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Pardaxin produces postjunctional muscle contraction in guinea-pig intestinal smooth muscle

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1 The action of pardaxin (PX), a toxin isolated from the secretion of the Red Sea flatfish, *Pardachirus marmoratus*, was studied on longitudinal muscle of guinea-pig ileum.

2 Pardaxin contracted the ileum and subsequently abolished muscle contraction to 5-hydroxytryptamine (5-HT), but did not affect the responses to acetylcholine (ACh) and substance P(SP).

3 Pardaxin-induced contraction was only partially suppressed by atropine and not affected by tetrodotoxin or morphine. Preparations desensitized to 5-HT or SP responded normally to pardaxin.

4 Pardaxin-induced contractions were normal in K^+-depolarizing Krebs Ringer solution and not affected by black widow spider venom.

5 It is concluded that the pardaxin-induced muscle contractions are not mediated through the release of neurotransmitters and do not involve 5-HT, SP or ACh receptors, but are due to a direct action on the muscle contractile mechanism.

Introduction

The Red Sea flatfish, *Pardachirus marmoratus*, has more than 200 glands located along its dorsal and anal fins (Clark & Chao, 1973). The fluid secreted from these glands has been found to be toxic to various teleosts (Clark & George, 1979). The secretion was shown to repel sharks (Clark, 1974; 1983; Gruber, 1982) suggesting that the biological significance of the secretion is the defence of the flatfish against its predators.

When the planar lipid bilayer membranes were used as a model system to study the nature of interaction between the secretion and lipid bilayers, there was a voltage-dependent conductance in discrete uniform steps due to the secretion, indicating formation of transmembranal channels (Moran *et al.*, 1978; Korchak, 1979). Some properties of the secretion have been studied using isolated organ preparations. In a frog neuromuscular preparation, the primary action of the secretion was to increase the number of neurotransmitters quantifiable as judged by the frequency of m.e.p.ps. However, muscle tremors observed in the presence of tubocurarine, and disruption of muscle fibres indicated that it also affects the muscle membrane directly (Spira *et al.*, 1976).

Pardachirus secretion contains several high and low molecular weight components. Using column gel chromatography, it was fractionated into several proteinic and non-proteinic fractions (Primor *et al.*, 1975). The principle toxin was purified and characterized as a single, helical, monomeric, acidic protein with four disulfide bridges and a mol. wt. of 13,100 daltons (Primor *et al.*, 1978; Primor & Tu, 1980). This toxin, named pardaxin (PX), was found to be primarily responsible for the toxicity of the secretion (Primor *et al.*, 1980; Primor & Lazarovici, 1981; Primor, 1983) and increases membrane permeability (Pal *et al.*, 1981a, b; Primor *et al.*, 1983; Zlotkin & Barnholz, 1983).

The secretion induced a contractile response of guinea-pig ileum and inhibited the stimulation induced by 5-hydroxytryptamine (5-HT). It was concluded that muscle contraction was due to the release of acetylcholine (ACh) from the nerve terminals (Parness & Zlotkin, 1976).

The main objective of the present study was to determine if pardaxin-elicited muscle contraction is due to its action via release of neurotransmitters or to a mechanism which does not involve neural pathways.

Methods

Adult short haired albino guinea-pigs (300–400 g) were killed by a blow on the head and bled.

Krebs-Ringer solution of the following composition (mM) was used: NaCl 118.0; KCl 4.7; $MgCl_2$ 1.2; $CaCl_2$ 2.5; NaH_2PO_4 1.2; $NaHCO_3$ 25.0 and glucose 11.0; kept at 37°C and bubbled with 95% O_2 plus 5% CO_2. Segments, approximately 3 cm long, were removed from the mid-ileum. All experiments were carried out on isolated strips of longitudinal muscle, which were separated from the underlying circular muscle by the method of Puig *et al.* (1978). These preparations include the myenteric plexus of Auerbach (Paton & Zar, 1968).

