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Research Article

Effects of Palytoxin on Guinea Pig Tracheal Strips

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The effects of palytoxin (PTX) on airway smooth muscle were investigated in opened rings of guinea pig trachea. Concentrations of PTX from 10 p*M* to 100 n*M* caused contractions of tracheal strips, with maximal contractions approximately 80% of those in response to 120 m*M* potassium. Tension increased to its maximum in approximately 5 min with 100 n*M* PTX, then decreased to or near resting tension over the next 60 min. Contractions were larger when the epithelium was removed. Exposure to high (100 n*M*) concentrations of PTX markedly reduced subsequent contractions to PTX but had less effect on potassium-induced contractions. In zero-calcium solution the rate of contraction was slowed but the maximal contraction was not reduced. The addition of a calcium channel blocker (verapamil) markedly reduced the contractions and a calcium chelator (EGTA) abolished them. Contractions to PTX in zero-calcium media suggested that tracheal cartilage was serving as a calcium source, as it has been previously reported to do. Potassium removal and sodium reduction also greatly reduced contractions. These data are consistent with other observations suggesting that PTX may form pores through which sodium leaks into the cell. PTX was also found to differ from ouabain in its mode of action.

KEY WORDS: Palytoxin; trachea; potassium depletion; smooth muscle contractions; guinea pigs.

INTRODUCTION

Palytoxin (PTX) ($C_{129}H_{223}N_3O_{54}$) is extracted from the zoanthid coelenterate *Palythoa tuberculosa* and other *Palythoa* spp. It is the most potent marine toxin known, with an i.v. LD_{50} in several experimental animals of 0.025–0.45 μg/kg (1,2). It causes depolarization of frog nerve fibers by increasing sodium conductance (3,4). In the erythrocyte, a nonexcitable cell, PTX induces K^+ loss from cells (5). Because ouabain alters the effects of PTX, the toxin has been proposed to transform (Na^+/K^+) ATPase into a pore, allowing passage of small ions (6). Although generally thought to inhibit (Na^+/K^+) ATPase (7,8), some researchers report that in neurons, PTX does not affect (Na^+/K^+) ATPase (9).

Palytoxin's effects have been extensively investigated in blood vessels (10–16) and a variety of other smooth muscle preparations. Nagase *et al.* (16) showed that 1 p*M* concentrations of PTX augment norepinephrine-induced contractions in isolated rat aorta. This was thought to be due to modification of synthesis or release of an endothelium-derived relaxing factor by PTX. Nagase and Karaki (17) reported that higher concentrations of PTX initiated the contractions of the rabbit aorta by releasing prostaglandins from endothelial and smooth muscle cells, releasing norepinephrine from neural stores, and depolarizing membranes.

The effects of PTX on strips of isolated airways tissue have not been reported. In the present study, we investigated the effects of PTX on a smooth-muscle-cartilage preparation from isolated guinea pig trachea, which has been reported to be a suitable model of human central and large airways (18) and thus a good indicator of effects on potential airway resistance.

MATERIALS AND METHODS

Animals and Tissue Preparation. Male barrier-raised, Hartley albino guinea pigs weighing 298 to 479 g (Charles River Labs, Wilmington, MA), were housed in a biosafety enclosure (Airoclean Engineering, Inc., Edgemont, PA). Following a blow on the head and exsanguination (19), the trachea from the larynx to the carina was removed, placed in cold aerated (95% O_2, 5% CO_2) Tyrode's solution, cleaned of fat and connective tissue, and cut longitudinally opposite the trachealis muscle. The trachea was then cut perpendicularly to the longitudinal axis into strips containing three rings of cartilage each. The epithelium was removed from some of the strips by rubbing with a moistened cotton swab. Strips which were not rubbed had an intact epithelium, while rubbed strips had approximately 90% of the epithelium removed as verified by sectioning, staining, and microscopic examination. Other strips were handled carefully to prevent damage to the epithelium. In this report unless otherwise stated, strips have epithelium present. Near each end of the strips, a silk mattress suture was placed through the tissue inside the two outermost pieces of cartilage. The lower end was left as a loop which was fixed to a hook in the bottom of the tissue bath. The loop at the upper end was connected to

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an isometric transducer (F-60, Narco Biosystems) above the bath. Tension was recorded by Physiograph MK-IV recorders. Tissues were suspended in aerated Tyrode's solution at a pH of 7.4 and maintained at 38°C.

