Br. J. Pharmacol. (1992), **106**, 307–314

The action of palytoxin on the isolated detrusor muscle of the rat

J. Posangi, *M.A. Zar & [1]J.B. Harris

School of Neurosciences and *Department of Pharmacological Sciences, University of Newcastle, Newcastle upon Tyne NE1 7RU

1 The effects of a coelenterate toxin, palytoxin (PTX) have been studied in the isolated detrusor muscle of the rat.

2 PTX (1–100 nM) initiated concentration-dependent contractions of the detrusor; the contraction led to an irreversible tachyphylaxis. Muscle desensitized to PTX continued to respond to acetylcholine (ACh) and excess K^+ but the contractions were reduced compared to pre-PTX contractions.

3 Contractions evoked by PTX were not affected by the presence of atropine (10 μM), indomethacin (10 μM) or tetrodotoxin (0.5 μM) but were greatly reduced by nifedipine (3 μM) and by the absence of K^+. PTX could not evoke contractions in the absence of Ca^{2+} or in tissues depolarized by exposure to excess K^+.

4 PTX abolished the neurogenic contractile responses to electrical field stimulation (EFS).

5 Combined treatment with atropine (10 μM) plus nifedipine (3 μM) abolished contractile responses to EFS and greatly reduced the contractile response to PTX.

6 The contractile response to PTX (100 nM) was reduced following exposure of the muscle to α,β-methylene ATP.

7 Exposure to PTX (100 nM) for 1–3 h reduced both the ACh content of the detrusor (by more than 80%), and the immunoreactivity of neuropeptide Y-containing nerve fibres compared to control.

8 It is concluded that the primary effect of PTX is to promote the release of endogenous motor transmitters, leading to their eventual depletion.

Keywords: Palytoxin; bladder; detrusor muscle

Introduction

Extracts of corals of the genus *Palythoa* have long been known to be toxic, and inhabitants of the Indo-Pacific have, in the past, used them as arrow- and spear-tip poisons. Palytoxin (PTX) appears to be the major active ingredient of these extracts (Moore & Scheuer, 1971; Beress *et al.*, 1983). It is a complex non-peptide with a calculated mol. wt. of approximately 3,300 and it has an LD_{50} in most species of 10–1000 ng kg^{-1}. Death is usually said to be caused by vasoconstriction of peripheral origin (Habermann, 1989).

Excitable cells exposed to the toxin are depolarized. In most cells the depolarization is not specifically Na^+-dependent, but seems instead to reflect a rather general increase in permeability to cations (Tesseraux *et al.*, 1983; Castle & Strichartz, 1988; Ikeda *et al.*, 1988; Muramatsu *et al.*, 1988; Rouzaire-Dubois & Dubois, 1990). In smooth muscle, PTX is spasmogenic. The origin of the activity is unclear and may vary between tissues; for example Shibata *et al.* (1986) have suggested that the contraction of the rabbit urinary bladder is mainly caused by the toxin-mediated release of metabolites of arachidonic acid; Ishida *et al.* (1985a) suggested that in the guinea-pig vas deferens, contractions are caused by the combination of a direct action of the toxin on the smooth muscle and the toxin-mediated release of noradrenaline; Ozaki *et al.* (1983) suggested that the PTX-induced contraction of the guinea-pig aorta is due to a Na^+-dependent depolarization of the smooth muscle, but Nagase & Karaki (1987) suggest that the contraction of the aorta of the rat and rabbit is caused by a combination of the release of prostaglandins from endothelium and smooth muscle, noradrenaline from nerve terminals and a direct depolarization of the smooth muscle.

Because PTX is known to have entered the food chain and given rise to human poisoning (Alcala *et al.*, 1988), it is important that its pharmacological activity is clarified. This paper describes our observations on the rat detrusor muscle.

Methods

Animals

All experiments were performed on tissues removed from adult male Wistar rats weighing 200–350 g and male guinea-pigs weighing 400–500 g. The animals were killed by concussion and decapitation.

Experimental procedures

Preparation for organ bath studies The lower abdomen was opened. The urinary bladder was held at its apex and the coat and connective tissue were cut away. The bladder was then removed and washed in Krebs-Henseleit solution (see below). Two lateral incisions were made in the bladder wall, and the tissue was opened into a rectangular sheet. Strips 1–1.5 × 0.2 cm were prepared with a pair of fine scissors. Single strips were suspended between parallel platinum electrodes in a 2 ml jacketed organ bath containing Krebs-Henseleit solution (composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 11) aerated with 95% O_2 + 5% CO_2 at 37°C, attached to an isometric transducer and a potentiometric recorder, at a resting tension of 0.5 g and allowed to equilibrate for 30 min before the start of further experimentation; during this period the preparation was repeatedly washed with fresh Krebs-Henseleit solution. The preparation of the strips has been described in detail by Luheshi & Zar (1990).

At the end of equilibration period, each strip was exposed to acetylcholine (ACh) 1 mM. The period of exposure lasted until the resulting contractile response had peaked (usually less than 1 min). The ACh-evoked tension was used as the

[1] Author for correspondence at: Muscular Dystrophy Group Research Laboratories, Newcastle General Hospital, Westgate Road, Newcastle upon Tyne NE4 6BE.

standard against which the contractile response evoked by exposure to PTX was assessed.

Electrical field stimulation Electrical field stimulation (EFS) was delivered through a Grass S88 electronic stimulator using trains of 5 pulses of 0.4 ms pulse duration at a frequency of 10 Hz, and at supramaximal voltage (50–70 V) repeated once every min.

Exposure to antagonists (atropine, indomethacin and nifedipine) was for at least 30 min, before their effect on the tissue response to ACh, PTX or EFS was determined. The only exception to this was exposure to tetrodotoxin (TTX) which lasted for 10 min before its effects were ascertained.

