Biochimica et Biophysica Acta, 688 (1982) 486–494
Elsevier Biomedical Press

BBA 71215

CHANGES IN ERYTHROCYTE PERMEABILITY DUE TO PALYTOXIN AS COMPARED TO AMPHOTERICIN B *

G. AHNERT-HILGER, G.S. CHHATWAL, H.-J. HESSLER and E. HABERMANN **

Rudolf Buchheim Institute of Pharmacology, Justus Liebig University, Frankfurterstrasse 107, D-6300 Giessen (F.R.G.)

(Received December 9th, 1981)

Key words: Permeability; Palytoxin; Amphotericin B; (Erythrocyte)

Palytoxin causes within minutes a temperature-dependent K^+ loss from human and rat erythrocytes which is followed within hours by haemolysis. It decreases the osmotic resistance in a concentration-dependent manner, so that osmotic influences are negligible for K^+ release but considerable in haemolysis. External K^+ inhibits the haemoglobin release and Rb^+ inhibits the release of K^+ and haemoglobin. Ca^{2+} (over 20 μM) and borate (over 5 μM) enhance the loss of K^+ and haemoglobin. With both Ca^{2+} and borate present, the efficacy of palytoxin is raised about 10000-fold. Under these conditions, about 15 palytoxin molecules per human cell trigger a 50% K^+ loss over a wide range of cell concentrations. The palytoxin effect is reversible. After depletion from K^+ by low concentrations of palytoxin, human cells can be refilled with K^+ and resealed. The pores formed by palytoxin are small. They allow the entrance of Na^+ and choline, whereas inositol is largely excluded and Ca^{2+}, as well as sucrose and inulin, are completely excluded. Amphotericin B resembles palytoxin in its ability to cause a considerable prelytic K^+ loss and to form small pores. However, it is about 1000-times weaker than palytoxin, is not inhibited by K^+ or Rb^+, is not activated by Ca^{2+} or borate, and has a negative temperature dependence. Thus palytoxin represents a novel type of cytolysin.

Introduction

Palytoxin is the common name for a group of closely related substances originating from various zoanthids of the species *Palythoa*. They consist of a long aliphatic, partially unsaturated chain with interspersed cyclic ether, hydroxyl and carbonyl groups, which starts with an amino group and ends with a hydroxyl group. The molecular weight is 2659 for palytoxin from the Tahitian *Palythoa* and 2677 for the two palytoxins from *Palythoa toxica* [1]. In appropriate concentrations palytoxin forces every contractile organ into contracture [5–7]. Since the raised sodium permeability is only partially suppressed by tetrodotoxin, general membrane damage may be assumed (for references see Ref. 2). Accordingly, palytoxin in nanomolar concentrations produces a slow haemolysis of the osmotic type. Haemoglobin release depends on the composition of the intracellular fluid and is preceded by complete K^+ loss [2]. Palytoxin is unique since it acts on erythrocytes in an ouabain-sensitive manner [4].

Nystatin [3] and its congener amphotericin B resemble palytoxin by their ability to trigger a prelytic K^+ loss. Like palytoxin, these antibiotics contain one primary amino group, many hydroxyl groups and a polyene structure. It was thus tempting to assume that amphotericin B might be

* Part of this work will be included in the thesis of H.-J. Hessler, Justus Liebig University, Giessen, F.R.G.

** To whom correspondence should be addressed.

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; EC_{50}, concentration of cytolysin which releases 50% of K^+ or haemoglobin.

0005-2736/82/0000–0000/$02.75

an, albeit less potent and structurally different, prototype of a group of cytolysins which includes palytoxin also.

We now have analysed the changes in permeability of rat and human erythrocytes under the influence of palytoxin, as compared to amphotericin B.

Materials and Methods

Palytoxin from the zoanthid *Palythoa caribaeorum* was a gift from L. Beress, Abteilung für Toxikologie, Christian-Albrechts-Universität, Kiel. Amphotericin B was from Serva, Heidelberg, Triton X-100 from Sigma, St. Louis. The various radioactive markers were obtained from Amersham-Buchler, Brunswick: [^{14}C]Inulin (6 mCi/mmol); [^{14}C]sucrose (5–15 mCi/mmol); $^{45}Ca^{2+}$ (10–40 mCi/mg); [^{14}C]inositol (225 mCi/mmol); $^{22}Na^{+}$ (25 μCi/mg).