Strips were subjected to electrical field stimulation with square-wave biphasic trains of pulses (duration 1 ms, supramaximal voltage of 40 V at a frequency of 0.1 Hz) delivered by a pair of platinum wire electrodes arranged along the walls of the bath and connected to a Grass S88 stimulator. The resting load on the preparations was 0.3 g. In some experiments the Krebs-Ringer solution was modified in that 80 mM NaCl was replaced by 80 mM KCl (Alberts *et al.*, 1982).

Isometric muscular contractions were recorded on a Grass 79D polygraph, using a Grass FT 03C force displacement transducer.

Only those preparations which responded to 5×10^{-7} M ACh by producing contractions larger than 1.5 g of tension were used. Preparations were equilibrated for at least 1 h with washes every 10 min before exposure to drugs.

Increasing concentrations of ACh, nicotine, substance P (SP) and 5-hydroxytryptamine (5-HT) were applied before and after administration of pardaxin (PX) (3×10^{-7} M) for a period of 10 min. In the experiments with agonists and antagonists, only one dose of PX per tissue was used. Contractile responses were measured in g of tension and expressed as a % of the contraction induced by ACh 5×10^{-7} M.

The toxic secretion of the Red Sea flatfish, *Pardachirus marmoratus* (Pisces; Soleidae), was prepared by the method described by Clark & Chao (1973) and stored in lyophylized form. Pardaxin (PX), the toxic component, and other fractions isolated from the secretion were obtained according to the method used by Primor *et al.* (1978). Black widow spider venom (BWSV) was prepared by homogenizing glands from black widow spiders (*Latrodectus tredecimguttatus*) in 0.15 M NaCl and kept frozen at − 18°C. The spiders were collected in the area of Jerusalem, Israel. The BWSV was diluted by adding 0.05 ml of the homogenate to 10 ml of bathing solution. This corresponds to 8 μg of the total glandular proteins per ml of bath as estimated by the method of Lowry *et al.* (1951). Concentrations of the salt form of drugs is given as molar ($mol\,l^{-1}$).

The following drugs were used: 5-hydroxytryptamine creatine sulphate (serotonin), acetylcholine chloride, atropine sulphate, physostigmine (eserine) sulphate, diphenhydramine, tetrodotoxin, nicotine sulphate and histamine dihydrochloride (Sigma Chemical Co., St Louis Mo., U.S.A.); morphine sulphate (Merck, Darmstadt, West Germany); substance P (Peninsula Laboratories, San Carlos, Ca., U.S.A.).

