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DELAYED HAEMOLYTIC ACTION OF PALYTOXIN

GENERAL CHARACTERISTICS

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1. Palytoxin is a haemolysin. The erythrocytes from various species can be classified into a sensitive and a hardly sensitive group. The former contain potassium as their main inside cation and are arranged according to their sensitivity as hog ⩾ rat, mouse > rabbit > guinea-pig > man. The latter, comprising those from sheep and cattle, have sodium as their main inside cation. In addition, chicken erythrocytes are relatively insensitive. 2. Haemolysis of rat erythrocytes is preceded by a lag period of 1–2 h. With increasing temperature the haemolysis proceeds more quickly but reaches the same final range between 25 and 42°C. The pH optimum in Britton-Robinson buffer supplemented with saline is between 7 and 8. Washing off palytoxin during the prelytic period reduces the haemolytic power. 3. The sensitivity of rat erythrocytes decreases with increase of osmolarity between 235 and 415 mosM. Accordingly, their osmotic resistance is lowered by palytoxin in a concentration-dependent manner. 4. With both rat and sheep erythrocytes, potassium loss by far precedes the haemolysis due to palytoxin. Potassium loss is measurable already after 1 min and increases with time. After 2 hours the quotient between the ED_{50} of haemolysis and that of potassium loss is around 200. Thus palytoxin is an unusually strong but slow haemolysin of the osmotic type. The extreme prelytic potassium loss and the correlation between susceptibility and potassium content of erythrocytes points towards the relevance of ionic fluxes.

Introduction

Palytoxin is the most potent marine toxin known [1,2]. It has been isolated from coelenterates of some zoanthid species (genus *Palythoa*). It is a large molecule ($C_{121}H_{207-9}O_{61}N_3$, M_r 2681) without repeating amino acid or sugar residues [3,4,1]. It depolarises every excitable organ investigated, such as cardiac [5–7], skeletal [5,7] and smooth muscle [8,9], as well as nerve fibres [10]. Consequently, contractile organs reach a state of contracture [2,5–9]. Palytoxin promotes the release of noradrenalin in the vas deferens [9]. Since some actions of palytoxin are decreased, but not abolished in low Na^+ medium or in the presence of tetrodotoxin, sodium influx is assumed to be enhanced [5,10]. $^{45}Ca^{2+}$ uptake into rabbit aortic strips is also increased [8] and direct damage of the electrochemical coupling has been assumed [7].

Because of its very high toxicity – LD_{50} intravenously in mammals is between 0.025 and 0.45 μg/kg [11] – we started with the hypothesis of palytoxin's being a neurotoxin. However, our present study indicates that palytoxin is in fact a potent cytolysin which has a slow course of action and causes a large prelytic potassium loss *.

Abbreviation Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2'-ethanesulphonic acid.

* The structure of palytoxin has been communicated after submission of this manuscript [14].

Materials

Palytoxin was prepared by one of us (L.B.) from the zoanthid *Palythoa caribaeorum*. Briefly, the toxin was extracted from the lyophilized animals with 50% ethanol/water, and purified by gel filtration on Sephadex G-50, chromatography on QAE- and SP-Sephadex, and finally by gel filtration in Biogel P6 (Beress et al., unpublished data).The LD_{50} (mouse) was between 0.25 and 0.5 μg/kg upon intravenous injection. Autopsy revealed an extreme dilatation of the right (but not of the left) heart ventricle and accumulation of fluid in the lung, which indicates a narrowing of the pulmonary vessels as the probable cause of death after intravenous injection.

The various palytoxin preparations regularly yielded two close ninhydrin positive spots (R_F 0.67 for the major and R_F 0.75 for the minor spot) when subjected to thin-layer chromatography on 20 × 20 cm cellulose plates in pyridine/water/*n*-butanol/acetic acid (10 : 12 : 15 . 13). Unstained strips corresponding to 10 μg palytoxin were cut into 0.5 cm segments, eluted with phosphate-buffered saline and the eluates tested for intravenous toxicity in mice and haemolytic activity (see below) at various dilutions. The maxima of both actions coincided with each other and the main ninhydrin-positive spot. Thus haemolysis is apparently due to palytoxin and not to any contaminant.

Melittin was prepared in this laboratory [12].

Erythrocytes. Unless indicated otherwise, rat erythrocytes were used. 3 ml blood was taken in 0.3 ml 10% sodium citrate, centrifuged at 1000 × *g* and the supernatant discarded. The erythrocytes were washed three times with phosphate-buffered saline, pH 7.4 (123 mM NaCl/16.4 mM Na_2HPO_4/3.7 mM NaH_2PO_4).

