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# Involvement of $(Na^+ + K^+)$-ATPase in binding and actions of palytoxin on human erythrocytes

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**Palytoxin (about 1 pM) increases the permeability of human erythrocytes. We now report its radiolabeling with $^{125}I$, followed by affinity purification on porcine kidney membranes. The resulting ligand binds fast and reversibly to intact erythrocytes. The $K_d$ from velocity and equilibrium measurements is $2 \cdot 10^{-11}$ M, and the number of binding sites about 200 per cell. Binding is promoted by divalent cations ($Ca^{2+} > Sr^{2+} > Ba^{2+}$) and by borate. It is inhibited by $K^+$ ($IC_{50}$ 2 mM), ouabain ($IC_{50}$ $3 \cdot 10^{-9}$ M) and ouabagenin ($IC_{50}$ $6 \cdot 10^{-6}$ M). Conversely, [$^3$H]ouabain is displaced by the substances and concentrations mentioned, and also by palytoxin ($K_i$ $3 \cdot 10^{-11}$ M). Dog erythrocytes, which are known to possess a very low $(Na^+ + K^+)$-ATPase activity, are resistant to and lack specific binding sites for palytoxin. Binding of $^{125}I$-palytoxin, like that of [$^3$H]ouabain, depends on the state of $(Na^+ + K^+)$-ATPase. ATP depletion decreases binding of both ligands to erythrocytes. Binding of $^{125}I$-palytoxin and [$^3$H]ouabain to red cell stroma is partially restored by ATP. In contrast to [$^3$H]ouabain, binding of $^{125}I$-palytoxin to red cell stroma is not promoted by $Mg^{2+}$ and $P_i$. The data show that (a) all known promoters and inhibitors of palytoxin action on human red cells do so by enhancing or decreasing its binding, (b) $(Na^+ + K^+)$-ATPase serves as a receptor for palytoxin, and (c) the antagonism by ouabain is competitive at the receptor level. They support our previous hypothesis that palytoxin increases human erythrocyte permeability by formation of pores through $(Na^+ + K^+)$-ATPase or its close vicinity.**

## Introduction

Palytoxin raises the permeability for small ions of skeletal muscles [1,2], smooth muscles [3–6], heart [7,8] and cultured nerve cells [9,10]. Human erythrocytes have turned out to be a particularly convenient model for palytoxin action. They lose their potassium long before haemoglobin [11]. Their response to the toxin is promoted by borate and $Ca^{2+}$, and is recorded in the picomolar range [12]. The initial lesion is small and reversible [13]. Many observations suggest that $(Na^+ + K^+)$-ATPase is involved in the actions of palytoxin. Ouabain [12] and $K^+$ [11] are inhibitory. ATP-depletion renders human erythrocytes less sensitive to the toxin. Red cell ghosts hardly respond to palytoxin when resealed in the absence of ATP, but they regain this property when ATP has been included into the resealing medium [14]. Dog erythrocytes, which are notorious for their defect in ATPase, are also resistant to palytoxin [12]. A possible role of the enzyme is further suggested by the competition of palytoxin for [$^3$H]ouabain binding to erythrocyte [12] and kidney [15] membranes. Finally the toxin inhibits ATP hydrolysis

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in cell membranes of different provenance [15,16].

Thus we have proposed that palytoxin raises the red cell permeability by formation of pores at or in close vicinity to $(Na^+ + K^+)$-ATPase [14]. To check the hypothesis, a radiolabeled tracer was prepared. It will be shown that the pharmacological properties of palytoxin on human red cells are strictly reflected by the binding properties of $^{125}$I-palytoxin.

## Substances

Palytoxin ($M_r$ 2680) from *Palythoa caribaeorum* [17] was iodinated according to Hunter and Greenwood [18]. In a siliconized vessel palytoxin solution (7 μl in water, 0.52 nmol) was combined with 10 μl 0.5 M sodium phosphate buffer (pH 7.5) and 20 μl carrier-free $Na^{125}I$ (2 mCi, about 1.2 nmol). Reaction was started with 5 μl chloramine T (4 nmol in water) and the same volume was added three times successively, 30 s apart. 30 s after the last addition, the reaction was stopped with 20 μl (100 nmol) sodium metabisulfite. Tris-HCl buffer (50 mM, containing 0.1% bovine serum albumin, pH 7.4) was added to a total of 250 μl. All dilutions of native or radiolabeled palytoxin below 1 μM had to be performed in the presence of 0.1% bovine serum albumin in order to avoid nonspecific adsorption.