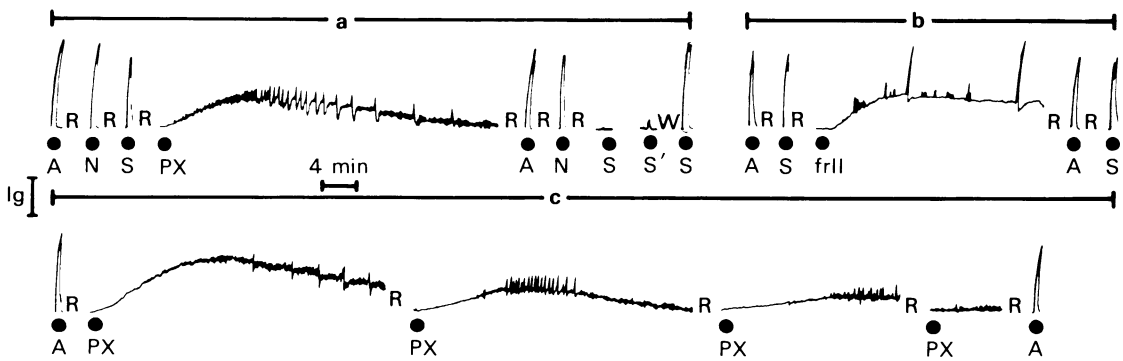

Figure 1 Effects of two fractions isolated from *Pardachirus marmoratus* secretion on guinea-pig ileum muscle contraction. (a) Pardaxin (PX) 2 $\mu g\,ml^{-1}$ (3×10^{-7} M), a toxic polypeptide. (b) Non-toxic polypeptide (Fraction II) 20 $\mu g\,ml^{-1}$. (c) Successive doses of PX with a single wash between each administration. Note PX inhibition to 5-hydroxytryptamine stimulation which could be restored after washing (W) with Krebs Ringer solution for 60 min. Acetylcholine (A) 5×10^{-7} M; nicotine (N) 8×10^{-6} M; 5-hydroxytryptamine (S) 2×10^{-6} M; (S') 2×10^{-5} M. (●) Indicates the time at which the drug was injected into the bath; (R) indicates rinse of the experimental bath from the remains of the previously administered drug. Each tracing is representative of 3 experiments. Note that PX abolished the response to 5-HT only.

Results

Effects on two fractions isolated from Pardachirus marmoratus *secretion on guinea-pig ileum muscle contractions*

Fractionation of *Pardachirus marmoratus* secretion on column Sephadex G-150 chromatography resulted in four major proteinic fractions, I, II, III (paradaxin (PX)), and IV (Primor *et al.*, 1978), which were individually tested on the strips of longitudinal muscle. PX produced a prolonged tonic contraction lasting 6–7 min, and the muscle tension returned gradually to the base line level (Figure 1a). On repeated administration of 3×10^{-7} M PX ($4\,\mu g\,ml^{-1}$) the contractile effect was seen to be tachyphylactic (Figure 1c). Fraction II ($20\,\mu g\,ml^{-1}$) elicited a small contractile response (Figure 1b) of about half that produced by PX.

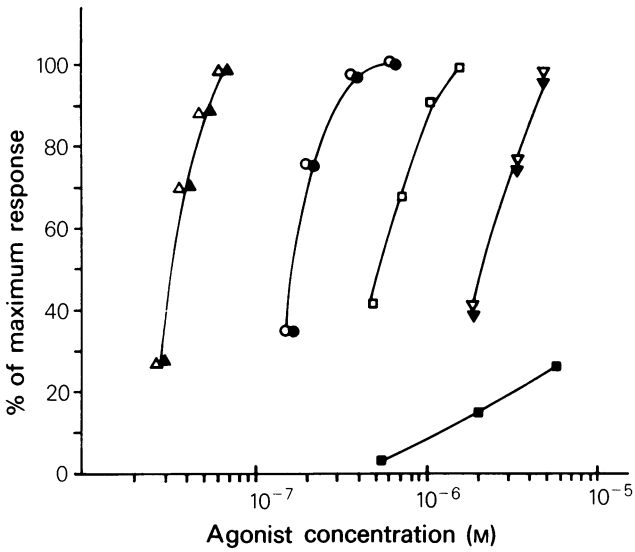

Figure 2 The muscle contraction in the dissected strip of guinea-pig ileum elicited by acetylcholine (ACh) (●, ○), nicotine (■, □), substance P (SP) (▲, △) and 5-hydroxytryptamine (5-HT) (▼, ▽) before and after administration of pardaxin (PX). Concentration-effect responses of agonists were measured following an exposure for a period of 10 min of PX (3×10^{-7} M). Ordinate: percentage of the maximum contraction elicited by ACh 5×10^{-7} M abscissa: log concentrations of ACh, nicotine, SP or 5-HT. Open symbols indicate the response to agonists before the administration of PX; closed symbols indicate the response after pretreatment with PX (PX was removed from the bath, by quickly washing the ileum twice, before the agonist was administered). Each point represents the mean value obtained in 3 different preparations.