Tension on the strips was increased in increments of 100 mg every 3 min to a total of 1 g, determined to be the optimal tension for maximal contractions. Incremental increases in tension eliminated many of the spontaneous tension changes which can exceed the potassium depolarization-induced contractions when the full tension is added at once (20). We also observed large spontaneous contractions following nonincremental tension addition in our preliminary studies. Strips were equilibrated for 1 hr with frequent changes of Tyrode's solution before exposure to a depolarizing concentration of potassium. After washing out the high-potassium solution, another hour of equilibration was allowed before addition of the next drug. All contractions were calculated as a percentage of the contraction to 120 m*M* K^+ added at the beginning of the experiment.

Addition of PTX. Strips were separately exposed to various concentrations (1 to 100 n*M*) of PTX, and tension changes recorded for 1 hr. Strips were also exposed to increasing concentrations of PTX, from 1 p*M* to 100 n*M*, in a cumulative fashion. Each concentration was left in contact with the strip until the tension change was maximal. A test drug (a muscarinic receptor antagonist, 1 μ*M* atropine; a histamine H_1 receptor antagonist, 1 μ*M* mepyramine; a calcium channel inhibitor, 1 μ*M* verapamil; a cyclooxygenase inhibitor, 10 μ*M* indomethacin; a lipoxygenase inhibitor, 10 μ*M* nordihydroguaiaretic acid; a sodium conductance blocker, 1 μ*M* tetrodotoxin; a Na^+/K^+ ATPase inhibitor, 10 μ*M* ouabain; or a combination of 10 μ*M* indomethacin plus 10 μ*M* nordihydroguaiaretic acid) was added to some baths 30 min before exposure to PTX. The concentrations employed have been demonstrated to antagonize/inhibit the respective receptors/enzymes in separate studies.

Inactivation of Responsiveness to Contractile Agents. Strips were exposed to 10 n*M* PTX for 30 to 60 min after a 1-hr equilibration. Two or eighteen hours later either 1 or 10 n*M* PTX, 100 μ*M* acetylcholine (ACh), or 10 μ*M* histamine was applied, the response was noted, and the drug and toxin were washed out. Ten minutes later 120 m*M* K^+ was added as a control and the contraction observed. When ACh and histamine were used as the test drugs, an initial response to the drug was obtained 10 min before the addition of PTX. In other experiments, 10 n*M* PTX was added to the tracheal strip followed 90 min later by 10 μ*M* ouabain without removing the PTX. Control strips were maintained that received no PTX, but received other drugs at the same time they were added to PTX-exposed strips, in both these and all other experiments.

PTX-Induced Contractions in Potassium-Free Tyrode's. Two types of studies were carried out in potassium-free Tyrode's. (i) After equilibration, Tyrode's solution was replaced by zero potassium (0-K^+) Tyrode's solution and 30 min later either 10 n*M* PTX, 10 μ*M* ouabain, 10 μ*M* histamine, or 100 μ*M* ACh was added to the bath and the tension changes were observed. (ii) After 30 min of exposure to 10 n*M* PTX, Tyrode's solution was replaced by PTX-containing 0-K^+ solution, and tension changes were observed. Similarly, after 20 min of exposure to ouabain, the effects of 0-K^+ solution were determined in the continued presence of ouabain.

The Interaction of PTX and Ouabain. The interactions were studied in three kinds of experiments: (i) 10 n*M* PTX was applied, followed 30 min later by 10 or 20 μ*M* ouabain without washing out the toxin; (ii) ouabain was given first, followed by PTX; and (iii) PTX and ouabain were given together.

PTX-Induced Contractions in Calcium-Free Media. Tyrode's solution was replaced with calcium-free (0-Ca^{2+}) Tyrode's solution (with two rinses) and 30 min later 10 n*M* PTX was added to the bath and tension changes were observed. In other experiments Tyrode's solution was replaced by 0-Ca^{2+} Tyrode's solution containing 0.5 m*M* EGTA and strips were equilibrated for 60 min before PTX was added. Control strips were incubated in Tyrode's solution.

