Palytoxin acidifies chick cardiac cells and activates the Na^+/H^+ antiporter

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The cardiotoxic action of palytoxin was investigated using embryonic chick ventricular cells. Under normal ionic conditions, palytoxin produced an intracellular acidification which is partially compensated for by the Na^+/H^+ antiporter thereby leading to an increased rate of ethylisopropylamiloride-sensitive $^{22}Na^+$ uptake. Under depolarizing membrane conditions, palytoxin produced a cellular acidification, a cellular alkalinization or no change in intracellular pH depending on the value of the extracellular pH. We propose that palytoxin acidifies cardiac cells by opening preexisting H^+ conducting pathways in the plasma membrane.

Palytoxin; Na^+/H^+ antiporter; Amiloride; Intracellular pH

1. INTRODUCTION

Palytoxin (PTX) is one of the most potent marine toxins known [1]. It has been isolated from marine coelanterate of some zoanthids species and it consists of a long aliphatic, partially unsaturated chain with interspersed cyclic ether, hydroxyl and carboxyl groups [2,3]. Palytoxin impairs the function of excitable cells such as neuronal cells [4,5] and smooth muscle cells [6,7]. In cardiac cells, PTX enhances the membrane permeability to Na^+ and leads to a sustained depolarization, arrhythmia and contracture [8–11] but the molecular mechanism of its action is still largely unknown [12].

Another interest of PTX is that it has tumor-promoting properties. In fibroblasts, PTX down-regulates epidermal growth factor action in a manner similar to phorbol esters [13]. However, unlike phorbol esters, PTX does not bind to or activate protein kinase C [14]. In this paper we show that in embryonic chick cardiac cells, PTX activates Na^+/H^+ exchange activity via a mechanism that is distinct from that of phorbol esters and that probably involves the opening of a membrane conducting pathway for H^+.

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Abbreviations: PTX, palytoxin; EIPA, ethylisopropylamiloride; BCECF, biscarboxyethylcarboxyfluorescein; pH_i, intracellular pH; pH_o, extracellular pH; NMG, *N*-methyl-D-glucamine

2. EXPERIMENTAL

PTX from *Palythoa caribaeorum* was kindly provided by Dr E. Habermann. Dilutions of PTX were prepared in 1% bovine serum albumin solutions and stored at −20°C until use. *Anemonia sulcata* toxin 2 was purified as previously described [15]. BCECF/AM and tetrodotoxin were purchased from Calbiochem. $^{22}NaCl$ (0.5 Ci/mg) was from Amersham. Nigericin and veratridine were from Sigma. Eagle's minimal essential medium and fetal bovine serum were from Gibco.

Chick ventricular cells were dissociated from 12-day-old chick embryos and maintained in culture as previously described [16]. The culture medium was Eagle's minimal essential medium supplemented with 5% charcoal-treated fetal bovine serum, 50 units/ml of penicillin and 200 μg/ml of streptomycin.

Incubation solutions used in biochemical experiments were derived from Earle's salt solution (140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose buffered at pH 7.4 with 25 mM Hepes-Tris). Na^+-free solutions were obtained by isoosmolar substitution by NMG-Cl or KCl. Cl-free solutions were obtained using methanesulfonate salts. When the external pH was changed, appropriate mixtures of Tris, Hepes and (2-[*N*-morpholino]ethane)-sulfonic acid were used.

For $^{22}Na^+$ uptake experiments, ventricular cells were seeded into 24 well tissue culture clusters and used after 2 days of culture. The culture medium was aspirated off and cells were incubated into a K^+-free Earle's salt solution supplemented with 2 μCi/ml of $^{22}Na^+$ and the desired toxins and inhibitors. At the end of the incubations, cells were washed 3 times with 0.1 M $MgCl_2$, harvested into 0.1 N NaOH and counted.

For pH_i measurements, freshly dissociated chick cardiac cells were loaded with 10 μM BCECF/AM for 1 h in culture medium at 37°C, centrifuged and resuspended into an Earle's salt solution. Flow cytometric analysis of the BCECF fluorescence was performed as described previously [17]. A model ATC 3000 cell sorter (Odam, Brücker) and an argon laser tuned at 488 nm were used. Fluorescence emission was detected at 520 and 640 nm and the ratio of the two fluorescence intensities was computed and collected cell-by-cell in real time. Cells were analyzed at a flow rate of 500–1000/s and means from 5000 measurements computed. Calibration was achieved using

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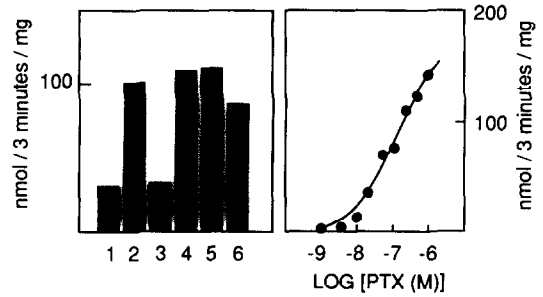