Immunohistochemistry The immunochemical technique used for the detection of neuropeptide Y (NPY) was similar to that described in detail by Iravani (1989). Tissues were fixed by immersion in 0.4% w/v *p*-benzoquinone in 0.01 M phosphate buffered saline (PBS) pH 7.4 for 2 h at 4°C and were then washed for at least 24 h with several changes of 0.01 M PBS containing 10% w/v sucrose and 0.01% w/v sodium azide (NaN_3). The tissues were then frozen in isopentane cooled with liquid nitrogen and frozen sections, 10 μm thick, were cut. The sections were placed on glass slides coated with a fine film of chrome-gelatin, to prevent detachment during further processing and were allowed 60 min to dry in air. The first layer antibody was rabbit anti-NPY IgG, diluted 1:200 with 0.01 M PBS containing 0.1% w/v bovine serum albumin (BSA) and 0.01% w/v NaN_3. This was applied to the sections for 16 h at room temperature in a covered Petri-dish, containing a moist filter paper in order to maintain humidity and thus prevent drying. Before the second layer antibody was applied, the preparations were washed in three changes of 0.01 M PBS at 10 min intervals. The second layer antibody, goat anti-rabbit IgG, conjugated with fluorescein isothiocyanate, was applied at a dilution of 1:50 in 0.01 M PBS containing 0.1% w/v BSA and 0.01% w/v NaN_3 for 2 h at room temperature. The preparations were washed in three changes of 0.01 M PBS every 10 min and mounted in DPX. The sections were then examined with a Nikon abophot microscope, equipped with an Incident Light Source (100 W) and V-2a filter block. Photographs were taken with a Nikon HFX-II camera, using Ilford PH5 film rated at 320 ASA and developed in Ilford Microphen for 8.5 min at 20°C.

Extraction of acetylcholine The method used for extraction of ACh was based on that of Paton & Zar (1968). After excision (see above), the rat urinary bladder was opened to form a rectangle and divided into two equal symmetrical pieces. On half was reserved for treatment with PTX and the other served as the control. Each half was cut into four strips to facilitate both the access of PTX to the deeper layers of the tissue and its subsequent removal from the tissue. The strips from each bladder-half were placed in a 2 ml bath containing Krebs-Henseleit solution aerated with 95% O_2 + 5% CO_2 at 37°C and washed repeatedly for 30 min. PTX was then added to one bath at a final concentration 100 nM. After 90 min incubation, both sets of tissue slices were washed for 60 min with changes of Krebs-Henseleit solution at 37°C every 10 min. The tissues from each bath were then carefully blotted to remove excess moisture, weighed, chopped into small pieces and placed in 2 ml of 10% trichloroacetic acid (TCA) at room temperature to extract ACh. The tissues were gently agitated for 90 min in TCA, and filtered through filter papers moistened with distilled water. The filtrates were shaken with 6 volumes of water-saturated ether in a separating funnel four times to remove the TCA. The ether was removed by bubbling the extracts with air at 60°C for 20 min and the residual acidity was neutralized to pH 7.0 with sodium bicarbonate. The final volume of the extract was determined.

Bioassay of acetylcholine The bioassay for ACh was carried out on a guinea-pig ileum preparation by comparison of the responses to various volumes of extract with those to known amounts of ACh. A 3.5–4 cm segment of ileum was removed from a humanely killed guinea-pig, flushed clean with Krebs-Henseleit solution, and suspended in a 5 ml organ bath. The bathing fluid contained morphine sulphate 13 μM and physostigmine sulphate 3 nM and was aerated with 95% O_2 plus 5% CO_2 at 30°C. The preparation was attached to an isometric transducer and a potentiometric recorder at a resting tension of 0.5 g and was allowed to equilibrate for 1–2 h before the assay was started. During this period the preparation was repeatedly washed with fresh Krebs-Henseleit solution containing morphine sulphate and physostigmine. The specificity of the assay for ACh was confirmed by demonstrating that atropine (10 μM) would completely block the response of the ileum to the extract, and that heating the extract to 100°C in NaOH (50 mM) for 2 min would also inactivate it.

Drugs

The drugs used and their sources were: acetylcholine chloride, atropine sulphate, indomethacin, α,β-methylene ATP (all from Sigma), morphine sulphate, EDTA, DPX mountant (BDH), nifedipine (Bayer), palytoxin (Prof L. Beress, University of Kiel, Germany), tetrodotoxin (Sankyo), physostigmine sulphate (Burroughs Wellcome & Co), rabbit anti-porcine neuropeptide serum and goat anti-rabbit IgG, (FITC) (Peninsula Laboratories Inc.). Solutions of drugs were usually made fresh on the day of their use. Stock solutions of tetrodotoxin and PTX were stored at − 20°C. All drugs except indomethacin and nifedipine were dissolved in distilled water. Nifedipine and indomethacin were dissolved in absolute ethanol to make stock solutions of 1 mM and 10 mM respectively. Because nifedipine is highly unstable if exposed to daylight or to ordinary laboratory light, all experiments involving the use of nifedipine were conducted in a laboratory illuminated solely with sodium light. Solutions of PTX were prepared with due regard to its toxicity; it was assumed that its molecular weight was precisely 3,300.

Statistics

All values are expressed as mean ± s.e.mean. Differences between means were compared by Student's *t* test. If the probability that a difference arose by chance was lower than 5% the difference was considered statistically significant. *n* refers to the number of strips of detrusor used, and only one strip from a bladder was used. Thus number of strips = number of bladders.

Results

Effect of palytoxin on muscle tone

At concentrations below 1 nM, PTX had no apparent effect on the tone of the rat isolated detrusor preparation, but concentrations greater than 1 nM evoked contractions. The contractile response to PTX was concentration-dependent and was maximal at 100 nM (Figure 1). A representative contraction to a single maximally effective dose of PTX (100 nM) is shown in Figure 2. The response had a latency of about 10 s and reached its peak in about 2 min. Once the peak was reached, the contraction was not maintained and despite the continued presence of the toxin in the bath, the preparation relaxed virtually to its original base-line in about 15 min. Persistent tachyphylaxis was a striking feature of the contractile action of PTX, and this is also illustrated in Figure 2. Following the decline of the initial contraction, the preparation was washed repeatedly with fresh Krebs-Henseleit solution; a second exposure to PTX, 100 nM failed to

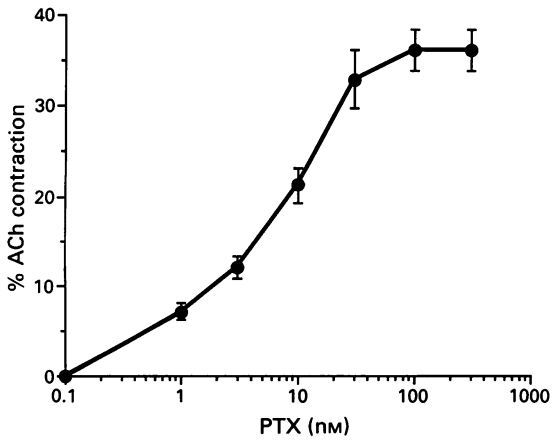

Figure 1 Dose-response curve showing the contractile action of palytoxin (PTX) on the rat isolated detrusor. The PTX-contractions are calculated as a percentage of contraction evoked by acetylcholine (ACh) 1 mM and each point represents mean of experiments on 5 strips of detrusor from 5 animals; s.e.mean shown by vertical bars.