PTX Addition in Reduced-Sodium Media. The sodium concentration of the Tyrode's solution was reduced from 149 to 12.1 m*M* and the osmolarity maintained by the addition of Tris or sucrose. Forty-five minutes later, 10 n*M* PTX was added to the bath and tension changes were observed. Control strips were maintained in Tyrode's.

Solutions. Composition of the Tyrode's solution was 124.8 m*M* NaCl, 2.7 m*M* KCl, 2 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$, 0.4 m*M* NaH_2PO_4, 23.8 m*M* $NaHCO_3$, and 11.1 m*M* glucose. Zero-calcium Tyrode's solution was made by omitting $CaCl_2$; zero-calcium Tyrode's solution with 0.5 m*M* EGTA [ethylene glycol bis (β-aminoethyl ether)-*N,N'*-tetraacetic acid] was also prepared. The 120 m*M* KCl-containing Tyrode's solution was made by decreasing the NaCl content by an amount equimolar to the KCl added. The reduced sodium PSS (12.1 m*M* Na^+) contained half of the $NaHCO_3$ and NaH_2PO_4 of the regular Tyrode's solution, and the deleted sodium (136.9 m*M*) was replaced with an equiosmolar amount of either sucrose or tris(hydroxymethyl)-aminomethane · H_2O · HCl (Tris). The pH of all solutions was adjusted to 7.4.

Indomethacin and nordihydroguaiaretic acid were dissolved in ethanol. The presence of <0.1% ethanol in the bath did not alter tissue responses, but vehicle was used in control baths. Palytoxin, from *Palythoa tuberculosa* (obtained from the Hawaii Biotechnology Group, Inc., Aiea, Hawaii), was 95% pure by UV and NMR spectrometry. It was dissolved in water and frozen until used. Other drugs were frozen as stock solutions and diluted fresh before use.

Concentration–response curves were compared using the SAS (Statistical Analysis Systems) procedure GLM (General Linear Model, SAS Institute Inc., Box 8000, Cary, NC 27511) with the repeated option for multivariate repeated-measures data. The program analyzes the overall change in profile as well as differences between profiles of treatments over the variable changing between measurements, i.e., time and drug concentration.

RESULTS

Guinea pig tracheal strips contracted significantly ($P < 0.05$) when exposed to PTX at concentrations as low as 10 n*M*. In preliminary experiments when tension was added nonincrementally, responses to PTX were large when spontaneous contractions had been large. When PTX was added

in a cumulative fashion, a dose-dependent contraction was observed (Fig. 1, left). Although an increased contraction is shown for the highest PTX concentration, it was rapidly followed by a relaxation to, or almost to, baseline. Tracheal strips with epithelium present contracted significantly ($P < 0.05$) less to 10 n*M* PTX.

Of the drugs tested (atropine, mepyramine, verapamil, indomethacin, nordihydroguaiaretic acid, tetrodotoxin, ouabain, or a combination of indomethacin plus nordihydroguaiaretic acid), only verapamil consistently altered PTX-induced tracheal contractions. In the presence of verapamil, the addition of 100 n*M* PTX resulted in a reduction in baseline tension (Fig. 1, right), while contraction resulted from 100 n*M* PTX addition in the absence of verapamil. Total tension generated to PTX addition was less ($P < 0.05$) in the presence of verapamil. In other studies (not shown) verapamil, added after PTX-induced contractions, caused relaxations which ranged from 30 to 100% (mean, 86%) of the original contraction.

Repeated or prolonged (≥120-min) exposures to 10 n*M* PTX sometimes, and ≥30-min single exposure to 100 n*M* PTX always, reduced subsequent contractions to PTX (not shown). Figure 2A shows the contractions to 100 μ*M* ACh, 10 μ*M* histamine, 120 m*M* KCl, and 10 n*M* PTX. Two hours later responses to the agonists were reduced and markedly more slowly developing (Fig. 2B). Eighteen hours after the initial PTX exposure, responses were further reduced and even more slowly developing, and the response to 10 n*M* PTX was absent or small and slowly developing (Fig. 2C). Contractions to ACh, histamine, and potassium at this time were within 20% of the initial contractions on the first day.