Fig. 1. Influence of PTX on $^{22}Na^+$ uptake by chick cardiac cells. (Left panel) Cells were incubated in a K^+-free, 145 mM Na^+ solution in the presence of the following compounds and rates of $^{22}Na^+$ uptake measured. (1) Control; (2) 10 μM *Anemonia sulcata* toxin 2 and 100 μM veratridine; (3) 10 μM *Anemonia sulcata* toxin 2, 100 μM veratridine and 1 μM tetrodotoxin; (4) 100 nM PTX; (5) 100 nM PTX and 1 μM tetrodotoxin; (6) 100 nM PTX and 10 μM EIPA. (Right panel) Dose-response curve for PTX action on $^{22}Na^+$ uptake. Experiments were performed in a K^+-free, 145 mM Na^+ solution supplemented with 10 μM EIPA. Time of uptake was 3 min.

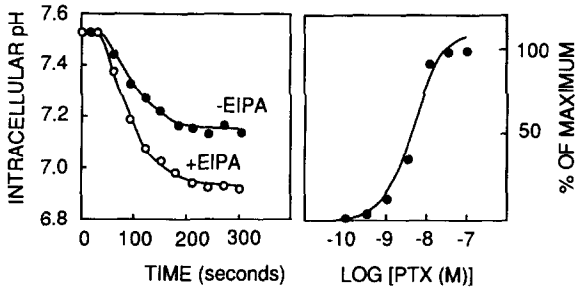

Fig. 2. PTX produced an acidification of chick cardiac cells. (Left panel) PTX (100 nM) induced changes in pH_i. Experiments were performed in a 140 mM Na^+ Earle's salt solution in the absence (•) or the presence (○) of 10 μM EIPA. Experiments shown in this panel were performed on the same batch of cells. (Right panel) Dose-response curve for PTX action on the pH_i of cells incubated in a 140 mM Na^+ Earle's salt solution. pH_i values were measured 6 min after the addition of the toxin. 100% represents a 0.5 pH unit intracellular acidification.

the nigericin/KCl clamping method [18]. The pK_a of intracellularly trapped BCECF was 6.95.

3. RESULTS AND DISCUSSION

The left panel of Fig. 1 shows that PTX produced a large increase in the rate of $^{22}Na^+$ uptake by chick ventricular cells. The action of 100 nM PTX was similar in amplitude to that of a mixture of 10 μM *Anemonia sulcata* toxin 2 and 100 μM veratridine, two toxins that open voltage-dependent Na^+ channels in chick cardiac cells [19]. The PTX-activated $^{22}Na^+$ uptake component was not altered by tetrodotoxin (1 μM), an inhibitor of voltage-dependent Na^+ channels. It was observed under conditions in which Na^+,K^+-ATPase activity was blocked using either 0.2 mM ouabain or K^+-free incubation solutions. Fig. 1 further shows that the PTX-activated $^{22}Na^+$ flux component could be partially suppressed by EIPA, an N-5 disubstituted derivative of amiloride that inhibits the Na^+/H^+ antiporter [20]. The $K_{0.5}$ value for PTX action on $^{22}Na^+$ uptake was 100 nM (Fig. 1, right panel). The $K_{0.5}$ value for EIPA reversion of PTX action was 0.5 μM (data not shown), consistent with the hypothesis of an action on Na^+/H^+ exchange activity [20]. Growth factors and phorbol esters are well known to activate Na^+/H^+ exchange possibly via phosphorylation reactions [21]. This leads to an increased rate of $^{22}Na^+$ uptake and to a cellular alkalinization that are both inhibited by amiloride derivatives [21]. Owing to the fact (i) that PTX, like phorbol esters, has tumor-promoting activity [16] and (ii) that phorbol esters activate Na^+/H^+ exchange and produce a cellular alkalinization of chick cardiac cells [22], pH_i measurements were performed to analyze in further detail the mechanism of action of PTX.

The left panel of Fig. 2 shows that PTX produced an intracellular acidification of cardiac cells rather than the cellular alkalinization expected for a phorbol ester-like action. The $K_{0.5}$ value for PTX action on pH_i was 5 nM (Fig. 2, right panel). EIPA (Figs 2 and 3) and Na^+-free conditions (using NMG as a substitute; Fig. 3A) potentiated the acidifying action of PTX. These results clearly indicated that the primary effect of PTX was to acidify cardiac cells and that the observed activation of the Na^+/H^+ antiporter was secondary to the initial acidification. This is an illustration of the well known protecting role of the cardiac Na^+/H^+ antiporter against intracellular acidifications [23].