Buffers. Two buffers were used. Part of the experiments was carried out in phosphate-buffered saline consisting of 135 mM NaCl and 20 mM sodium phosphate (pH 7.4). After having observed the activation by Ca^{2+} and borate (see p. 490) further experiments were performed in buffer A consisting of 150 mM NaCl/10 mM Hepes/1 mM $CaCl_2$/0.5 mM borate brought to pH 7.4 with NaOH. All dilutions of palytoxin and amphotericin B contained 0.1% bovine serum albumin to protect from nonspecific adsorption.

Human or rat erythrocytes were used as indicated. Haemolysis was always performed with rat cells. Human erythrocytes are less sensitive than those of the rat [2], however better known in many respects. The cells were washed three times with saline and brought to final concentrations (v/v) of 0.1–0.3% in the respective buffer. Before dilution the erythrocyte volume was determined from a 20–50% (v/v) suspension by centrifugation at $10000 \times g$ for 5 min using haematocrit tubes.

EC_{50} data. To obtain the EC_{50} data, palytoxin or amphotericin B solutions were diluted stepwise by a factor of 2 or 3 before incubating with a 50-fold volume of erythrocyte suspension. Incubation was performed at 37°C, except in those experiments given in Fig. 1. The cells were shaken with a constant frequency of 390/min. A concentation-response experiment with palytoxin or amphotericin B was always run in parallel under standard conditions, to compensate for day-to-day variations (see Ref. 2) which were particularly remarkable with haemolysis. Duration of incubation for K^+ release was 30 min and for haemolysis 4 h, if not otherwise stated.

Potassium. Potassium was measured by atomic absorption spectrometry (Unicam SP 90 A) in an aliquot of the centrifuged ($3000 \times g$) samples, and haemoglobin was determined in an Eppendorf spectrophotometer at either 405 or 578 nm. A semilogarithmic concentration-effect diagram was constructed from the release data over a wide range of palytoxin concentrations. Release by Triton X-100 was set as 100% and the EC_{50} was interpolated graphically. The slope of the concentration-response plots was given by about four data points.

Pore size. Pore size was measured by determining the entry of radioactive markers into the erythrocytes under the influence of palytoxin or amphotericin B. In Eppendorf vessels, 5 μl palytoxin or amphotericin B in buffer A containing 0.1% bovine albumin was mixed with 10 μl or about 50 nCi of the labelled permeant, diluted in 1 mM unlabelled permeant. The reaction was started by adding 200 μl of a 20% (v/v) human erythrocyte suspension in buffer A. After incubation at 37°C for 30 min 250 μl dibutylphthalate was added. Centrifugation (30 s at $8000 \times g$) resulted in the separation of cell sediment (bottom), dibutylphthalate (middle) and supernatant. The supernatant was removed and the tubes were rinsed three times with water. After the third washing dibutylphthalate was largely removed and the cells were lysed with 0.5 ml water. To prevent nonspecific adsorption of the marker, the lysate was adjusted to 0.5 M of the unlabelled permeant (except with inulin, where it was 0.005 M). The haemoglobin was precipitated by heating (10 min at 95°C) after addition of 0.5 ml buffer A. After centrifugation ^{3}H, ^{14}C and ^{45}Ca were measured in an aliquot of the supernatant in a liquid scintillation counter. Intracellular $^{22}Na^{+}$ was measured directly in a crystal well counter. Corrections were made for the inulin space of the sediment which was about 4%. The colour quench was negligible.

Electrophoresis. High-voltage electrophoresis was performed at 4°C on thin-layer (0.1 mm thick)

cellulose plates (Polygram cel 300, Macherey and Nagel, Düren). In one series, pyridine/acetic acid/water (5:1:94, v/v) (pH 6.0) was used with or without 1 mM borate. In the second series, carbonate/bicarbonate, pH 9.0 or borate/NaOH (pH 9.0) (both 20 mM) were used. Palytoxin was applied at the center of the plate and the electrophoresis carried out at a constant voltage of 1000 V for 20 min. Lysine, glutamic acid and alanine were used as standards. The mobility was determined after staining with ninhydrin.

Results

1. Dependence of K^+ loss and haemolysis on time, temperature and osmotic pressure

Palytoxin causes a complete, temperature-dependent K^+ loss from the erythrocytes followed by haemolysis. With high palytoxin concentrations (3 ng/ml), K^+ loss from the rat cells starts in the first minute and proceeds quickly (Fig. 1). Its velocity rises significantly with temperature. Particularly at low temperature, a lag period is evident. Like palytoxin, amphotericin B releases K^+ long before haemoglobin. However, amphotericin B is by weight substantially less potent than palytoxin with respect to K^+ release and haemolysis (Fig. 2). Also, as shown in Fig. 1, no lag period is evident and the velocity of K^+ release falls with increase in temperature.