Methods

Haemolysis was performed, if not otherwise stated, by incubating 1 ml erythrocytes (0.1%, v/v in phosphate-buffered saline) with 0.02 ml palytoxin (in phosphate-buffered saline containing 0.1% bovine serum albumin) at 37°C in a shaking water bath. After various time intervals the tubes were centrifuged for 2 min at 1000 × *g*. The extinction of the supernatant was measured at 405 nm or at 578 nm in an Eppendorf photometer.

TABLE I

pH DEPENDENCE OF PALYTOXIN HAEMOLYSIS

Rat erythrocytes were prepared in Britton-Robinson buffer (150 mM NaCl/6 mM H_3BO_3/6 mM CH_3COOH/6 mM H_3PO_4, adjusted with 0.2 M NaOH) of the pH indicated. Incubation was stopped after 1 h or 4 h by centrifugation.

Medium pH	ED_{50} (ng/ml) after incubation for	
	1 h	4 h
5.0	>200	>200
6.0	>200	0.355
7 0	237	0.016
8.0	5	0.013
9.0	43 4	0 200

Potassium and sodium were measured in the diluted supernatant using an atomic absorption spectrophotometer (Unicam SP 90 A).

TABLE II

PALYTOXIN HAEMOLYSIS OF ERYTHROCYTES FROM DIFFERENT ANIMAL SPECIES

Haemolysis was measured using 0 1% (v/v) erythrocyte suspensions as described in Methods. To estimate the absolute concentration of Na^+ and K^+, erythrocytes from the species indicated were prepared in Na^+- and K^+-free buffer (150 mM choline hydrochloride/10 mM Hepes adjusted to pH 7.4 with 0 1 M LiOH), centrifuged and lysed in water. The cell volume was determined using haematocrit tubes centrifuged at 10 000 × *g* for 5 min.

Animal species	ED_{50} (ng/ml)		Intracellular concentrations (mM)	
	4 h inc.	8 h inc.	Na^+	K^+
Sensitive				
Hog	0.75	0.45	4	115
Rat	1.23	0 56	2	101
Mouse	1.6	0.89	5	104
Rabbit	3 5	2.2	10.5	86.8
Guinea-pig	15 8	1.0	5.1	102
Man	173 (ED_{25})	4 0	6 2	97.8
	ED_{25} ng/ml			
Less sensitive				
Cattle	579	119	58 6	18 1
Chicken	>1 000	163	4 7	92
Sheep	>1 000	100	78	11

Results

The haemolysis by palytoxin is a slow process. As shown in Fig. 1, it was first noticed between 1 and 2 h and increased then quickly with time. The ED_{50} of the toxin varied in different erythrocyte preparations. Its mean value after 4 h incubation at 37°C was 1.2 ± 0.5 ng/ml (n = 10). Lowering the temperature slowed down the haemolytic process (Fig. 1) and below 15°C no haemolysis could be seen even after 8 h incubation with 200 ng/ml palytoxin. At 42°C haemolysis proceeded more quickly than at 37°C but reached approximately the same final ED_{50} after 8 h incubation.

To determine the pH dependence of palytoxin haemolysis a universal buffer system according to