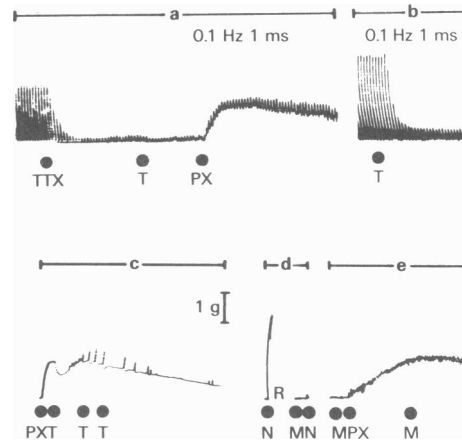

Figure 3 Effect of atropine (T), tetrodotoxin (TTX) and morphine (M) on pardaxin (PX)-induced muscle contraction in the dissected strip of guinea-pig ileum. (a) Muscle contraction produced by PX 3×10^{-7} M in the presence of 10^{-6} M of atropine (T) 10^{-6} M and TTX 10^{-6} M during electrical stimulation (0.1 Hz, 1 ms). (b) Atropine (10^{-6} M) inhibited the electrically (0.1 Hz, 1 ms) evoked contractions. (c) Repeated doses of atropine (10^{-6} M) were added during the course of PX (3×10^{-7} M)-induced muscle contraction. (d) Morphine (3×10^{-6} M) inhibited contractile responses to the exogenous application of nicotine (N) 8×10^{-6} M. (e) Muscle contraction produced by PX (3×10^{-7} M) in the presence of morphine (M) 3×10^{-6} M. (●) Indicates the time at which the drug was injected into the bath; (R) indicates rinse of the experimental bath from the remains of the previously administered drug. Each tracing is representative of 3 experiments. Note the transient inhibitory effect of the first dose of atropine.

Effect of pardaxin on muscle contractions induced by acetylcholine, nicotine, substance P, and 5-hydroxytryptamine

Concentration-response curves to ACh, nicotine, SP and 5-HT were obtained before and after the muscle had been exposed to PX (3×10^{-7} M) for 10 min. PX caused a marked shift to the right of the concentration-response curve to 5-HT, together with a depression in the maximal response. However, there was no antagonism of the responses to ACh, SP or nicotine (Figure 2). The response to 5-HT was restored after washing for 40–60 min (Figure 1a). Following administration of fraction II ($20\,\mu g\,ml^{-1}$), no inhibition of 5-HT stimulation was observed (Figure 1b).

Effect of atropine and tetrodotoxin on pardaxin-induced muscle contractions

Atropine (1×10^{-6} M) or tetrodotoxin (TTX) (1×10^{-6} M) abolished the twitch response of the ileum to electrical stimulation, but did not suppress the PX-induced contraction (Figure 3a, b). A transient inhibition of the PX-induced contractions was observed with the first dose of atropine, but subsequent doses did not affect the contraction (Figure 3c). Furthermore, atropine (1×10^{-6} M) together with TTX (1×10^{-6} M) administered before PX did not reduce the PX-induced contraction (Figure 3a). A tenfold higher concentration of atropine (1×10^{-5} M) also produced a transient inhibition of PX-induced contraction similar to that observed with the lower concentration.

Effect of pardaxin in preparations desensitized to substance P and 5-hydroxytryptamine

Preparations were desensitized by applying four doses of SP (4×10^{-8} M) or 5-HT (5×10^{-5} M) for 4 min each. The response of the ileum to PX (3×10^{-7} M) was unaltered in the desensitized preparations, suggesting that PX-induced contraction is not mediated via the mechanisms activated by 5-HT or SP (Figure 4).

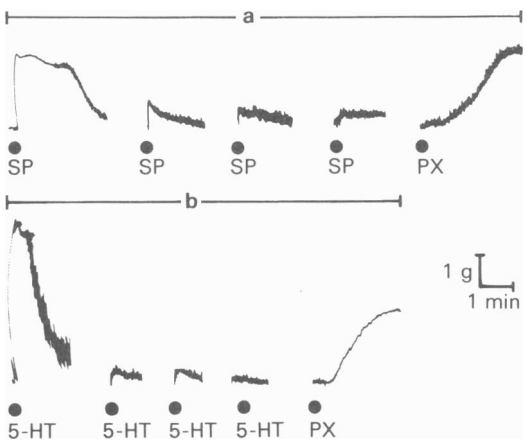

Figure 4 Muscle contraction produced by pardaxin (PX) in the dissected strip of guinea-pig ileum following desensitization to substance P (SP) and 5-hydroxytryptamine (5-HT). (a) Response to PX 3×10^{-7} M in a preparation desensitized to substance P (SP). Desensitization was induced by four subsequent incubations with 4×10^{-8} M SP for four min each. (b) Response to PX 3×10^{-7} M in a preparation desensitized to 5-HT. Desensitization was induced by incubation for five min with 5-HT (5×10^{-5} M). (●) Indicates the time at which the drug was injected into the bath; (R) indicates rinse of the experimental bath from the remains of the previously administered drug. Each tracing is representative of 3 experiments.