Following exposure of strips to 100 n*M* PTX for 1 hr, responses to agonists were smaller and often absent. There were no contractions to histamine at 2 or 18 hr after 100 n*M* PTX exposure ($n = 5$), none to 100 n*M* PTX readdition ($n = 5$), and greatly reduced, slower responses to ACh and 120 m*M* potassium (data not shown). After exposure of strips to 100 n*M* PTX for 100 min, 10 μ*M* ouabain elicited almost no contraction (Fig. 2D).

The effects of 10 p*M*, 100 p*M*, 10 n*M*, or 100 n*M* PTX on the contractile tension of trachealis were observed for 1 hr (Fig. 3). With 10 and 100 p*M* PTX, there were no significant effects over time between the two dose levels of palytoxin and no significant change in tension over time. With the two higher PTX concentrations, there was evidence for significant effects. The individual effects of each of these independent variables were not significantly affected by the other. Addition of 100 n*M* PTX always caused an initial contraction followed by a relaxation beginning approximately 5 min after onset of the contraction. The mean maximal tension observed was approximately 80% of that produced by the 120 m*M* potassium control.

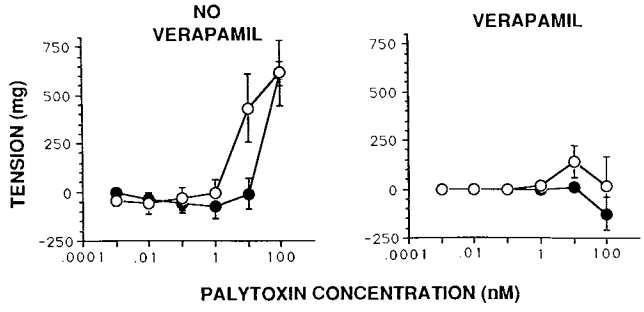

Fig. 1. Effect of 1 μ*M* verapamil on contractile tension in guinea pig trachea induced by cumulatively increasing concentrations of palytoxin (PTX). Epithelium present (●); epithelium removed (○). Each point is the mean ± SE for 7–15 observations.

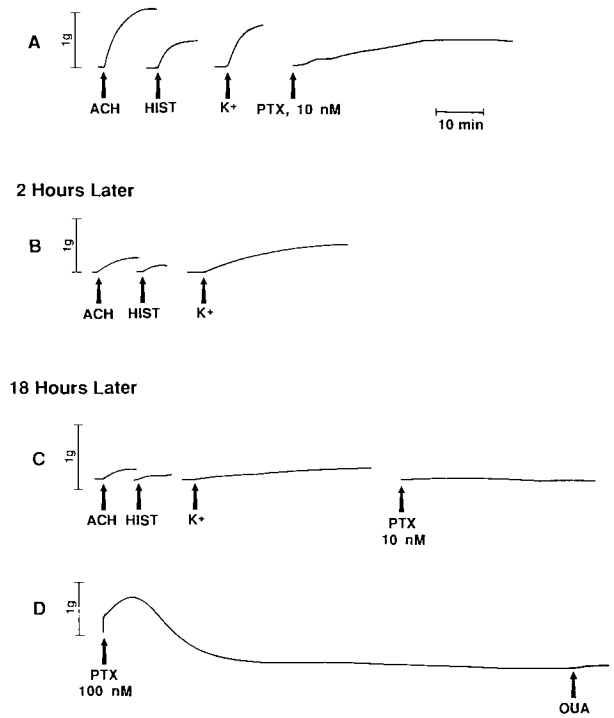

Fig. 2. Representative recordings showing the inactivation of responsiveness of guinea pig tracheal strips with epithelium following exposure to a high concentration (100 n*M*) of palytoxin (PTX). (A) Initial contraction to 100 μ*M* acetylcholine (ACh), 10 μ*M* histamine (Hist), 120 m*M* potassium (K^+), and 10 n*M* palytoxin (PTX). (B) Contraction to ACh, Hist, and K^+ 2 hr later. (C) Contraction to all four agonists 18 hr later. (D) Contraction to 100 n*M* PTX and to ouabain (OUA) 100 min later.