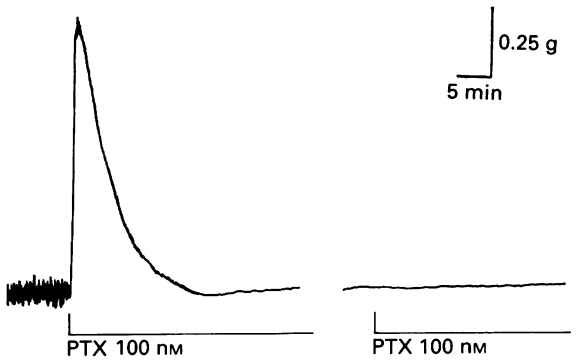

Figure 2 Contractile effect of palytoxin (PTX) (100 nM) on the rat isolated detrusor. The interruption in the record represents a 20 min interval, during which the preparation was washed repeatedly with fresh Krebs-Henseleit solution. Note the decay of the initial contractile response to PTX and the total insensitivity of the preparation to a repeat exposure to PTX.

evoke any contraction (Figure 2). In other experiments, repeated washes with Krebs-Henseleit solution lasting for periods up to 2 h, following an initial exposure to PTX 100 nM, failed to reverse the tachyphylaxis.

The tension generated in response to exposure to a fixed concentration of PTX was significantly greater when the concentration was achieved by a single bolus rather than by cumulatively-rising doses (mean response to PTX, 100 nM, as % of the response to ACh 1 mM, $= 58 \pm 3$, $n = 8$, and 36 ± 2, $n = 5$, with single and cumulative applications respectively).

Effect of palytoxin on spontaneous activity

The isolated detrusor preparations invariably exhibited a modest degree of spontaneous activity. PTX enhanced the spontaneous activity in a concentration-dependent manner by increasing both the force and frequency of spontaneous contractions (Figure 3). At a concentration of 100 nM or more, it caused an increase in resting tension, and spontaneous activity slowly ceased (Figure 3a,b). The presence of the cyclo-oxygenase inhibitor, indomethacin (10 μM), in the bathing medium prevented neither spontaneous activity nor the effect of PTX.

Effect of palytoxin on contractions evoked by electrical field stimulation

Electrical field stimulation (EFS) evoked consistent and reproducible contractile responses. These contractile responses were blocked by exposure to TTX (0.5 μM) and were therefore neurogenic. Figure 4a,b shows parts of the records from two experiments demonstrating the effect of the PTX on motor transmission. Preparation (a) was exposed to PTX in cumulatively increasing concentrations of 0.1 nM, 1 nM and 10 nM. PTX, 0.1 nM, had no effect on the contractile response to EFS; at 1 nM the response was slightly reduced and at 10 nM, PTX totally blocked the response to EFS. Preparation (b) was exposed to PTX, 100 nM, by a single application; it abolished transmission as with PTX 10 nM, but the effect was achieved more rapidly.

Effect of palytoxin on acetylcholine-evoked contractions

A typical experiment demonstrating the effect of PTX on ACh-evoked contractions is shown in Figure 5. After obtaining a control response to ACh (1 mM), the preparation was washed (5 min; 3 changes of bathing fluid) and then exposed to PTX (100 nM). The contractile response to PTX was allowed to subside fully. The preparation was exposed once

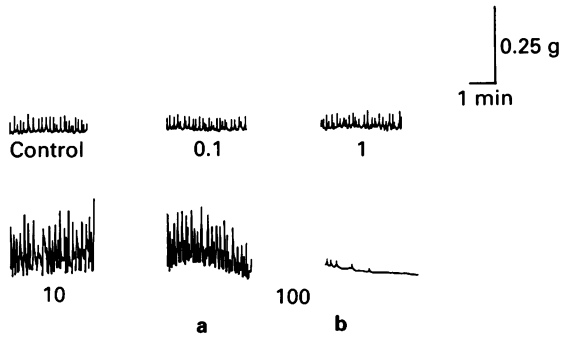

Figure 3 Effect of palytoxin (PTX) on the spontaneous activity of the rat isolated detrusor. The different panels show the spontaneous activity of a detrusor strip before (control) and during exposure to PTX 0.1, 1, 10, and 100 nM. Exposure to PTX, 100 nM, produced a rapid contraction and the spontaneous activity during the contracted state is shown in (a); subsequently during the continued presence of PTX, the preparation relaxed with gradual, but complete extinction of spontaneous activity (b).

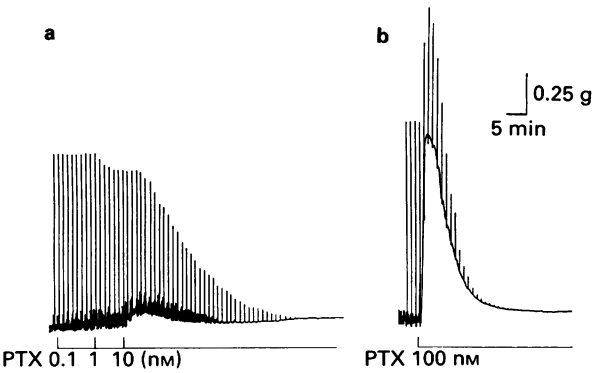

Figure 4 Effect of palytoxin (PTX) on the neurogenic response of rat isolated detrusor to electrical field stimulaton. The nerves were stimulated by trains of 5 pulses of supramaximal voltage, 0.4 ms duration at 10 Hz once every min. Preparation (a) was exposed to cumulatively increasing concentrations of PTX (0.1, 1 and 10 nM) while preparation (b) was treated with a bolus of 100 nM PTX. Note the concentration-dependence of the action of PTX on neurogenic contractions.