The time-dependence of palytoxin action was also followed by taking concentration-response diagrams at different incubation times (human erythrocytes, buffer A, 37°C). The EC_{50} (pg/ml) was 2.8 after 10 min, 1.25 after 20 min, 0.9 after 40 min, 0.6 after 80 min and 0.45 after 160 min.

To see if the increase in permeability due to palytoxin is specific for K^+, we replaced Na^+ in the external medium by choline. Under these conditions the release of the small amounts of Na^+ present in human cells can be measured together with K^+. Both ions leave the cells with the same

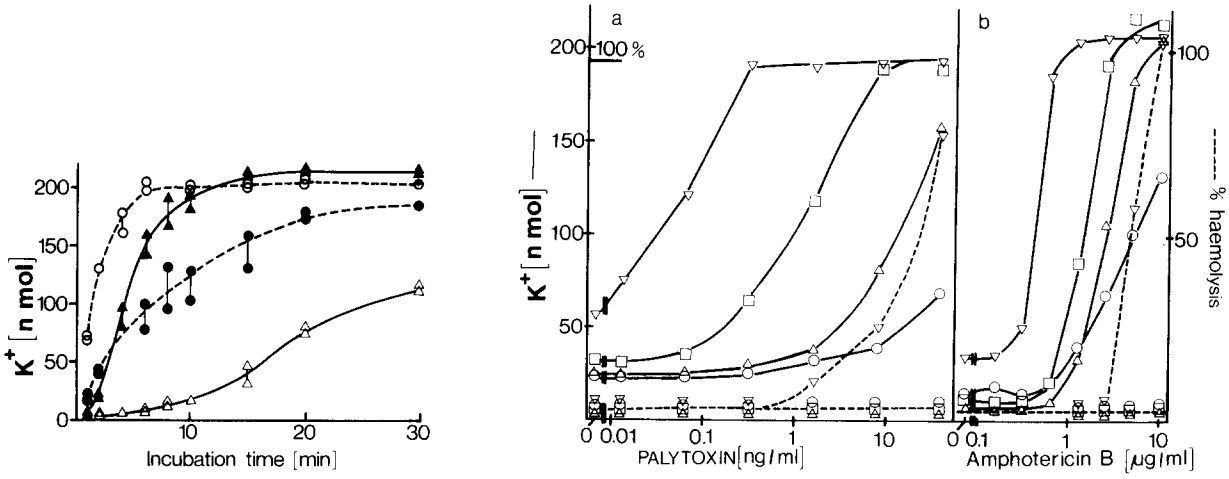

Fig. 1. (Left.) Temperature- and time-dependence of K^+ loss due to palytoxin and amphotericin B. Rat erythrocytes in phosphate-buffered saline (0.19–0.21%, v/v) were incubated at 7°C (△,○) and 37°C (▲,●) with 3 ng/ml palytoxin (—) or 3 µg/ml amphotericin B (- - - - - -). The release was stopped at the times shown by rapid (15–20 s) centrifugation at 10000× *g*. Each point represents a single value. The ordinate represents nmol K^+ released. Total K^+ was 210 nmol. K^+ loss was also tested on temperatures between 7°C and 37°C but is not shown to aid clarity. There was no maximum or minimum between the extremes shown.

Fig. 2. (Right.) K^+ loss due to palytoxin and amphotericin B by far precedes haemolysis. Rat erythrocytes in phosphate-buffered saline were kept for 20 min at 37°C. Then they were treated with palytoxin (a) or amphotericin B (b) in the concentrations given on the abscissa. Incubation was stopped by rapid centrifugation (15–20 s) at 10000× *g* at: ○, 1 min; △, 3 min; □, 10 min; ▽, 2 h. Potassium (solid line) and haemoglobin (broken line) were estimated in the supernatant. Each point represents a single value. The erythrocyte suspension used for the experiments in (a) was 0.15% (v/v) and contained 180.9±3.5 ($n=4$) nmol K^+ per ml. The data for (b) were 0.19% and 215.5±8.8 ($n=4$) nmol K^+.