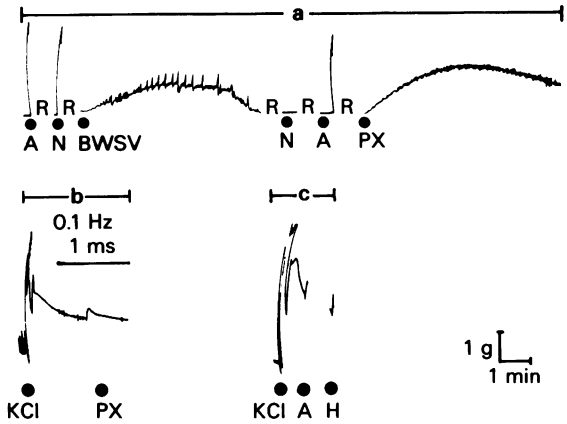

Figure 5 Effect of black widow spider venom (BWSV) and high potassium on pardaxin (PX)-induced muscle contraction in the dissected strips of guinea-pig ileum. (a) Muscle contraction produced by PX 3×10^{-7} M following incubation for 20 min with BWSV. Note that BWSV abolished the contractile response to exogenous nicotine (N) 8×10^{-6} M while the response to ACh (A) 5×10^{-7} M remains. (b) Response to PX 3×10^{-7} M in the presence of high potassium (80 mM). Note that in a high potassium solution the preparation is not able to respond with contractions to electrical stimulations (0.1 Hz, 1 ms). (c) Response to ACh (5×10^{-7} M) and histamine (H) 5×10^{-7} M in the presence of potassium 80 mM. Note that in high potassium, responsiveness to PX, ACh and histamine were reduced to a similar degree. (●) Indicates the time at which the drug was injected into the bath. (R) Indicates rinse of bath with Krebs-Ringer solution (a) and with Krebs-Ringer solution containing 80 mM KCl (c) to remove the previously administered drug. Each tracing is representative of 3 experiments.

Effect of physostigmine on pardaxin-induced muscle contractions

Physostigmine is known to produce rapid spasmodic contractions of the ileal longitudinal muscle. This effect is caused by its action on the muscle membrane (Cox & Lomas, 1972). Therefore its specific use as an anticholinesterase agent to enhance ACh-induced contractions is invalid.

In the present study, physostigmine, at a concentration of 4×10^{-6} M, did not enhance contractions induced by PX (1.5×10^{-7} M). However, at a concentration of 2.4×10^{-5} M, it caused PX to produce a series of prolonged and spasmodic contractions.

Effect of diphenhydramine on pardaxin-induced muscle contractions

Diphenhydramine (2×10^{-7} M) had no effect whether it was administered before or during the course of contractions produced by PX (3×10^{-7} M).

Effect of morphine on pardaxin-induced muscle contractions

Morphine (3×10^{-6} M) suppressed nicotine-induced muscle contractions (Figure 3d), but did not reduce the contraction to PX (Figure 3e).

Effect of Black widow spider venom on pardaxin-induced muscle contractions

In guinea-pig ileum, BWSV has been shown to deplete neurotransmitters and to cause disruption at cholinergic and non-cholinergic prejunctional nerve terminals (Frontali *et al.*, 1973; Einhorn & Hamilton, 1974). Following BWSV, the ileum loses its responsiveness to nicotine without losing that to ACh (Primor, 1980).