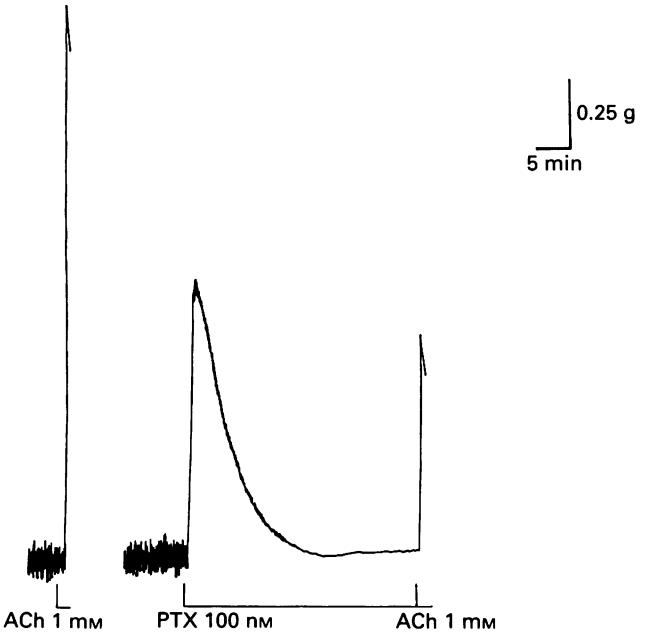

Figure 5 Effect of palytoxin (PTX) on acetylcholine (ACh)-evoked contractions of rat isolated detrusor. The preparation was exposed to ACh, 1 mM, and after obtaining the peak contraction, ACh was washed out and the preparation allowed to relax to its pre-ACh resting tension. The preparation was then exposed to PTX, 100 nM, and when the tone was stable, it was exposed to ACh 1 mM once again.

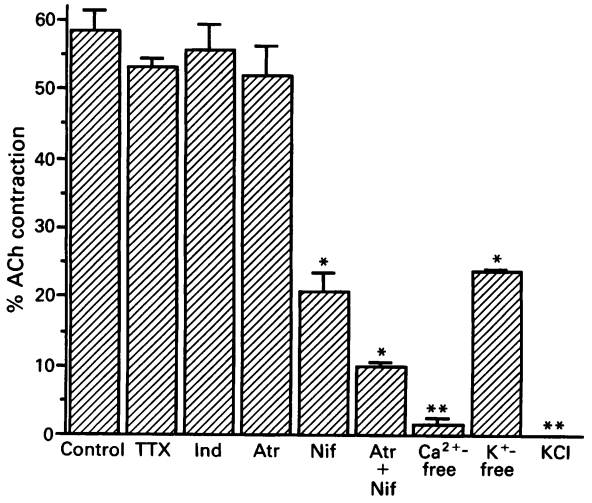

Figure 6 The effect of tetrodotoxin (TTX) 0.5 μM ($n = 4$), indomethacin (Ind) 10 μM ($n = 4$), atropine (Atr) 10 μM ($n = 4$), nifedipine (Nif) 3 μM ($n = 4$), Atr 10 μM + Nif 3 μM ($n = 5$), absence of Ca^{2+} ($n = 5$), absence of K^+ ($n = 3$) and excess K^+ 250 mM ($n = 3$) on palytoxin (PTX)-evoked contractions of rat isolated detrusor. PTX-evoked contractions have been expressed as percentage (mean with s.e.mean shown by vertical bars) of the acetylcholine (ACh)-evoked contraction. Control ($n = 8$) represents experiments in which contractions to PTX were evoked without any pretreatment. Significant differences from the control are depicted by $^*P < 0.01$ and $^{**}P < 0.001$.

more to ACh (1 mM) in the continued presence of PTX (100 nM). ACh still evoked a contraction, but in a series of 5 experiments the amplitude of contraction was reduced by 56 ± 2%. A similar result was obtained when PTX was washed out of the tissue bath before the second exposure to ACh. Control experiments, in which no PTX was used, showed that there was no change in the amplitude of the response to ACh during three successive exposures.

Effect of pharmacological agents on palytoxin-evoked contractions

TTX 0.5 μM, indomethacin 10 μM or atropine 10 μM all failed to affect PTX-evoked contractions compared to PTX-evoked contractions of untreated controls (Figure 6). The Ca^{2+}-channel antagonist, nifedipine (3 μM), reduced the size of the PTX-evoked contraction by 60% (Figure 6). The time course of the PTX-contraction was also changed in nifedipine-treated preparations. This is shown in Figure 7, which illustrates an experiment on two detrusor strips from the same rat. Strip (a) was treated with nifedipine (3 μM) for 30 min, before being exposed to PTX (100 nM); the second strip, (b), was not treated with nifedipine and served as the control. Strip (b) responded to PTX with a brisk contraction, which reached its peak in under 2 min and then subsided by about 90% in approximately 15 min. Strip (a) responded sluggishly to PTX; the initial large, brisk contraction was absent and it was replaced with a smaller slower, but sustained contraction which reached its peak in about 40 min.

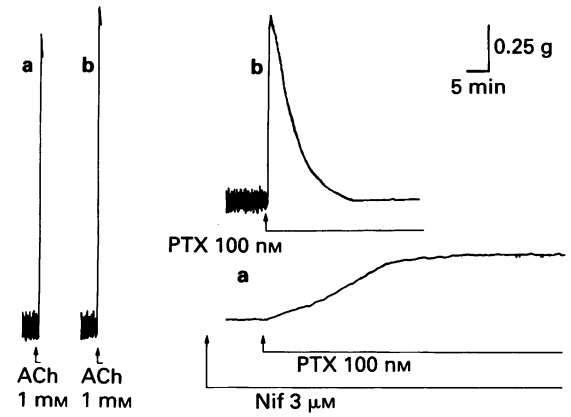

Figure 7 Effect of nifedipine (Nif) on palytoxin (PTX) evoked contraction of rat isolated detrusor. Strips (a) and (b) were exposed to acetylcholine (ACh) 1 mM for 30 s. Strip (a) was then treated with nifedipine for 30 min before exposing it to PTX. Strip (b) was exposed to PTX alone without any nifedipine treatment. Note the absence of phasic contraction by PTX in the nifedipine-treated strip (a).

Effect of absence of Ca^{2+}

Preparations incubated in Ca^{2+}-free Krebs-Henseleit solution (no added Ca^{2+}; 1.2 mM EDTA), lacked spontaneous activity and were virtually unresponsive to PTX (100 nM) (Figure 6).

Effect of absence of K^+

PTX (100 nM) still caused a contraction of the detrusor muscle when incubated in K^+-free Krebs Henseleit solution but the contractions were 60% smaller than those obtained by PTX 100 nM in the presence of K^+ (Figure 6).