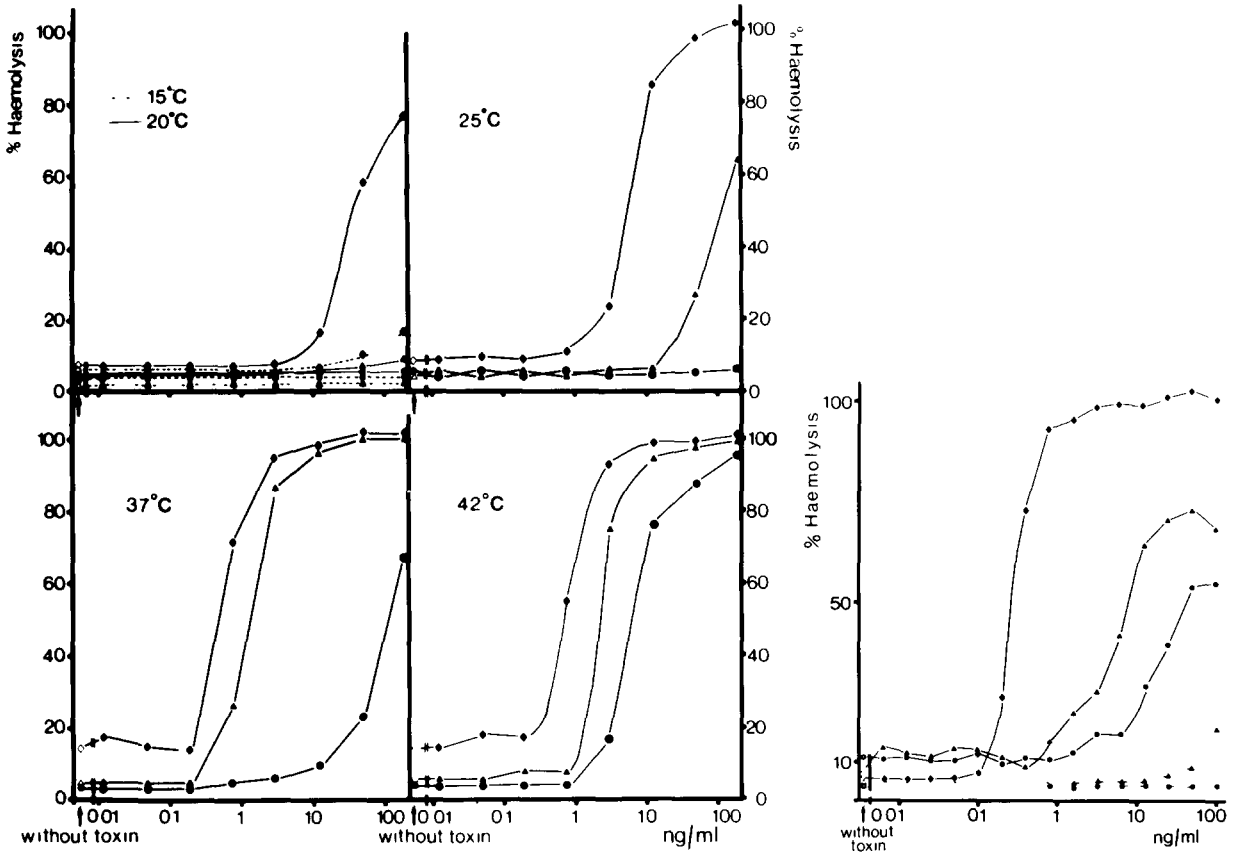

Fig. 1. Time, concentration and temperature dependence of palytoxin haemolysis. Rat erythrocytes were prepared as described in 'Methods' and incubated for 2 h (●), 4 h (▲) or 8 h (♦) at the temperatures indicated. The abscissa gives the final palytoxin concentration. Each point represents a single determination. All experiments were performed with one erythrocyte suspension.

Fig. 2. Slowing of the time course of palytoxin haemolysis by washing. Rat erythrocytes were kept with various palytoxin concentrations (abscissa) for 10 min or 60 min and centrifuged and their supernatants were replaced by fresh phosphate-buffered saline. Incubation was then continued till 4 h. A third series was incubated with palytoxin for 4 h without interruption. As indicated by the dotted lines, haemoglobin was absent in the supernatant obtained after 10 (● · · · · · · ●) and negligible after 60 (▲ · · · · · ▲) min. The further progression of haemolysis was considerably retarded by removal of palytoxin after 10 min (●——●) or 60 min (▲——▲) as compared with the result of the continuing exposure (♦——♦). Percent haemolysis (ordinate) always refers to the amount of haemoglobin present at the start of the experiment. To exclude mechanical effects due to centrifugation an additional series was centrifuged after 1 h, resuspended in the supernatant and incubated for further 3 h There was no change in the concentration response curve, except that the basal haemolysis was increased to 10%.

Britton and Robinson (Table I) was used instead of phosphate-buffered saline. The pH optimum was found to be between 7 and 8 upon 4 h incubation. The addition of borate had decreased the ED_{50} of palytoxin by a factor of 80.

Besides rat erythrocytes, palytoxin also haemolysed erythrocytes from various other species (Table II) which could be separated into two groups according to their sensitivity. Sensitive erythrocytes were from hog, rat, mouse, rabbit, guinea-pig and man, all of which have K^+ as the main inside cation. The hardly sensitive erythrocytes were from chicken, which are nucleated, and from cattle and sheep, which have Na^+ as their main inside cation.