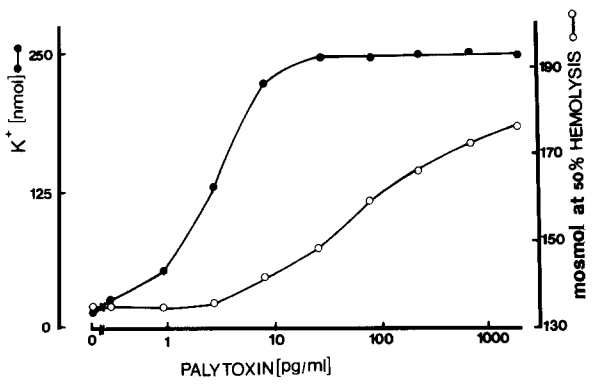

Fig. 3. Decrease in the osmotic resistance, as compared to K^+ loss. Human red cells (1 ml in buffer A, $2.5 \cdot 10^{-7}$ cells/ml) were incubated with 20 μl of various palytoxin concentrations for 30 min at 37°C as described in Materials and Methods. Then 100-μl aliquots were transferred to tubes containing saline of different osmolarities, incubated for 10 min at 37°C and centrifuged, and the haemoglobin was measured in the supernatant. A second series was run in parallel for measuring K^+ release. The K^+ release with final palytoxin concentrations (abscissa) above 10 pg/ml was complete.

EC_{50} of palytoxin [4]. The restricted specificity of permeants is also evident from our experiments on pore size, since even the permeation of inositol is enhanced (see p. 492).

Osmotic influences were studied on human cells in the presence of Hepes, borate and Ca^{2+} (see Materials and Methods). Independent of the external cation (choline or Na^+), the K^+ loss due to palytoxin did not depend on the osmolarity of the medium between 200 and 420 mosmol. The EC_{50} was 2.5 pg/ml throughout in Na^+ medium, and between 3.9 and 4.2 in the presence of choline. Accordingly, K^+ loss started before the decrease of osmotic resistance, which, however, became prominent with rising palytoxin concentrations (Fig. 3). Under the conditions of haemolysis, where about 1000-times higher concentrations of the cytolysin are applied for 4 h, palytoxin had considerably diminished the osmotic resistance. Palytoxin haemolysis largely decreased with rising osmolarity [2].

2. Inhibition by K^+ and Rb^+

Since K^+ release by far precedes haemolysis, addition of external K^+ was thought to delay the haemoglobin loss. Thus Na^+ was replaced stepwise by equivalent amounts of K^+ or Rb^+ in phosphate-buffered saline. Already 2.3 mM K^+ depressed the haemolysis by a factor of 8, and 36.4 mM K^+ abolished it. The same was true for Rb^+, although this ion was slightly less potent (Table I). In contrast, the haemolysis due to amphotericin B was even enhancced with increasing concentrations of both cations.

Assuming a basically similar action of Rb^+ and K^+ we have assessed the influence of Rb^+ on K^+ release. The efficacy of palytoxin in rat erythrocytes is decreased already with 2.5 mM Rb^+,

TABLE I

EFFECTS OF K^+ AND Rb^+ ON THE HAEMOLYSIS DUE TO PALYTOXIN AS COMPARED TO AMPHOTERICIN B

Rat erythrocytes were prepared in phosphate-buffered saline or buffer in which Na^+ had been partially replaced by K^+ or Rb^+. Haemoglobin was estimated in the supernatant after 4 h at 37°C. Erythrocyte concentrations (v/v) of 0.1% (palytoxin) or 0.2% (amphotericin B) were used in four different experiments (each column represents one experiment). EC_{50} values for palytoxin are in ng/ml; those for amphotericin B in μg/ml.

	$[K^+]$ (mM)	EC_{50}	$[Rb^+]$ (mM)	EC_{50}
Palytoxin	0	0.75	0	1.2
	2.3	6.1	2.5	5.8
	9.1	61.3	10	24.4
	36.4	>250	40	224 (EC_{25})
			123	>250
Amphotericin B	0	9	0	5
	2.5	9.3	2.5	3.1
	10	9.3	10	3.3
	40	7.2	40	2.9
	123	2.1	123	2.1

TABLE II

CONTRARY EFFECTS OF Rb^+ ON PALYTOXIN- AND AMPHOTERICIN B-INDUCED K^+ LOSS IN RAT ERYTHROCYTES

Rat erythrocytes were prepared in phosphate-buffered saline or buffer in which Na^+ was equivalently replaced by Rb^+. After 30 min incubation at 37°C K^+ loss was estimated in the diluted supernatant. In two different experiments (palytoxin, amphotericin B) the erythrocyte concentrations were 0.2%. The potassium standards, as required in atomic absorption spectrometry, were made up with the respective Rb^+ concentrations.