Effect of excess K^+

Preparations 'desensitized' to PTX contracted when exposed to excess K^+ (KCl 250 mM) but the K^+-evoked contraction was smaller than that produced in preparations not previously exposed to PTX (Figure 8a). Depolarization of the detrusor strip by excess K^+ (KCl 250 mM) evoked a large, brisk contraction which gradually subsided fully in about 30 min during the continued presence of excess K^+. The addition of PTX (100 nM) following the high K^+ failed to evoke any contractile response (Figures 8b and 6).

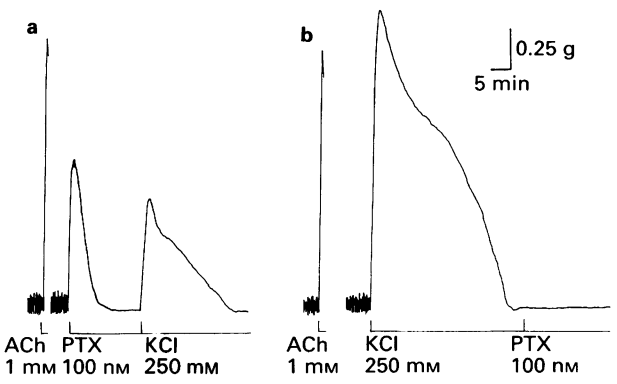

Figure 8 Effect of palytoxin (PTX) treatment on a K^+-evoked contraction (a) and of K^+-induced depolarization on PTX-evoked contraction (b) in rat isolated detrusor.

Effect of palytoxin following postsynaptic block of motor transmission

It has been reported recently that motor transmission in the detrusor muscle of the rat can be fully blocked by a combination of atropine (10 μM) plus nifedipine (3 μM) (Zar *et al.*, 1990). A total of five experiments were done in which the action of PTX (100 nM) was examined in the combined presence of atropine (10 μM) plus nifedipine (3 μM). Records from typical experiments are shown in Figure 9; electrically-evoked motor transmission was abolished and PTX (100 nM) caused a sluggish and a much smaller contraction than in the absence of nifedipine and atropine (compare Figure 9a,b).

The effect of desensitization of purinoceptors on the response to palytoxin

There is considerable evidence that ATP or an ATP-like substance functions as a non-cholinergic motor transmitter in the rodent urinary bladder (Burnstock *et al.*, 1978). In five preparations purinoceptors were desensitized by exposing the tissue to α,β-methylene ATP (α,β-MeATP) (2 × 10 μM). After densensitization (Figure 10), the contraction caused by PTX (100 nM), was 12 ± 3% of the contractile response evoked by ACh (1 mM) compared to a typical PTX-induced response of 58 ± 3% ($n = 8$) of that evoked by ACh (1 mM).

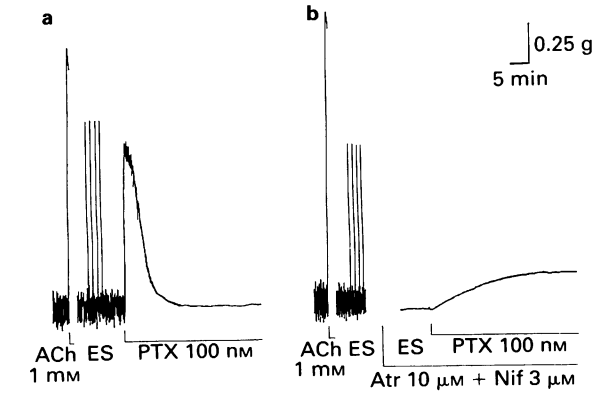

Figure 9 Effect of atropine + nifedipine on contractions of rat isolated detrusor evoked by electrical field stimulation (ES) and by palytoxin (PTX). Both preparations, (a) and (b) from the same animal. After obtaining a contractile response to acetylcholine (ACh) and ES, strip (b) was treated with atropine (Atr) 10 μM plus nifedipine (Nif) 3 μM for 30 min before repeating ES and exposure to PTX. Note the loss of phasic responses to both ES and the phasic contractile response to PTX. Parameters of ES; trains of 5 pulses, supramaximal voltage, 0.4 ms duration, 10 Hz once every min.

Effect of palytoxin on neuropeptide Y-containing innervation

Control preparations of rat detrusor muscle incubated for 3 h in normal Krebs-Henseleit solution were used for the immunocytochemical demonstration of NPY-containing axons. Fluorescent images demonstrated the dense plexus of varicose nerves extending throughout the muscle. After exposure to PTX (100 nM) for 1 h, NPY-reactivity was greatly reduced, and by 3 h, NPY-reactivity was virtually non-existent (Figure 11).

Palytoxin-induced release of acetylcholine

The acetylcholine content of detrusor muscles was reducd by 82 ± 6% (range 74–98%, $n = 4$) following exposure to PTX (100 nM) for 90 min compared to control.

Discussion

The observation that palytoxin (PTX) was a powerful spasmogen in the detrusor muscle of the rat urinary bladder was not unexpected since a wide range of other smooth muscles contract when exposed to PTX, including the urinary bladder of the rabbit (Ito *et al.*, 1976; 1977; Ohizimu & Shibata, 1980; Ozaki *et al.*, 1983; 1987; Ishida *et al.*, 1985a,b; Shibata *et al.*, 1986). The response of the rat muscle was Ca^{2+}-dependent, resistant to TTX and exhibited profound tachyphylaxis.

There is considerable evidence that PTX impairs the physiological integrity of excitable tissues, primarily by increasing the permeability of the plasma membranes to a range of small cations and, in some cases, compounds such as inulin (Habermann, 1989). This increase in permeability results from the opening of cation selective channels (Rouzaire-Dubois & Dubois, 1990 for example) and leads to non-specific depolarization (Dubois & Cohen, 1977; Pichon, 1982; Muramatsu *et al.*, 1984). It is superficially attractive to suggest, therefore, that PTX is a general cytotoxin, and to interpret its wide range of pharmacological actions in this light. It is clear, however, that the toxin is not a non-specific

