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THE POTENT DEPOLARIZING ACTION OF PALYTOXIN ISOLATED FROM *Palythoa Tubercurosa* ON THE ISOLATED SPINAL CORD OF THE FROG

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1 The effects of palytoxin (PTX) isolated from *Palythoa tubercurosa* were tested on the isolated intra-arterially perfused spinal cord of the frog. The resting and evoked potentials were recorded by means of a sucrose-gap technique.

2 PTX caused a marked depolarization of both ventral and dorsal roots. The minimum effective concentration was extremely low, approximately 10^{-11} M. During the depolarization the evoked ventral and dorsal root potentials were markedly reduced in amplitude. The ventral root reflex was first augmented and then decreased.

3 The depolarization caused by PTX was markedly reduced when the preparation was perfused with NaCl –deficient medium. However, tetrodotoxin (10^{-7} M) only slightly inhibited the depolarization.

4 In a high Ca^{2+} medium (3.6 mM), the time required to reach the maximum depolarization evoked by PTX was significantly prolonged. In contrast, in a low Ca^{2+} medium (0.9 mM), PTX caused a marked depolarization soon after application. In a Ca^{2+}-free, Mg^{2+} (9.0 mM) medium, PTX caused rhythmic oscillatory potentials in both ventral and dorsal roots.

5 The potency of N-acetyl PTX was one hundredth that of the parent compound.

6 It is suggested that PTX may interfere with the stabilizing action of Ca^{2+} on the neuronal membrane, consequently facilitating Na^{+} permeability. The primary amine in the PTX molecule may be important for its pharmacological action.

Introduction

Palytoxin (PTX) is a substance isolated from a zoanthid, *Palythoa sp.* (Moore & Scheuer, 1971; Kimura & Hashimoto, 1973; Kaul, Farmer & Ciereskо, 1974; Hirata, Uemura, Ueda & Takano, 1979). The molecular structure of the toxin has not been determined fully, however, the molecular weight of the compound was estimated to be 3300 and was neither a polypeptide nor a polysaccharide (Moore, Dietrich, Hatton, Higa & Scheuer, 1975). To date, many pharmacological studies have shown the toxin to be extremely potent on cardiac muscle (Rayner, Sanders, Harris, Lin & Morton, 1975; Deguchi, Urakawa & Takamatsu, 1976; Ito, Karaki & Urakawa, 1979), vascular smooth muscle (Ito, Karaki & Urakawa, 1977), intestinal smooth muscle (Deguchi *et al.*, 1976) and the membrane potential of skeletal muscle (Deguchi *et al.*, 1976), heart muscle (Weidmann, 1977) and myelinated fibres (Dubois & Cohen, 1977). However, the effect of the toxin on the central nervous system has not been studied.

The present study was therefore undertaken to investigate the effect of PTX on the central nervous system using the isolated spinal cord of the frog (Kudo, 1978).

Methods

Isolated, intra-arterially perfused spinal cord of the bullfrog

Bullfrogs (*Rana catesbiana*), weighing 100 to 200 g, (73 animals) were obtained from September 1978 to May 1979. The technique for preparing the isolated, intra-arterially perfused spinal cord was similar to that described previously (Kudo, Abe, Goto & Fukuda, 1975). Frogs were cooled in crushed ice until anaesthetized and the spinal cord was isolated with dorsal and ventral roots, and arranged in a chamber kept at 16°C. A glass cannula (about 200 μm in tip diameter)

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was inserted into the anterior spinal artery and used to perfuse the spinal cord with amphibian Ringer solution of the following composition (mM): NaCl 115, KCl 2.7, $CaCl_2$ 1.8, glucose 5.5 and $NaHCO_3$ 3.0, pH 7.6. NaCl-deficient solution was prepared by substituting 170 mM sucrose for 85 mM NaCl.

Recording of the root potentials and root reflexes by a sucrose-gap method

The sucrose-gap method applied in the present study has been described by Kudo *et al.* (1975). Potential differences between the spinal cord and the peripheral root stumps which were divided by a sucrose stream were detected by a pair of Ag–AgCl electrodes and recorded by a two pen d.c. recorder (Technicorder, Type 3047, Yokokawa). An adjacent dorsal root was stimulated by a pair of Ag–AgCl electrodes (0.1 Hz, duration 0.5 ms, 3 to 8 V, Nihonkohden SEN 31301). The evoked ventral and dorsal root potentials and reflexes were displayed on an oscilloscope (Nihonkohden VC-9), and photographed (five superimposed traces). The membrane conductance in the motoneurone was detected according to the method described by Nicoll, Padjen & Baker (1976) by applying constant current pulses across the sucrose-gap placed on the ventral root through a bridge circuit (WPI M707).

Materials

Drugs used were palytoxin, isolated from *Palythoa tubercurosa* (supplied by Dr Hirata and Dr Scheuer), N-acetylpalytoxin (supplied by Dr Hirata and Dr Uemura) and tetrodotoxin (TTX, Sankyo, Japan). The estimated chemical component of purified PTX supplied by Dr Hirata was $C_{118}\ H_{211}\ N_3\ O_{63}$ (mol. wt 2681.1 ± 0.35) and that of the N-acetyl derivative was $C_{120}\ H_{213}\ N_3\ O_{64}$ (mol. wt. 2723.1 ± 0.35) (Macfarlane, Uemura, Ueda & Hirata, 1980). All agents were dissolved in Ringer solution and applied through the anterior spinal artery by exchanging the perfusing medium with a solution containing test agents.