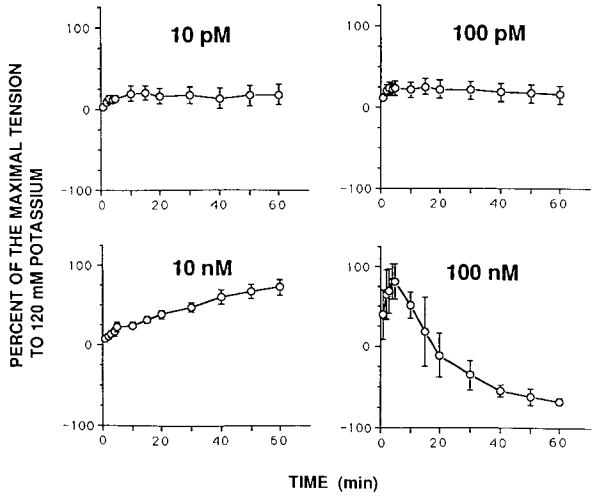

Fig. 3. Tension changes with time induced by a single concentration of PTX added to separate guinea pig tracheal strips with epithelium. Each point is the mean ± SE for 3–11 observations.

Potassium depletion (0-K^+) caused an initial increase in the contractile tension of tracheal strips, followed by a slow, steady rate of tension decline (Fig. 4). Palytoxin (10 n*M*) caused no contraction in 0-K^+ Tyrode's solution (Fig. 4C). On the other hand, ouabain caused contractions in each of five strips, with the contraction preceded by a relaxation in two of the five strips (strips shown did not relax first) (Fig. 4A). Histamine caused no contraction (n = 6) (Fig. 4D), while ACh caused a small contraction in each of five strips (Fig. 4B).

Replacement with 0-K^+ Tyrode's, still containing either PTX or ouabain, caused relaxation of trachealis muscle strips previously contracted with 10 n*M* PTX (Fig. 5A) or 10 μ*M* ouabain (Fig. 5B) in regular Tyrode's. The results of studies of giving PTX before, after, or simultaneously with ouabain were inconclusive, in that no consistent interactions were observed (not shown).

After 30-min exposure to 0-Ca^{2+} Tyrode's solution, tension developed in response to 10 n*M* PTX (Fig. 6B) more slowly than in the control strip (Fig. 6A) (n = 5 each), but the maximal tension developed was approximately the same. On the other hand, 1-hr incubation of the tracheal strip in 0-Ca^{2+} Tyrode's containing 0.5 m*M* EGTA totally eliminated the contractile response to PTX (Fig. 6C).

Reduction of sodium concentration to 12 m*M* by replacement with either Tris (Fig. 7B) or sucrose (Fig. 7C) almost abolished the contraction caused by PTX.

DISCUSSION

In preliminary experiments with trachealis muscle, before using stepped tension application to reduce spontaneous contractions, PTX contractions were usually larger when the spontaneous activity was great. Similarly, guinea pig taenia coli, when isometric tension was applied, responded to PTX in proportion to the magnitude of a spontaneous minute rhythm, if present (10).

Larger contractions to 10 n*M* PTX in tracheal strips with epithelium removed may have resulted in the release of an airway epithelial-derived relaxing factor following application of PTX, since such factors have been reported in guinea pig tracheal epithelium (20–24). Involvement of a relaxing factor in the action of PTX has also been demonstrated in the rabbit (17) and rat aorta (16). Alternatively, this could also have resulted from increased release of a contractile factor from nonepithelial tissue which is under regulatory control by the epithelium, by increased access of the PTX, or by decreased PTX metabolism.

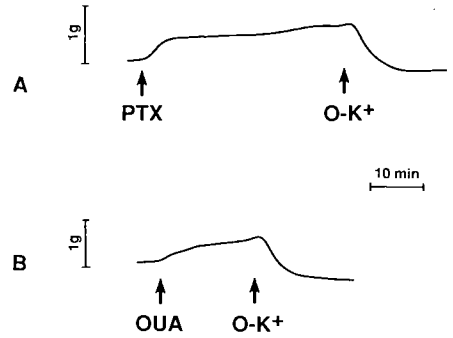

Fig. 5. Representative recordings showing the effects of removing potassium (0-K^+) on contractile tension of guinea pig tracheal strips with epithelium already contracted with (A) 10 n*M* palytoxin (PTX) or (B) 10 μ*M* ouabain (OUA).