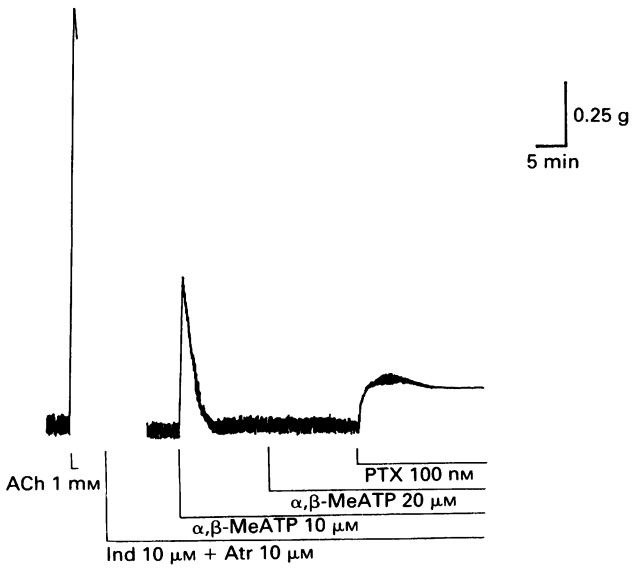

Figure 10 Effect of α,β-methylene ATP (α,β-MeATP) desensitization on palytoxin (PTX)-evoked contraction in rat detrusor. After obtaining a control acetylcholine (ACh) response, the preparation was treated with indomethacin (Ind) + atropine (Atr) for 30 min before exposure to α,β-MeATP first at 10 μM and then at 20 μM. Note the successful induction of α,β-MeATP desensitization and the small contractile response to PTX following α,β-MeATP-densensitization.

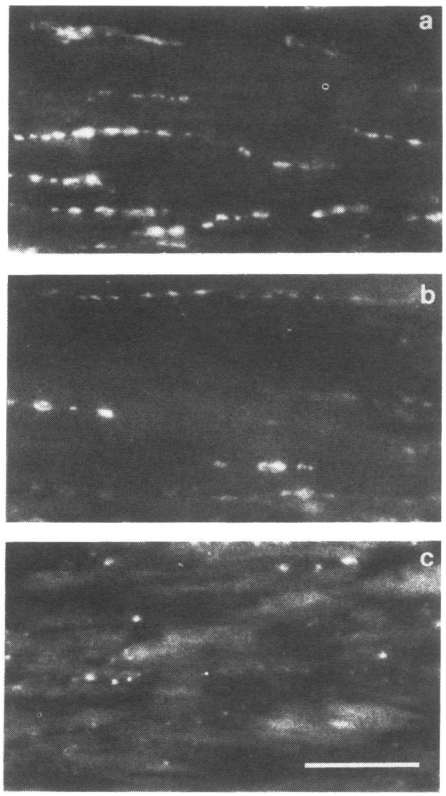

Figure 11 Fluorescence micrographs of neuropeptide Y (NPY)-immunoreactive nerve fibres in longitudinal sections of rat detrusor after treatment with palytoxin (PTX) (100 nM) for 60 min (b) and 180 min (c). Section (a) was not treated with PTX and served as control. Note the decrease of NPY-immunoreactivity after PTX-treatment. The scale bar represents 25 μm for all three plates.

cytotoxin. In our own experiments, for example, when tachyphylaxis rendered the preparations totally unresponsive to PTX, exposure to ACh (1 mM) and high K^+ still evoked a contraction even though the amplitude of the responses was reduced by 40–50% in each case compared to pre-PTX treatment. Moreover, PTX does not affect the growth of bacteria and yeasts in culture (unpublished observations reported in Habermann, 1989).

Motor transmission in the rodent detrusor muscle is now widely recognised to be partly cholinergic and partly non-cholinergic in nature. The precise identity of the non-cholinergic component is unclear, but neuropeptide Y (NPY) and ATP or an ATP-like compound are the most likely candidates. It is possible that both are involved (Ambache & Zar, 1970; Burnstock *et al.*, 1972; Brading & Williams, 1990; Luheshi & Zar, 1990). In the rabbit detrusor, there is some evidence that metabolites of arachidonic acid play a role in motor transmission (Johns & Paton, 1976; Andersson *et al.*, 1980), but this is not the case in the rat (Ambache & Zar, 1970). PTX was shown to deplete the rat detrusor of ACh and NPY. We believe the transmitter-depleting effect of PTX represents its primary mode of action. This accounts for the ability of PTX to block motor transmission in the detrusor of the rat. It also explains the contractile effect of PTX on first exposure and the subsequent tachyphylaxis; it is consistent with the observation that the contraction of the tissue in response to PTX can be largely prevented by the presence of atropine and nifedipine, a combination of drugs known to block the motor response of the rat detrusor to electrical field stimulation. Indomethacin partially blocks the response of the rabbit detrusor muscle to PTX (Shibata *et al.*, 1986), but had no effect on the response of the rat detrusor muscle. This difference is consistent with the postulated role played by metabolites of arachidonic acid in the rabbit, but not the rat detrusor (see above). In our investigation, the drastic reduction in the ability of PTX to evoke a contraction of detrusor following APT-desensitization is consistent with the postulated role of ATP as a major non-cholinergic motor neurotransmitter in this tissue (Burnstock *et al.*, 1978; Brading & Williams, 1990).

The effect of PTX on the force and frequency of spontaneous mechanical activity in the rat detrusor is also consistent with the view that the primary action of the toxin is to initiate the release of transmitter from motor nerve terminals. The spontaneous activity in this tissue is thought to be initiated by the spontaneous low-level release of transmitter (Kock & Pompeius, 1963). We suggest that exposure to PTX increases the amount of transmitter released, thus enhancing spontaneous activity and initiating an increase in resting tension. This initial phase of enhanced activity and increase in tone would be expected to subside as the available transmitter is depleted from stores in the nerve terminal, and to cease when the amount released falls below the threshold required to initiate mechanical activity in the muscle.

The effect of the toxin was not blocked by TTX, so it may be concluded that fast, depolarization-dependent Na^+ channels are not a necessary component involved in PTX-initiated transmitter release. The reduction of Ca^{2+} from 2 mM to zero blocked the motor response of the muscle to PTX, but preliminary experiments on the mechanism by which PTX induces transmitter release (Posangi, Harris & Zar, unpublished) suggest that Ca^{2+} is not an essential requirement for PTX-induced transmitter release. We would suggest therefore, that it is the mechanical response initiated by the liberated transmitter that is Ca^{2+}-sensitive.