[Rb^+] (mM)	Palytoxin EC_{50} (ng/ml)	Amphotericin B EC_{50} (μg/ml)
0	0.1	1.3
2.5	0.22	1.1
10	0.64	1.4
40	6.5	1.2
123	12	0.82

TABLE III

ENHANCEMENT OF K^+ LOSS FROM RAT ERYTHROCYTES DUE TO PALYTOXIN IN THE PRESENCE OF Ca^{2+} IN THE EXTERNAL MEDIUM

Rat erythrocytes (0.21% (v/v) final concentration) were prepared in Hepes-buffered saline. After 30 min incubation with or without Ca^{2+} or EGTA in the concentrations (μM) given, and with varying amounts of palytoxin, K^+ was estimated in the supernatant.

[Ca^{2+}] (μM)	EC_{50} for K^+ loss (pg/ml)
0	690
31.3	160
62.5	94
125	54
250	40
500	21
0+500 μM EGTA	850

whereas that of amphotericin B is not affected over a wide range of Rb^+ concentrations, and is slightly increased with 123 mM Rb^+ (Table II). The K^+ loss from human erythrocytes in phosphate-buffered saline is slightly less sensitive to Rb^+. The EC_{50} is increased from 1.2 to 2.4 ng/ml by 5 mM Rb^+, and to 50 ng/ml by 20 mM Rb^+.

3. Activation by Ca^{2+} and borate

Since Ca^{2+} has been repeatedly reported to be involved in the palytoxin effects on isolated organs, we have looked for possible interactions on erythrocytes. The K^+ loss due to palytoxin is enhanced by the presence of Ca^{2+} in the external medium at a concentration as low as 31 μM (Table III). Conversely, EGTA slightly inhibits the K^+ release. With human cells, activation by Ca^{2+} is up to 100-fold (the EC_{50} of palytoxin for K^+ loss dropped from 7.5 ng/ml to 65 pg/ml), and again a slight decrease of potency occurs upon addition of $1 \cdot 10^{-4}$ M EGTA. The presence of Ca^{2+} (6 mM) also potentiates the haemolysis of rat cells about

TABLE IV

INFLUENCE OF BORATE ON K^+ LOSS AND HAEMOLYSIS DUE TO PALYTOXIN AND AMPHOTERICIN B

Rat erythrocytes, prepared in phosphate-buffered saline, were incubated with different borate concentrations and varying amounts of palytoxin or amphotericin B. K^+ loss was estimated after 30 min, and haemolysis after 4 h at 37°C. In the two different experiments (palytoxin, amphotericin B) the final erythrocyte concentration was always 0.2% (v/v). n.d., not determined.

[Borate] (mM)	Palytoxin EC_{50} (pg/ml) for		Amphotericin B EC_{50} (μg/ml) for	
	K^+ loss	haemolysis	K^+ loss	haemolysis
6	4	7	n.d.	n.d.
3	n.d.	n.d.	0.45	1.7
1.5	4.5	10.5	n.d.	n.d.
0.38	5	14	n.d.	n.d.
0.09	12	29	n.d.	n.d.
0	130	600	0.45	1.7

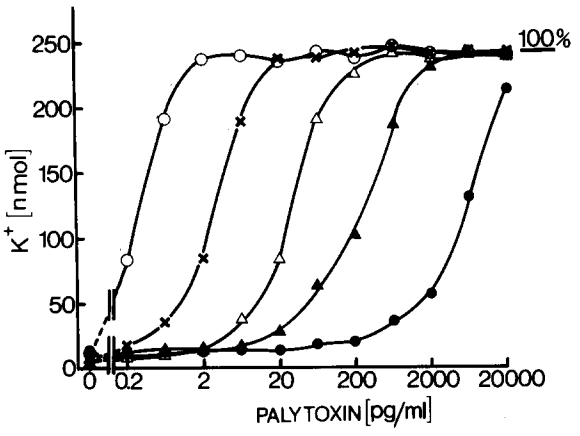

Fig. 4. Combined effects of borate and calcium ions on the palytoxin-induced K^+ loss from human erythrocytes. Palytoxin was diluted in 0.02 mM Hepes-buffered saline containing 0.1% bovine serum albumin, so that the desired amount of the cytolysin was contained in 0.02 ml. The same buffer (0.5 ml) without additive (●), or with 0.25 mM $CaCl_2$ (▲), with 2.5 mM $CaCl_2$ (△), with 1 mM borate (×), or with both 2.5 mM $CaCl_2$ and 1 mM borate (○) was added, followed by 0.5 ml erythrocyte suspension ($5 \cdot 10^7$ cells/ml) in Hepes-buffered saline. After 30 min at 37°C, the cells were spun down and K^+ determined in the supernatant. No trace of haemolysis occurred under these conditions.

1000-fold (not shown). Amphotericin B differs from palytoxin in that neither haemolysis nor K^+ loss is affected by the presence of Ca^{2+} over a wide range of concentrations.