Results

Effects on resting and evoked potentials in dorsal and ventral roots

At concentrations greater than 10^{-11} M, PTX depolarized the resting root potentials and Figure 1a and b show the characteristic actions of PTX (10^{-8} M). The depolarization developed gradually and reached its maximum in approximately 15 min. The amplitudes of the evoked root potentials were reduced during the depolarization (Figure 1a). Figure 2 shows the dose-response relationships for PTX on the resting root potentials. The ventral root reflex was markedly augmented in the initial 5 min after the application. However, the reflex potentials were found to be diminished 20 min later (Figure 1b). PTX (10^{-8} M) increased the membrane conductance by 200 to 300%.

Influence of tetrodotoxin and Na-deficient solution

After treatment of the spinal cord with TTX (10^{-7} M), the depolarizations of ventral and dorsal roots evoked by PTX (10^{-8} M) in four experiments were reduced to an insignificant degree by 9.3 ± 4.7% and 8.8 ± 4.3%, respectively.

Table 1 The influences of NaCl-deficient solution and different external Ca^{2+} concentrations on the depolarization and the attenuation of evoked root potentials by palytoxin (PTX, 10^{-8} M)

Root	*Medium*	*Max. depolarization* (mV)	T_1 (min)	T_2 (min)	*% attenuation of root potentials*
Dorsal	Normal (1.8 mM Ca^{2+})	6.9 ± 1.2	11.4 ± 2.4	16.0 ± 3.3	83.2 ± 9.2
	1/4 NaCl	2.2 ± 0.6*	17.3 ± 5.1	19.7 ± 11.4	32.7 ± 16.8*
	0.9 mM Ca^{2+}	5.5 ± 0.6	3.9 ± 1.0*	>60	93.8 ± 6.3
	3.6 mM Ca^{2+}	5.2 ± 1.1	17.5 ± 2.9	8.3 ± 1.8	57.5 ± 20.0
Ventral	Normal (1.8 mM Ca^{2+})	6.2 ± 1.1	13.4 ± 3.9	23.8 ± 5.8	79.4 ± 10.7
	1/4 NaCl	2.7 ± 0.7*	12.7 ± 3.9	16.0 ± 7.2	26.7 ± 14.8*
	0.9 mM Ca^{2+}	4.4 ± 1.1	5.3 ± 1.5	>60	87.5 ± 12.5
	3.6 mM Ca^{2+}	5.4 ± 0.8	18.0 ± 1.7	12.8 ± 1.1	69.0 ± 23.7

Each value is the mean ± s.e. ($n = 4$).
T_1: The time required for a maximum depolarization to develop; T_2: the time required for restoration of the resting root potentials to half of the maximum depolarization.
*$P < 0.05$ (Student's t test).

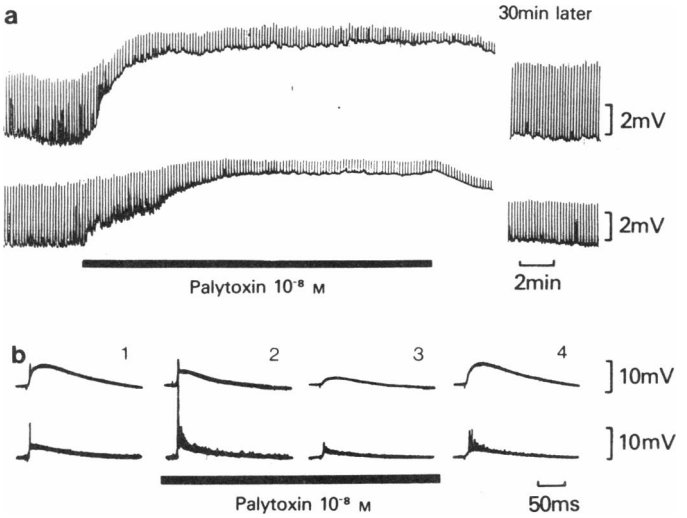

Figure 1 The effect of palytoxin (PTX) on the resting and evoked root potentials in the frog spinal cord. (a) A representative recording of the effect of PTX on root potentials. Root potentials were recorded by means of the sucrose-gap technique. The upper trace shows the dorsal root potentials of 10th dorsal root. The lower trace shows the ventral root potentials of the 9th ventral root. The stimulation of the dorsal root was at a rate of 0.1 Hz (duration 0.5 ms; intensity 8 V). PTX (10^{-8} M) was perfused for 20 min. (b) Effects of PTX on the reflex and root potential complexes recorded from the 10th dorsal root and 9th ventral root in response to the stimulation of the ipsilateral 9th dorsal root. The recordings consist of 5 superimposed tracings. (1) control; (2) 2 min after the application of PTX; (3) 20 min after the application; (4) 30 min after the perfusion with control medium.