To study the reversibility of its action, erythrocytes were treated with different concentrations of palytoxin. After 10 or 60 min the medium was replaced with toxin-free buffer and the incubation was continued till 4 h (Fig. 2). At the end of the total incubation, 50% haemolysis required the initial presence of 100 ng/ml palytoxin when the washing was done after 10 min, and 10 ng/ml when the treated erythrocytes were washed after 60 min. The ED_{50} after 4 h presence of palytoxin was 0.27 ng/ml. Thus the continuous presence of palytoxin in solution appears to be necessary for the time course of haemolysis. Removal of palytoxin slows it down considerably.

The haemolysis by palytoxin depended strongly on the osmolarity of the incubation buffer. The ED_{50} after 4 h incubation increased 200-fold when the osmolarity was raised from 235 to 415 mosM (Table III). Accordingly pretreatment with palytoxin decreased the osmotic resistance (Fig. 3).

Whereas the haemolysis by palytoxin became manifest after a lag period of 1 h or longer, release of K^+ started within the first min, had reached an ED_{50} of less than 10 ng/ml already after 10 min, and proceeded thereafter with increasing incubation time. As shown in Fig. 4 (left) the ED_{50} for K^+ loss after 1 h incubation was below 0.5 ng/ml, whereas the ED for haemolysis under the same conditions was above

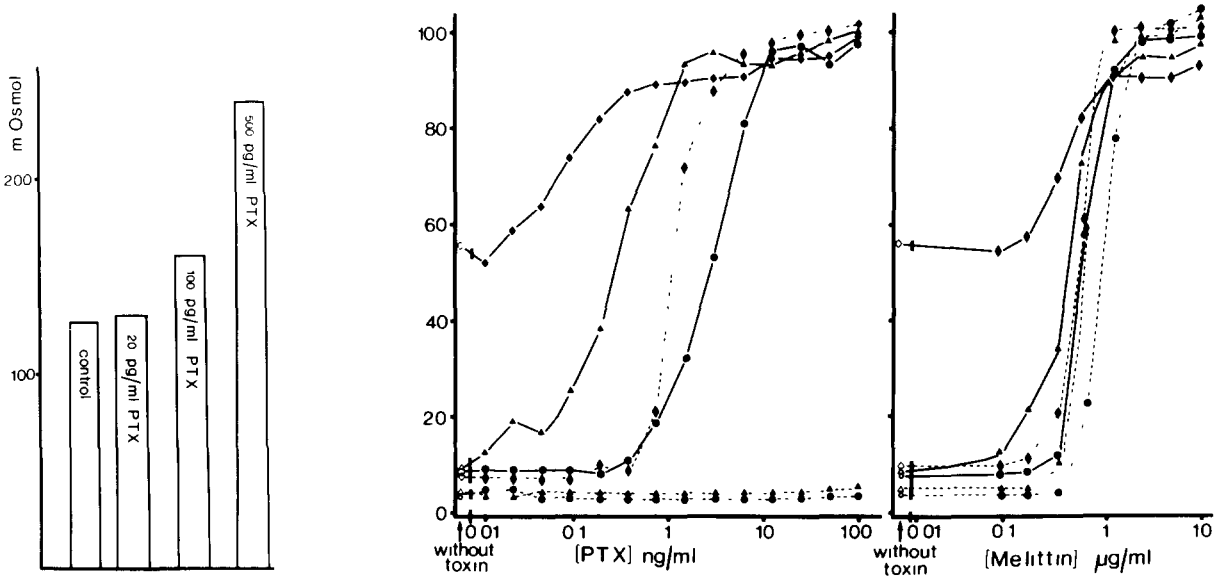

Fig. 3. Palytoxin decreases the osmotic resistance of rat erythrocytes. After 4 h treatment at 37°C in normal phosphate-buffered saline (295 mosM) with the sublytic palytoxin (PTX) concentrations indicated, the erythrocytes were transferred to phosphate-buffered saline varying from 95 to 270 mosM. Incubation was stopped after 10 min at 37°C. The ordinate gives the osmolarity leading to 50% haemolysis.

Fig. 4. Potassium loss and haemolysis in rat erythrocytes exposed to palytoxin (PTX) or melittin. Rat erythrocytes in phosphate-buffered saline were treated with either of the toxins for 8 min (•), 1 h (▲) or 8 h (♦). After centrifugation, haemoglobin and potassium were estimated in the supernatants. Each point represents a single value. (Ordinate is percentage haemolysis or K^+ loss.) Note the dissociation between potassium loss (———) and haemolysis (- - - - - -) with palytoxin but not with melittin All experiments were performed with one erythrocyte suspension.