In the guinea pig taenia coli, Ozaki *et al.* (25) found that neither tetrodotoxin, indomethacin, tripelennamine (an H_1 receptor antagonist), nor atropine altered PTX-induced contraction. Other investigators have found that tetrodotoxin does not reduce the PTX-induced increase in sodium permeability (3,4,10,11,26–28). None of the antagonists listed above which were used by Ozaki *et al.* (25) consistently altered the responses to PTX in the present experiments. In the rat and rabbit aorta, however, indomethacin partially antagonizes PTX contractions, indicating that PTX releases a cyclooxygenase product that contracts arteries. Since both indomethacin and nordihydroguaiaretic acid inhibit PTX-induced contractions of the rabbit urinary bladder (29), the effects of PTX in bladder include formation and release of a

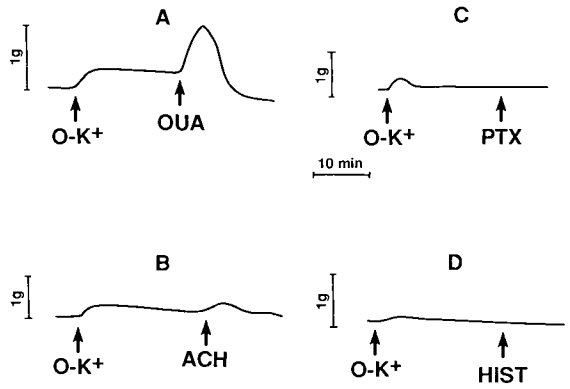

Fig. 4. Representative recordings showing the effects of potassium deletion (0-K^+) on subsequent contractions of guinea pig tracheal strips with epithelium to (A) 10 μ*M* ouabain (OUA), (B) 100 μ*M* acetylcholine (ACh), (C) 10 n*M* palytoxin (PTX), and (D) 10 μ*M* histamine (HIST).

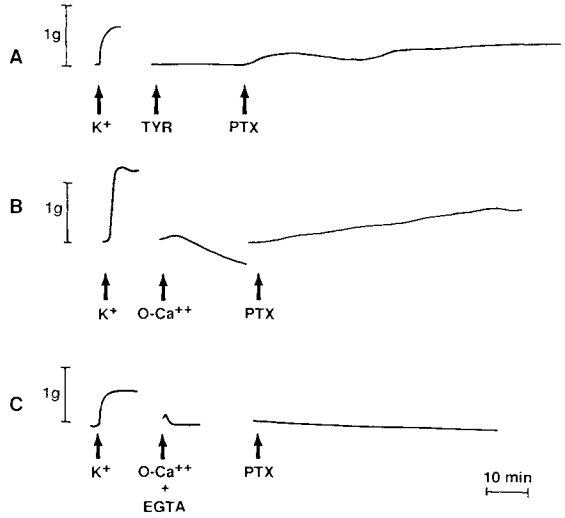

Fig. 6. Representative recordings showing the effects of calcium depletion (0-Ca^{2+}) on contractile tension of guinea pig tracheal strips to 10 n*M* palytoxin (PTX): (A) in Tyrode's containing Ca^{2+}; (B) after 30 min in 0-Ca^{2+} Tyrode's; (C) after 1 hr in 0-Ca^{2+} Tyrode's with 0.5 m*M* EGTA.