Preparations desensitized to PTX contracted when exposed to K^+ and to ACh. The contractions elicited by the two antagonists were smaller than before exposure to PTX. The observations show that desensitization to PTX does not reflect an inability of the muscle to contract and it is reasonable to infer that the acute exposure of smooth muscle to PTX does not result in the destruction of the tissue. The reduction in amplitude of K^+- and ACh-induced contractions may indicate, however, that the toxin causes a limited degree of damage. No ultrastructural studies have yet been made on smooth muscle exposed to PTX but it is probably relevant that Tesseraux *et al.* (1983) have shown that the toxin damages skeletal muscle after exposure *in vitro*. An alternative explanation is that part of the motor response of the detrusor muscle to K^+ and ACh is indirect and results from the depolarization of motor-nerve terminals. If transmitter stores have already been depleted by exposure to PTX (see above) it might be expected that the indirect component of the response to K^+ and ACh would be impaired.

The transmitters responsible for mediating motor transmission in the detrusor muscle of the rat have not been formally identified. ACh is a known transmitter but there is good evidence that both ATP (or an ATP-like substance) and NPY are also involved in motor transmission. Nifedipine inhibits the non-cholinergic components of transmission and a proportion of the cholinergic component (Iravani *et al.*, 1988; Bo & Burnstock, 1990). In our experiments nifedipine blocked the effects of PTX by 60% and the PTX-induced contraction could be further inhibited by the combined application of nifedipine and atropine. The data may be interpreted as suggesting that atropine blocked the nifedipine-insensitive component of cholinergic transmission. Atropine alone failed to affect PTX-induced contractions. Why this was so is difficult to understand, because PTX clearly caused the release of ACh from the tissue. It is possible that the three candidate transmitters in the detrusor (ACh, ATP, NPY) interact in a subtle fashion and that the physiological significance of the individual transmitters in an experimental environment varies according to the conditions of the experiment. Thus, PTX may cause the rapid release of the non-cholinergic transmitters, but cause only the very slow release of ACh. It may be notable in this regard that the PTX-

induced contraction of the detrusor was initiated after a delay of only 10 s and reached its peak by 2 min. This very brisk response was selectively blocked by nifedipine. The nifedipine-insensitive component rose slowly to reach its peak after 10–15 min. It is attractive to suppose that this latter response was caused by ACh. Until more is known of the details of the neuromuscular physiology of the detrusor muscle of the rat, it is not profitable to speculate further.

Our observations on the pharmacological activity of PTX do not address directly its mechanism of action. Recently, however, Rouzaire-Dubois & Dubois (1990) have studied the activity of PTX on neuroblastoma-glioma NG108-15 hybrid cells using patch clamping. It was shown that PTX activated cation-selective (but non-specific) channels in the plasma membrane. It was also shown that the current consequent upon the opening of the channels was maintained with low doses (<1 nM) of PTX but declined in the continued presence of high doses (>10 nM) of the toxin. After high doses of PTX, the cells were refractory to further doses, even after prolonged washing. These data (and those of others who have also shown that PTX opens cation selective channels in excitable cells; see Rouzaire-Dubois & Dubois, 1990 for a summary) are not inconsistent with our own suggestions that the release of transmitter from motor nerve terminals is the primary mechanism of action, but they do suggest an alternative explanation for the onset of tachyphylaxis. Rouzaire-Dubois & Dubois (1990) have also noted that the rate of onset of channel opening and the size of the total current were dose-dependent. They interpreted this as suggesting that the binding of PTX to its 'receptor' on the plasma membrane was probably co-operative. They then interpreted the fall in the current with time, in the presence of high doses of toxin, and the tachyphylaxis as indicating that the toxin-receptor complex leaves the membrane and is either internalised or enters the external medium. If re-incorporation of the receptor is low, there will be a continuing down-regulation of the system. Clearly, such a mechanism would also explain all of our experimental data. The mechanism would not necessarily be expected to lead to the release of all available transmitter, although our data clearly indicate that, within the limitations of the techniques we have used, all transmitter stores are depleted.

The cell surface receptor that binds PTX has not been identified, but Na^+-K^+ ATPase is a strong candidate (Habermann, 1989). Whether PTX renders the ATPase an ion 'channel' is less clear. It is of interest, however, that PTX is a powerful inhibitor of Na^+-K^+ ATPase activity (Haberman, 1989), and inhibition of Na^+-K^+ ATPase is considered a primary step involved in the physiology of transmitter release in the autonomic nervous system (Paton *et al.*, 1971).

So far, discussions on the mechanism of action of PTX have concentrated on the role of ATPase and the opening of ion-channels. It is striking that very little work has been done on the morphology of tissue exposed to PTX. Tesseraux *et al.* (1983) showed that PTX caused severe damage to skeletal muscle exposed to concentrations as low as 1 nM and that the damage involved the sarcoplasmic reticulum, mitochondria and contractile elements. It seems highly probable that one explanation for the total depletion of transmitter and the irreversibility of the effects of high doses of PTX is that in addition to its specific activity, PTX causes significant structural damage to neuronal tissue.

J.P. is supported by The Asian Development Bank through the Indonesian Government.

References

ALCALA, A.C., ALCALA, L.C., GARATH, J.S., YASUMARA, D. & YASUMOTO, T. (1988). Human fatality due to ingestion of the crab *Demania reynaudii* that contained a palytoxin-like toxin. *Toxicon*, **26**, 105–107.

AMBACHE, N. & ZAR, M.A. (1970). Non-cholinergic transmission by post ganglionic motor neurones in the mammalian bladder. *J. Physiol.*, **210**, 761–783.

ANDERSSON, K.E., HUSTED, S. & SJÖGREN, C. (1980). Contribution of prostaglandin to the adenosine triphosphate-induced contraction of rabbit urinary bladder. *Br. J. Pharmacol.*, **70**, 443–452.

BERESS, L., ZWICK, J., KOLKENBROCK, H.J., KAUL, P.N. & WASSERMANN, O. (1983). A method for the isolation of the Caribbean palytoxin (C-PTX) from the coelenterate (zoanthid) *Palythoa caribaeorum*. *Toxicon*, **21**, 285–290.

BO, X. & BURNSTOCK, G. (1990). The effects of Bay K 8644 and nifedipine on the responses of rat urinary bladder to electrical field stimulation, $\beta\gamma$-methylene ATP and acetylcholine. *Br. J. Pharmacol.*, **101**, 494–498.

BRADING, A.F. & WILLIAMS, J.H. (1990). Contractile response of smooth muscle strips from rat and guinea-pig urinary bladder to transmural stimulation; effects of atropine and α,β-methylene ATP. *Br. J. Pharmacol.*, **99**, 493–498.

BURNSTOCK, G., DUMSDAY, B. & SMYTHE, A. (1972). Atropine-resistant excitation of the urinary bladder: the possibility of transmission via nerves releasing a purine nucleotide. *Br. J. Pharmacol.*, **44**, 451–461.