TABLE V

NUMBER OF PALYTOXIN MOLECULES REQUIRED TO MAKE ONE HUMAN RED CELL LOSE 50% OF ITS K^+ CONTENT

The calculation is done on the basis of EC_{50} for K^+ loss from different concentations of erythrocytes. Incubation was performed for 30 min in buffer A with the six different erythrocyte concentrations given in the first column.

Erythrocyte concentration (cells/ml) ($\times 10^{-6}$)	EC_{50}		
	pg/ml	molecules/ ml ($\times 10^{-8}$)	molecules/ cell
830	50	118	14
415	30	68	16
210	14.5	34	16
105	7	16	15
53	3.4	8	15
26	2.3	5	19

Previously [2] we had noticed that replacement of phosphate-buffered saline by a buffer containing borate had much increased the haemolytic potency of palytoxin. As shown in Table IV, loss of K^+ and haemoglobin from rat cells due to palytoxin rise with the borate concentrations. EC_{50} values for K^+ loss became as small as 4 pg palytoxin/ml. The effects were still discernible with 10 μM borate. The results were essentially the same whether the erythrocytes or palytoxin were preincubated with borate before dilution. The borate effect cannot be mimicked, either on haemolysis or on K^+ loss, with amphotericin B (Table IV) or melittin (not shown). In human cells suspended in phosphate-buffered saline, the EC_{50} of palytoxin decreased from 700 pg/ml to 200 pg/ml in the presence of 5 μM borate, and to 44 pg/ml with 50 μM borate. In contrast, phenyl boronate (50 μM) was without effect.

Borate and Ca^{2+} appear to activate differently, since the effects of near-optimal concentrations of both are additive (Fig. 4). By that combination, the

TABLE VI

REVERSIBILITY OF PALYTOXIN- AND AMPHOTERICIN B-INDUCED PERMEABILITIES

A human erythrocyte suspension (total K^+, 198 nmol) in buffer A containing sucrose, 120 mM NaCl, 10 mM, Hepes, 30 mM sucrose, 0.5 mM borate and 1 mM Ca^{2+} was preincubated with palytoxin or amphotericin B for 30 min at 37°C. The cells were centrifuged down and K^+ measured in the supernatant. K^+ loss was complete upon exposure to the lysins. The cells were then washed with buffer A containing sucrose, suspended in 120 mM KCl/30 mM sucrose and incubated at 37°C for 45 min for resealing. The cells were sedimented, washed three times with buffer A containing sucrose, suspended in the same buffer and incubated for 30 min at 37°C. Finally the cells were centrifuged down and lysed with 1 ml of 0.1% Triton X-100. K^+ was determined in the lysate.

Preincubation with	K^+ loss due to the cytolysins (nmol)	Total K^+ in cells after washing, resealing and incubation (nmol)
Palytoxin (100 pg/ml)	196	135
Palytoxin (50 pg/ml)	195	134
Amphotericin B (10 μg/ml)	198	200
Buffer	5	198

EC_{50} of palytoxin for K^+ loss could be lowered about 10000-fold. About 15 molecules of palytoxin are sufficient to make one human red cell lose its K^+ by 50% (Table V) under the conditions of optimal activation. This statement holds over a wide range of erythrocyte concentrations, indicating that nonspecific binding of palytoxin to intact erythrocytes is negligible.

The effect of borate might be due to a chemical interaction with palytoxin, for instance through its many OH groups. We did high-voltage electrophoresis of palytoxin on a thin-layer cellulose plate in the presence and absence of borate. The mobility of palytoxin was not affected by borate, either at pH 6 or at pH 9, which argues against a stable complex.

4. Reversibility of the palytoxin effects

Polyene cytolysins can be used to open the cell membrane for alkali cations. The membrane reseals upon removal of the lysins [3]. Palytoxin behaves in a basically similar manner (Table VI). However, whereas the barrier function of the cell membrane can be recovered completely after amphotericin B, some leakiness remains after exposure to palytoxin.