An acidifying effect of PTX was observed when external Cl^- had been substituted by methanesulfonate, in the presence of 0.2 mM ouabain or of 20 mM bicarbonate (data not shown), indicating that it was not dependent on Cl^- or bicarbonate ions and that it did not require an active Na^+,K^+-ATPase. Important information about the mechanism by which PTX could affect pH_i was obtained in experiments performed under depolarizing membrane conditions. Fig. 3A shows that depolarizing membrane conditions with pH_o 7.4 reduced to a large extent the action of PTX on pH_i. We observed, however, that when pH_o was 6.0, PTX still produced a large intracellular acidification (Fig. 3B). Conversely when pH_o was 8.3, PTX produced the opposite effect, i.e. an intracellular alkalinization (Fig. 3C). Considering that PTX has no ionophore properties by itself [24], these observations could mean that PTX opened a preexisting H^+ conductive pathway in the plasma membrane of cardiac cells. This hypothesis would also be consistent with the observation that the acidification produced by PTX was larger when cells were incubated in the absence of Na^+ than in the presence of Na^+ and EIPA (Fig. 3A). Removal of external Na^+ prevents the partial depolarisation induced by PTX [11] and is therefore expected to increase

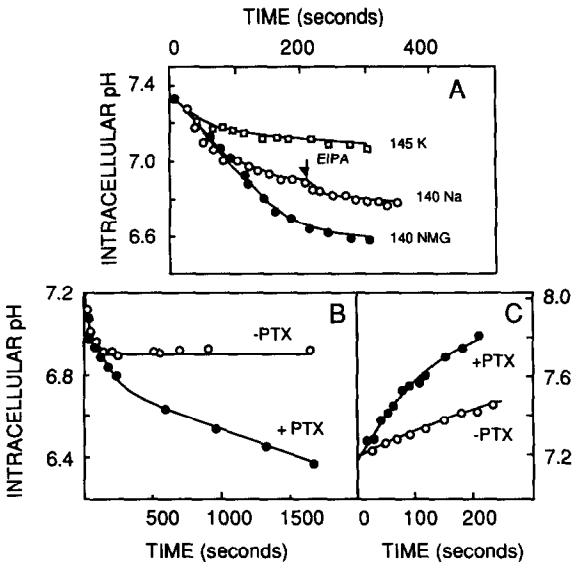

Fig. 3. The action of PTX on the pH_i of K^+ depolarized cardiac cells. (Panel A) Cells were incubated into a complete Earle's salt solution (140 Na, ○), in a Na^+-free, 140 mM NMG solution (●) or into a 145 mM K^+ solution (□). PTX (100 nM) was added at time zero. The arrow indicates the addition of 10 μM EIPA to cells incubated in a complete Earle's salt solution. (Panels B and C) Cells were incubated in 145 mM K^+ solutions at pH 6.0 (panel B) or 8.3 (panel C) in the presence (●) or the absence (○) of 100 nM PTX. Experiments shown in this figure were performed on the same batch of cells.

the driving force for H^+ entry into the cells via putative H^+ channels. Voltage-dependent H^+ currents have previously been reported in snail neurons [25] and in axolotl oocytes [26]. In addition, H^+ conductive pathways may be present in brain synaptosomes [27] and in various epithelial cells [28] but no evidence has yet been presented for their presence in cardiac cells.

PTX also increased $^{22}Na^+$ uptake by chick cardiac cells via a pathway that was insensitive to tetrodotoxin and to EIPA (Fig. 1, left panel). This pathway could be the non-selective cationic channel that has recently been shown to be activated by PTX in rat cardiomyocytes [29,30] and for which no inhibitor is yet available. The question whether the same channel is responsible for the increased H^+ and ethylisopropylamiloride-insensitive Na^+ transports cannot be answered easily for unitary H^+ currents are too small to be detected in patch clamp recordings. The observation that the $K_{0.5}$ value for PTX action on $^{22}Na^+$ uptake (100 nM) was higher than the $K_{0.5}$ value for PTX action on pH_i (5 nM) could suggest that independent events are involved.

The mechanism of action of PTX was also studied using aortic smooth muscle cells of the A7r5 cell line and endothelial cells from rat brain microvessels. In both cell types we observed: (i) that under physiological external ionic conditions, PTX produced a cellular acidification and stimulated a $^{22}Na^+$ uptake component that could be inhibited by EIPA; and (ii) that under depolarizing membrane conditions, PTX increased or decreased pH_i depending on the value of pH_o. It seems therefore that H^+ channels, the putative targets for PTX action in chick cardiac cells, are widely distributed. PTX may be a valuable tool for analyzing the properties and functions of such channels.

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