o-activated K⁺ channels.

The K⁺ efflux due to PTX was markedly decreased by ouabain in rabbit aorta (19) and erythrocytes (15). Other monoglycosides such as cymarins or convallatoxin showed 10 to 15 times more potent inhibitory effects

than ouabain, and triglycosides such as digoxin and digitoxin were about 40 times less potent than ouabain in rabbit erythrocytes (16). In the ileal muscle, the PTX-induced K⁺ efflux was also inhibited by cardiac glycosides (cymarins, ouabain and digitoxin). The order of the inhibitory effects was cymarin > ouabain = digitoxin in the ileal muscle. The reason why digitoxin showed similar potency as ouabain in the ileal muscle was not clarified. These results support the previous suggestion that the binding site of PTX is the Na⁺,K⁺-ATPase (17).

In summary, the K⁺ efflux due to PTX was Ca²⁺_o-insensitive, TEA-sensitive and partly dependent on Ca²⁺_i, whereas the K⁺ efflux due to CCh was Ca²⁺_o and TEA-sensitive in guinea-pig ileal longitudinal smooth muscle. Cardiac glycosides inhibited the PTX-induced K⁺ efflux, supporting the previous suggestion that the binding site for PTX is Na⁺,K⁺-ATPase.

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