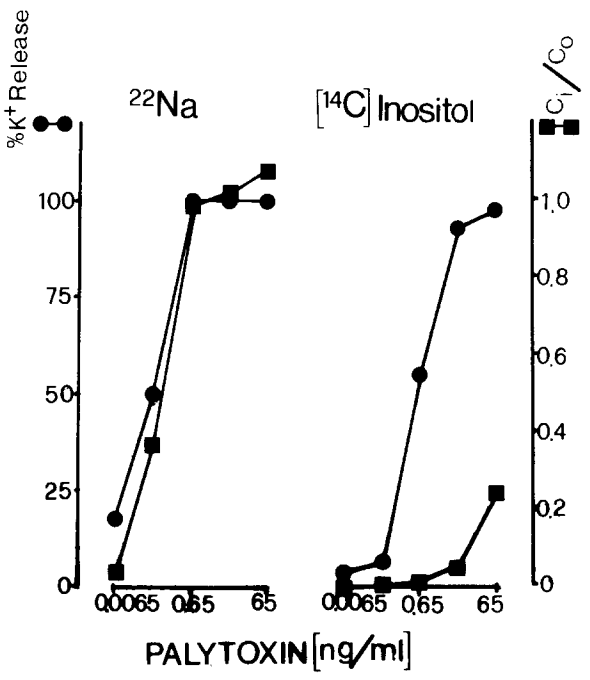

Fig. 5. Influx of ^{22}Na and [^{14}C]inositol into human red cells in relation to K^+ loss due to palytoxin. The erythrocyte suspension (hematocrit = 20% in buffer A) was incubated with the markers and various palytoxin concentrations, and the influx was measured as given in Materials and Methods. The K^+ loss due to palytoxin was determined in a parallel series of experiments. ●—●, K^+ release; ■—■, c_i/c_o (see Table VII for further data).

TABLE VII

INFLUX OF LABELLED MARKERS INTO HUMAN RED CELLS UNDER THE INFLUENCE OF PALYTOXIN AND AMPHOTERICIN B

Palytoxin 6.5 ng/ml and amphotericin B 80 μg/ml are equipotent in terms of K^+ loss from the erythrocytes (not shown). For experimental details see Materials and Methods. The experiments with palytoxin and with amphotericin B were performed with different cell suspensions.

Marker	Molecular weight	Effective radius (Å)	Relative marker concentrations (c inside/c outside) in the presence of				
			Palytoxin (ng/ml)			Amphotericin B (μg/ml)	
			65	6.5	0	80	0
[^{14}C]Inulin	5500	14.4	0.036	0.032	0	0.006	0
[^{14}C]Sucrose	542	4.4	0.074	0.065	0.045	0.085	0.08
[^{14}C]Inositol	191	3.6	0.264	0.072	0.027	0.098	0.044
$^{45}Ca^{2+}$	45	4.1	0.038	0.023	0	0.040	0.039
$^{22}Na^+$	22	3.6	1.096	1.036	0.018	0.862	0.013

5. Pore size

To determine the pore size we suspended human cells in buffer A containing radioactive permeants of different molecular sizes together with palytoxin or, for comparison, amphotericin B. Under the conditions chosen (30 min, 37°C, 20% human erythrocytes (v/v)), both palytoxin and amphotericin B form very small pores. Influx of inulin, sucrose or Ca^{2+} was too small to be measurable. Palytoxin, dependent on its concentration, facilitates the equilibration of inositol, which indicates that the pore size is not invariant. As expected, the entrance of Na^+ closely follows the efflux of K^+ (see Table VII, and Fig. 5).

Discussion

We have chosen erythrocytes as a model for the study of the mode of action of palytoxin. The general characteristics of its haemolytic potency have been described, and a large prelytic K^+ loss is prominent [2]. As shown previously, ouabain inhibits the K^+ efflux due to palytoxin from erythrocytes in a so far unknown way. Once palytoxin has contacted the erythrocyte, its surface activity might trigger perturbances in the cell membrane [4]. The present study deals with the conditions and consequences of the membrane alterations.

Palytoxin differs from other cytolysins not only by its high potency and by its ouabain sensitivity but also by its chemical structure. Thus the selection of another cytolysin for reference will always be arbitrary. We have compared palytoxin with amphotericin B because the latter also causes a prelytic potassium loss. Morever, the chemical structure resembles that of palytoxin at least with respect to the single NH_2 group, the multitude of OH groups, and the distinction of hydrophilic and hydrophobic domains. However, palytoxin molecules have no ring structure. Palytoxin, in contrast to amphotericin B, fails to release glucose from liposomes prepared with phosphatidylcholine (with or without cholesterol) as well as with lipid extracts of human erythrocytes (unpublished observation), indicating that it does not react with cholesterol or phospholipids. Also, amphotericin B effects are insensitive to ouabain. The similarities and differences between palytoxin and amphotericin B are compiled in Table VIII.