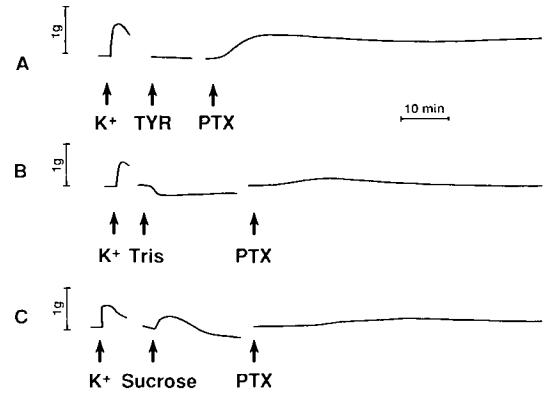

Fig. 7. Representative recordings showing the effect of reduced sodium concentration (12 m*M*) on PTX (10 n*M*) contractions of guinea pig tracheal strips. (A) Tyrode's (TYR); (B) tris substitution; (C) sucrose substitution.

contracting factor. The data reported here, demonstrating a lack of effect by these compounds, do not support a role for an arachidonic acid metabolite in trachealis muscle contraction to PTX.

In the rat and rabbit aorta (17), guinea pig taenai coli (25), and rabbit urinary bladder (29) as in the trachealis muscle reported here, verapamil or nifedipine antagonized PTX-induced contractions. This suggests that these contractions involve the influx of extracellular calcium into the smooth muscle cells.

Preexposure of rats or mice to PTX can afford protection against subsequently administered PTX (30). However, adrenalectomized animals do not develop any degree of protection, while adrenalectomized animals pretreated with hydrocortisone are protected. This protection seems to be steroid dependent and not due to the inactivation observed in our studies of the trachealis responses. Dubois and Cohen (3) found the effects of PTX on frog myelinated nerve fibers to be irreversible with respect to both sodium permeability and effects on the action potential. They concluded that the inactivation of responsiveness to PTX or histamine following exposure to a high concentration of PTX results from an irreversible increase in sodium permeability leading to a marked intracellular sodium accumulation and a decreased responsiveness to extracellular calcium. In the present studies, following an inactivating exposure to 100 n*M* PTX, the trachealis muscle still contracted slightly to ACh but not to histamine. These results were expected as ACh contraction of the trachealis muscle involves primarily intracellular calcium (31,32), while contraction to histamine relies more heavily on extracellular calcium (32,33).

Removal of extracellular potassium reduces the activity of Na^+/K^+ ATPase. In the present experiments, as in umbilical artery, removal of K^+ caused a small contraction (12). However in the trachealis, the response to PTX was almost abolished, while that to ouabain was little affected; almost the opposite occurred in the human umbilical artery. When potassium was deleted from a strip contracted by either PTX or ouabain, the trachealis rapidly relaxed in our experiments. Similarly, after contraction by ouabain and subsequent partial relaxation of the human umbilical artery, potassium caused a relaxation (12). These data indicate that PTX and ouabain differ in their modes of action.

In experiments with calcium deletion without the addition of a calcium chelator, the contractile responses of human umbilical artery (12) and guinea pig vas deferens (34) to PTX are almost abolished. This is in marked contrast to our findings in guinea pig tracheal strips. In 0-Ca^{2+} Tyrode's, PTX contractions were not reduced but typically achieved maximal height more gradually. Raeburn *et al.* (35,36) reported that in trachea with cartilage present, the cartilage can serve as a calcium source. This may explain the present findings that the addition of a calcium chelator abolished contractions.

Contractile tension developed to PTX is markedly reduced in physiological solutions in which most of the sodium is replaced by sucrose, tris, or lithium. This was observed by Ozaki *et al.* (25) in the guinea pig taenia coli with sucrose replacement. It was also observed by Ozaki *et al.* (13) in the guinea pig aorta, where replacement of sodium with either lithium or tris decreased contractile responses to PTX to approximately 30% of control. In the present experiments sodium depletion abolished the contractile response of the trachealis to PTX.

CONCLUSION

Palytoxin was a potent constrictor of trachealis muscle. The contraction was not dependent on arachidonic acid metabolites. PTX may, however, have caused release of an epithelial-derived relaxing factor. PTX-induced contractions of trachealis muscle were less dependent on extracellular calcium than are PTX-induced contractions of noncartilaginous tissues reported previously. High concentrations of PTX irreversibly inactivated responses to reapplied PTX, as judged by lack of recovery 18 hr later. The toxin's effects were consistent with the postulated mechanism of forming a pore for the influx of sodium into smooth muscle cells. PTX was also found to differ from ouabain in its mode of action.

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