TABLE III

DEPENDENCE ON OSMOLARITY OF PALYTOXIN HAEMOLYSIS

Rat erythrocytes were prepared in phosphate-buffered saline of 295 mosM and diluted to 0.1% in buffers with the osmolarities given. They were incubated with different palytoxin concentrations for 2 or 4 h.

Osmolarity of the buffer (mosM)	ED_{50} (ng/ml) after incubation for:	
	2 h	4 h
235	31.6	0.43
275	47.3	0.36
295	145	1.2
325	188 (ED_{25})	2.7
415	>200	81.8

100 ng/ml. In contrast, the difference between prelytic K^+ loss and haemolysis due to melittin was small and did not change with time (Fig. 4). After about 4 h potassium leaks out from the palytoxin-free controls, which forbids an extension of the experiment over longer periods. Table IV compiles the release of potassium from rat and sheep erythrocytes which are known to contain preferentially K^+ or Na^+ as cations, respectively (see also Table II). As with haemolysis, the sheep erythrocytes were again about 100-times less sensitive than those of rats.

TABLE IV

K^+ LOSS FROM RAT AND SHEEP ERYTHROCYTES

The erythrocytes (0.1% v/v) were incubated in phosphate-buffered saline with various palytoxin concentrations. After the times given, the incubation was stopped by centrifugation and both the K^+ loss and haemolysis were estimated in the supernatant. Till 60 min no haemolysis could be detected with 100 ng/ml in rat erythrocytes of 800 ng/ml in sheep erythrocytes, respectively.

Incubation time (min)	ED_{50} (ng/ml)	
	Rat	Sheep
8	3.3	335
60	0.32	39.8

Discussion

Palytoxin is a powerful, however atypical, haemolysin. Depending on the conditions, its ED_{50} is between 10 pg and 2 ng/ml, whereas that of melittin is around 500 ng/ml. Palytoxin exerts its effects on isolated organs over a comparable concentration range. In the case of the guinea-pig isolated atria, 2.5–5.0 ng/ml raised the contractility [7], 10 ng/ml led to the contraction of the guinea-pig vas deferens [9] and was active on the guinea-pig papillary muscle [8]. Still smaller concentrations (100 pg/ml) were found to be effective on electrically driven guinea-pig atria, isolated guinea-pig ileum strips and excised frog rectus abdominus muscles [5]. It may be added that palytoxin also inhibits the accumulation of [^{3}H]choline into crude rat synaptosomes, promotes the release of radioactivity from synaptosomes preptreated with [^{3}H]choline and increases the accessibility of occluded lactic dehydrogenase over concentration ranges between 10 and 100 pg/ml (unpublished data). Taken altogether, palytoxin appears to be an universal cytolysin directed against excitable and, as our present study shows, non-excitable membranes.

Palytoxin is unique as a haemolysin not only as to its potency but also as to its mode of action. It is not a protein and therefore not an enzyme. Nevertheless, haemolysis proceeds extremely slowly. Apparently haemolysis is a late process triggered by a primary lesion. The early damage manifests itself by the immediate start of a prelytic potassium loss which becomes complete long before the haemolysis starts. The positive correlation of haemolysis with temperature may be explained in various ways. The early potassium release is already shown to depend on temperature (unpublished data). Moreoever, long-lasting exposure to increased temperatures may add a physical damage to the palytoxin effects and potentiate them in that way. There is no evidence that palytoxin belongs to the haemolysins acting better in the cold, like lysolecithin or digitonin (see Ref. 13).

The crucial role of ionic fluxes is evident from other observations. The haemolysis is of the osmotic type, i.e., the ED_{50} of palytoxin decreases with the osmolarity, and erythrocytes pretreated with palytoxin under standard conditions become more sensitive towards a hypoosmotic environment applied

thereafter. Moreover, the pronounced species specificity of palytoxin haemolysis can be correlated with the ion content of erythrocytes. Particularly palytoxin-sensitive are just those erythrocytes from mammals which contain mostly potassium as their intracellular cation. Bovine and sheep erythrocytes are much less sensitive and possess more sodium than potassium as intracellular cations. Work is in progress to elucidate the role of ions in palytoxin haemolysis.

The high potency of palytoxin indicates that not much of it is 'used up' during haemolysis. Minimal binding is also evident from our so far futile attempts to inactivate palytoxin by high concentrations of erythrocytes or liposomes of varying composition (unpublished data).

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