TABLE VIII

PALYTOXIN AND AMPHOTERICIN B: A COMPARISON

	Palytoxin	Amphotericin B
Structure		
Overall	linear	ring
Polyene sequences	rare	frequent
Hydrophobic domain	present	present
Hydroxyl groups	frequent	frequent
Amino group	single	single
Carboxyl group	substituted	free
Reaction with erythrocytes		
Prelytic potassium loss	yes	yes
Reversibility	partial	complete
Formation of small pores	yes	yes
Potency range	fM to pM	nM
Binding site(s)	ouabain-sensitive	sterols
Activation by borate or Ca^{2+}	yes	no
Inhibition by K^+ or Rb^+	yes	no
Temperature-dependence	positive	negative

As to the osmotic influences, we have reported previously that the palytoxin haemolysis is of the osmotic type. However, the decrease in osmotic resistance depends on the palytoxin concentration used. K^+ release starts at palytoxin concentrations not yet measurably altering the osmotic resistance, and is independent of the osmolarity over a wide range.

At low palytoxin concentration a distinct membrane alteration is prominent which can be described best by pore formation. As with the pores due to amphotericin B, their diameter is small, just permitting the exit of potassium and the entrance of Na^+ or choline (see also Ref. 4). There is some dependence of pore size on the palytoxin concentration, as seen with inositol, the size of which is apparently at the upper limit. The data on pore size should be regarded as preliminary, since they have been obtained with relatively high concentrations of erythrocytes. The increase due to palytoxin of extracellular K^+ might disturb the permea-

bility (see below), and binding to haemoglobin might influence the c_i/c_o ratio. Work on efflux of markers from resealed ghosts is in progress. We have calculated that about 15 palytoxin molecules have to be present in order to deplete on human erythrocyte of half its K^+. It is generally agreed that one human red blood cell is equipped with between 200 and 1200 ouabain-binding sites. Even if we assume that all palytoxin added is bound to the cells through ouabain-binding sites, only a fraction of them has to be occupied in order to trigger the K^+ loss (see also Ref. 4).

Two arguments speak against an irreversible interaction between palytoxin and its receptor. First, palytoxin can be washed off and the cells resealed. In this respect palytoxin resembles amphotericin B again; however, the efficacy of resealing is less. We conclude that the binding equilibrium is not completely shifted to the palytoxin-receptor complex. The K_i of palytoxin against ouabain is in the range of 10 nM, which also argues against any extreme affinity [4].

In the course of the experiments mentioned, we became aware of the unique sensitivity of palytoxin effects, both haemolysis and K^+ release, to K^+ and Rb^+ (which inhibit) and Ca^{2+} and borate (which activate). As described in the Results section, the influences are conspicuous. An appropriate combination of Ca^{2+} and borate can raise the palytoxin sensitivity of erythrocytes by a factor of up to 10000, whereas amphotericin B just retains its efficacy. So far we are able to offer no explanation but only speculation. K^+ and Rb^+ inhibit the binding of ouabain to its receptor. Since palytoxin competes with ouabain [4], it might be subject to the same inhibition. However, K^+ also inhibits the ouabain-resistant effects of palytoxin on mast cells [8]. Ca^{2+} is known to prepare erythrocytes for K^+ loss under various circumstances, for instance during resealing of erythrocyte ghosts [9], or by way of the well-known Gardós effect [10,11]. Perhaps Ca^{2+} does the same for K^+ loss due to palytoxin. It should be mentioned that the pores due to palytoxin do not allow the entance of measurable amounts of Ca^{2+}, which argues against an exchange reaction, and that palytoxin is active in the absence of extracellular Ca^{2+} albeit to a lesser extent. However, what intracellular free Ca^{2+} concentration is required to raise K^+ permeability is not known. It might be below our present detection limit, and Ca^{2+} might be shifted between intracellular subcompartments. Ca^{2+} also enhances the effects of palytoxin on mast cells [8]. The borate effect is not restricted to the action of palytoxin on erythrocytes. Borate in concentrations between 10 and 1000 μM also increases the sensitivity of mast cells to palytoxin [8] and raises the potency of palytoxin on the mouse hemidiaphragm (Dreyer, F., unpublished data). Previous injection of borate augments the lethality of palytoxin in mice (Chhatwal, G.S., unpublished data). Borate might interact either with the many OH groups of palytoxin, or it might 'prepare' the membranes for the palytoxin effects. Our electrophoresis experiment argues for the latter. Loading with borate should have changed the mobility of palytoxin, which was not the case. Surface activity of palytoxin was also not changed by borate [4].

Acknowledgements

We are indebted to Mrs. M. Laux and E. Neudert for their skillful technical assistance, and to Dr. U. Schummer, Institute of Microbiology, for his aid in atomic absorption spectrometry. G.S.C. was a fellow of the Alexander von Humboldt Stiftung, Bonn. Work was supported by a grant of the Deutsche Forschungsgemeinschaft.

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