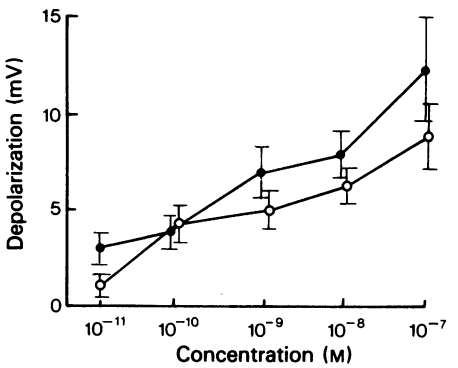

Figure 2 The dose-response relationships for palytoxin (PTX) on the resting root potentials: (●) depolarization caused by PTX in the dorsal root; (○) depolarization caused by PTX in the ventral root. Each point represents the mean of 4 separate experiments, calculated from the value obtained 20 min after the application; vertical lines show s.e. mean.

In NaCl-deficient solution, the depolarizations evoked by PTX (10^{-8} M) were significantly reduced as were the attenuation of evoked root potentials (Table 1).

Influence of external calcium ion concentration

Although the depolarizing action of PTX (10^{-8} M) on the resting root potentials was influenced by the extracellular Ca^{2+} concentration, the maximum depolarization was unchanged at external Ca^{2+} concentrations of 0.9, 1.8 and 3.6 mM. However, in the highest Ca^{2+} medium a much longer time was required for the maximum depolarization to develop and recovery occurred rapidly after washing. The attenuation of the evoked root potentials was significantly reduced in high Ca^{2+} medium. On the other hand, in low Ca^{2+} medium, the depolarization could not be reversed by prolonged washing (Table 1).

The effect of PTX (10^{-8} M) on the resting root potentials were seen in the Ca^{2+}-free and Mg^{2+} (9.0 mM)-containing medium. In such a medium, PTX evoked rhythmic oscillatory potentials in both ventral and dorsal roots.

Effects of N-acetyl-palytoxin

N-acetyl-PTX in a concentration of 10^{-8} M caused only a slight depolarization of the ventral (0.9 ± 0.2 mV; $n = 3$) and dorsal root (1.2 ± 0.2 mV; $n = 3$). At higher concentrations (10^{-6} M) it caused a marked depolarization of both the ventral (5.5 ± 0.7 mV;

$n = 2$) and dorsal roots (7.3 ± 2.3 mV; $n = 2$). Qualitatively the depolarization evoked by N-acetyl-PTX was the same as that of the parent compound.

Discussion

Recent experiments on cardiac and smooth muscle preparations demonstrated the potent excitatory actions of PTX (Deguchi *et al.*, 1976; Ito *et al.*, 1977; 1979). The present experiments provide further evidence of the potent actions of PTX on excitable membranes. In the frog spinal cord the depolarizing action of PTX is detectable at a concentration as low as 10^{-11} M. In keeping with the experiments of Dubois & Cohen (1977) on frog myelinated fibres, the present experiments show the action of PTX to be sodium-dependent, but virtually uninfluenced by TTX.

Rayner *et al.* (1975) found that PTX facilitates ^{45}Ca uptake into the rabbit ventricle strip and attributed the cardiac stimulant action of the toxin to an increased Ca^{2+} flux. Ito *et al.* (1977) also found PTX to enhance ^{45}Ca uptake into vascular smooth muscle. In addition, in the guinea-pig papillary muscle the positive inotropic action of PTX was potentiated in a high Ca^{2+} medium (Ito *et al.*, 1979). Recently Ohizumi & Shibata (1980) have shown PTX to facilitate Ca^{2+}-dependent catecholamine release from the hypogastric nerve ending in guinea-pig vas deferens. In the present study, it is interesting to note that the time required for a maximum depolarization to develop in ventral and dorsal roots after PTX treatment was significantly prolonged by increasing the external Ca^{2+} and almost complete recovery from the depolarization was obtained within 30 min of washing. On the other hand, the depolarization caused by PTX in low Ca^{2+} medium reached its maximum immediately after the application and barely recovered after prolonged washing. The rhythmic oscillatory potentials evoked by PTX in Ca^{2+}-free medium containing Mg^{2+} (9.0 mM) were similar to those seen in the frog spinal cord perfused with Ca^{2+}-free medium (Kim, Kudo & Fukuda, 1978). Thus in the frog spinal cord, Ca^{2+} flux may not play a primary role in the development of the depolarization caused by PTX. However, Ca^{2+} is important in the stabilization of the neuronal membrane (Brink, 1954; Frankenhaeuser & Hodgkin, 1975), and the toxin-induced depolarization of the neuronal membrane may be related to an antagonistic action of PTX on the stabilizing effect of Ca^{2+}.

The depolarizing action of the N-acetyl derivative of PTX was found to be about one hundredth of the parent compound. Since only the primary amine group was acetylated (Macfarlane *et al.*, 1980), the dramatic reduction of the potent depolarizing action of PTX by N-acetylation strongly suggests that the active site of the toxin includes this amino group.

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