In the present experiments, BWSV was used in an attempt to deplete the neurotransmitters in order to determine if PX acted on their release or directly on the muscle membrane. The PX-induced contraction was similar in muscles which had or had not been pretreated (10 min) with BWSV 8.0 μg protein ml^{-1} (Figure 5a). Conversely, preincubation for 20 min with PX (3×10^{-7} M) did not reduce the contraction to BWSV (8.0 μg protein ml^{-1}).

Exposing the ileum to high-potassium (80 mM) Krebs-Ringer solution contracts the muscle, and abolishes the response to field stimulation. Even though muscles so treated were already contracted, PX elicited a small additional contraction (Figure 5b) similar in size to that produced by ACh or histamine (Figure 5c).

Discussion

In this study, PX contracted the ileum and this contractile response was tachyphylactic. Furthermore, PX specifically inhibited the responses to 5-HT. Therefore, PX elicits the same responses on longitudinal muscle as does *Pardachirus* secretion on intact ileal segments (Parness & Zlotkin; 1976). In that study, atropine blocked secretion-induced muscle contraction. Thus the present finding that atropine failed to block PX-induced contraction remains puzzling. Another toxin, isolated from tiger snake venom, also causes the ileum to contract and this contraction is not blocked by atropine (Harris & Zar, 1978).

Previous studies indicated that there are at least two separate neuronal pathways in guinea-pig ileum. One involves the excitation of postganglionic cholinergic neurones, the release of ACh, and muscle contraction (Schultz & Herz, 1976). Drugs which act via this pathway are expected to be inhibited by atropine and morphine. The other pathway is resistant to antagonism by atropine and morphine and involves the release of 5-HT, which then acts through the enteric nerves to release SP. SP probably acts, in part, as a final transmitter (Gintzler & Scalisi, 1982) and by the release of ACh (Yau & Youther, 1982). Thus there are several ways PX might induce muscle contraction.

Firstly, PX may disrupt nerve terminals and cause a release of neurotransmitters. If this were PX's mode of action, one would expect to observe a loss of response to nicotine. In addition, morphine, atropine and diphenhydramine would be expected to suppress PX-induced contraction. However, there was only a transient effect of atropine. The response to nicotine was unaffected either by *Pardachirus* secretion (Parness & Zlotkin, 1976) or by PX. In addition, morphine and diphenhydramine did not suppress the PX-induced muscle contraction. A second possible mechanism is that PX releases 5-HT or SP, or acts on their receptors. Contraction of the ileum induced by 5-HT and SP are known to be reduced by TTX (Sakai *et al.*, 1979; Huidobro-Toro *et al.*, 1982). However, neither PX nor *Pardachirus* secretion (Parness & Zlotkin, 1976) was affected by TTX. High concentrations of 5-HT and SP are known to produce desensitization to their respective effects (Huidobro-Toro & Foree, 1980; Holzer & Petsche, 1983). However, in preparations desensitized to 5-HT and SP, PX maintained its activity. This suggests that PX does not act via receptors to these neurotransmitters.

In previous studies BWSV has been found to induce a massive influx of calcium, resulting in the release of neurotransmitters (Grasso *et al.*, 1980; Meldolesi, 1982; Nicholls *et al.*, 1982). This mechanism might lead to a release of all types of neurotransmitters (Tzeng & Siekevitz, 1978; Fritz *et al.*, 1980). Hence, as BWSV depletes neurotransmitters, it has been used to investigate the pre- and postjunctional action of drugs (Primor, 1980). The observation that BWSV-induced contraction does not suppress the contraction to PX is further evidence that PX does not cause the release of neurotransmitters. Depletion of nerve terminal transmitters as the sole mechanism of *Pardachirus* secretion-induced contraction of the ileum was previously suggested by Parness & Zlotkin (1976).

The third way PX might act is by inducing contraction by a direct action. High potassium eliminates the ability of the enteric nervous system to cause muscle contraction but PX, even in the presence of 80 mM

potassium, elicited an additional contraction. This contraction, similar in size to those produced by ACh or histamine could only have resulted from a direct action on the muscle membrane. Thus, the data suggest that PX acts directly on the muscle membrane to produce a contraction.

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