BURNSTOCK, G., COCKS, T., CROWE, R. & KASAKOV, L. (1978). Purinergic innervation of the guinea-pig urinary bladder. *Br. J. Pharmacol.*, **63**, 125–138.

CASTLE, N.A. & STRICHARTZ, G.R. (1988). Palytoxin induces a relatively non-selective cation permeability in frog sciatic nerve which can be inhibited by cardiac glycosides. *Toxicon*, **26**, 941–951.

DUBOIS, J.M. & COHEN, J.B. (1977). Effect of palytoxin on membrane potential and current of frog myelinated fibres. *J. Pharmacol. Exp. Ther.*, **201**, 148–155.

HABERMANN, E. (1989). Review article: Palytoxin acts through Na^+, K^+-ATPase. *Toxicon*, **27**, 1171–1187.

IKEDA, M., MITAMI, K. & ITO, K. (1988). Palytoxin induces a non selective cation channel in single ventricular cells of rats. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **337**, 591–593.

IRAVANI, M.M. (1988). The physiological and pharmacological profile of neuropeptide Y in peripheral autonomically-innervated organs. *PhD. thesis, University of Newcastle upon Tyne, England.*

IRAVANI, M.M., LUHESHI, G.N. & ZAR, M.A. (1988). Inhibition of non-cholinergic motor transmission in isolated rat urinary bladder by nifedipine. *J. Physiol.*, **401**, 69P.

ISHIDA, Y., KAJIWARA, A., TAKAGI, K., OHIZUMI, Y. & SHIBATA, S. (1985a). Dual effect of ouabain on the palytoxin-induced contraction and norepinephrine release in the guinea-pig vas deferens. *J. Pharmacol. Exp. Ther.*, **232**, 551–556.

ISHIDA, Y., SATAKE, N., HABON, J., KITANO, H. & SHIBATA, S. (1985b). Inhibitory effect of ouabain on the palytoxin-induced contraction of human umbilical artery. *J. Pharmacol. Exp. Ther.*, **232**, 557–560.

ITO, K., KARAKI, H., ISHIDA, Y., URAKAWA, N. & DEGUCHI, T. (1976). Effect of palytoxin on isolated intestinal and vascular smooth muscles. *Jpn. J. Pharmacol.*, **26**, 683–692.

ITO, K., KARAKI, H. & URAKAWA, N. (1977). The mode of contractile action of palytoxin on vascular smooth muscle. *Eur. J. Pharmacol.*, **46**, 9–14.

JOHNS, A. & PATON, D.M. (1976). Evidence for a role of prostaglandins in atropine-resistant transmission in the mammalian urinary bladder. *Prostaglandins*, **11**, 595–597.

KOCK, N.G. & POMPEIUS, R. (1963). Studies on the nature of the rhythmic activity of the human bladder. *Investigate Urology*, **1**, 253–261.

LUHESHI, G.N. & ZAR, M.A. (1990). Inhibitory effect of streptozotocin-induced diabetes on non-cholinergic motor transmission in rat detrusor and its prevention by sorbinil. *Br. J. Pharmacol.*, **101**, 411–417.

MOORE, R.E. & SCHEUER, P.J. (1971). Palytoxin: a new marine toxin from a coelenterate. *Science*, **172**, 495–498.

MURAMATSU, I., UEMURA, D., FUJIWARA, M. & NARAHASHI, T. (1984). Characteristics of palytoxin-induced depolarization in squid axons. *J. Pharmacol. Exp. Ther.*, **231**, 488–494.

MURAMATSU, I., NISHIO, M., KIGOSHI, S. & UEMURA, D. (1988). Single ionic channels induced by palytoxin in guinea-pig ventricular myocytes. *Br. J. Pharmacol.*, **93,** 811–816.

NAGASE, H. & KARAKI, H. (1987). Palytoxin-induced contraction and release of prostaglandins and nor-epinephrine in the aorta. *J. Pharmacol. Exp. Ther.*, **242,** 1120–1125.

OHIZUMI, Y. & SHIBATA, S. (1980). Mechanism of the excitatory action of palytoxin and N-acetylpalytoxin in the isolated guinea-pig vas deferens. *J. Pharmacol. Exp. Ther.*, **214,** 209–212.

OZAKI, H., NAGASE, H., KARAKI, H. & URAKAWA, N. (1987). Effects of palytoxin on contractile response and calcium movement in guinea-pig taenia coli. *Comp. Biochem. Physiol.*, **86C,** 387–393.

OZAKI, H., TOMONO, J., NAGASE, H. & URAKAWA, N. (1983). The mechanism of contractile of palytoxin on vascular smooth muscle of guinea-pig aorta. *Jpn. J. Pharmacol.*, **33,** 1155–1162.

PATON, W.D.M., VIZI, E.S. & ZAR, M.A. (1971). The mechanism of acetylcholine release from parasympathetic nerves. *J. Physiol.*, **215,** 819–848.

PATON, W.D.M. & ZAR, M.A. (1968). The origin of acetylcholine release from guinea-pig intestine and longitudinal muscle strips. *J. Physiol.*, **194,** 13–33.

PICHON, Y. (1982). Effects of palytoxin on sodium and potassium permeabilities in unmyelinated axons. *Toxicon*, **20,** 44–47.

ROUZAIRE-DUBOIS, B. & DUBOIS, J.M. (1990). Characterization of palytoxin-induced channels in mouse neuroblastoma cells. *Toxicon*, **28,** 1147–1158.

SHIBATA, S., SATAKE, N., UEDA, S., OHIZUMI, Y., FLORES, F. & PAULINO, R. (1986). The contractile action of palytoxin in the isolated rabbit urinary bladder. *Eur. J. Pharmacol.*, **127,** 129–133.

TESSERAUX, I., HARRIS, J.B. & WATKINS, S.C. (1983). Physiological and morphological effects of palytoxin (*Palythoa caribaeorum*) on skeletal muscle. In *Toxins as Tools in Neurochemistry*. ed. Hucho, F. & Ovchinnikov, Y.A. pp. 91–100. Berlin, Germany: Walter de Gruyter & Co.

ZAR, M.A., IRAVANI, M.M. & LUHESHI, G.N. (1990). Effect of nifedipine on the contractile response of the rat bladder. *J. Urol.*, **143,** 835–839.

(Received December 13, 1991
Revised January 21, 1992
Accepted February 26, 1992)