Aliquots of the radiolabeled sample were kept aside for comparison with native palytoxin, and the remainder was subjected to gel filtration on a 1 × 40 cm Sephadex G-10 fine column with the Tris-buffered albumin solution mentioned. By that way palytoxin was separated from unreacted iodide and minor labeled contaminants.

Porcine kidney membranes [19] were used for the final affinity purification. Their 48 000 × *g* sediment (20 mg protein) was resuspended in 3 ml Tris (50 mM)-borate (1 mM) buffer equilibrated with HCl to pH 7.4. $^{125}$I-Palytoxin (20–30 μCi or ≈ 30 pmol, purified by gel filtration) was added and shaken for 30 min at 37°C. The sediment (20 min, 48 000 × *g*, 4°C) was washed twice with each 6 ml Tris-borate containing 1% bovine serum albumin. $^{125}$I-Palytoxin, together with unlabeled palytoxin, was dissociated from the pellet by overnight treatment with 2 ml 0.1 M acetic acid at 4°C. The mixture was centrifuged at 4°C and 48 000 × *g*, and the neutralized supernatant preserved at 4°C for not longer than one week.

Ouabain, ouabagenin, digitoxin, digitoxigenin and valinomycin were from Sigma, St. Louis (U.S.A.). [$^3$H]Ouabain (23–37 Ci/mmol) was from Amersham Buchler, Braunschweig (F.R.G.).

Erythrocytes were prepared from fresh human blood containing 1% trisodium citrate. The sedimented red cells were three times washed with saline and resuspended in the desired buffer. If not otherwise stated the suspension was 1% (v/v, initial concentration) for binding studies. For the measurement of potassium release, the cell concentration was 1.5%. All solutions were made in NHBC buffer, consisting of NaCl (150), Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10), $CaCl_2$ (1), borate (0.5, all in mM) brought to pH 7.4 with NaOH.

Red cells were depleted from their ATP [20] by a prolonged (12 h at 37°C) incubation without glucose, followed by 3 h treatment with inosine and iodoacetamide (5 mM each). Controls contained 11 mM glucose but no metabolic poisons.

Erythrocyte membranes were prepared according to Dodge et al. [21]. The nearly white pellet was resuspended to 5 mg/ml protein in 7 mM sodium phosphate (pH 7.4) and kept at −20°C until used.

## Methods

*Binding studies.* In the standard assay with $^{125}$I-palytoxin human erythrocytes (1% v/v in NHBC, 200 μl) were added to a mixture of tracer (2–25 fmol or 150–500 Bq, 50 μl in NHBC) and NHBC (50 μl) with or without the agents to be studied. The two latter solutions contained 0.1% serum albumin as a protective colloid. All experiments were performed in triplicate. After 30 min at 37°C the reaction was terminated with 3 ml ice-cold NHBC-albumin and the mixture immediately filtered with suction through Whatman GF-C. The glass fibre filter was quickly rinsed twice with 3 ml each of ice-cold NHBC. The retained radioactivity was measured in a Kontron autogamma counting system. By definition nonspecific binding occurred in the presence of $3 \cdot 10^{-8}$ M (final concentration) unlabeled palytoxin.

Binding of [$^3$H]ouabain was determined as de-

scribed for $^{125}$I-palytoxin. However, incubation times were prolonged to 4.5 or 6 h because of the different association kinetics of ouabain. The volume of the erythrocyte suspension was increased ten times because of the lower specific radioactivity of the tracer whose concentrations were also raised. Since the red cells are destroyed by the filtration process, haemoglobin does not interfere with [$^3$H] measurements. $K_i$ was calculated according to Cheng and Prusoff [39] from $IC_{50}/(1 + L \cdot K_A)$ where $IC_{50}$ was the concentration of inhibitor which inhibited $^{125}$I-palytoxin binding by 50%, $L$ the final concentration of the tracer and $K_A$ its association constant.

Filtration was compared with centrifugation to separate free from bound $^{125}$I-palytoxin. There was no difference with respect to specific binding, but the nonspecific binding was much higher (about 50% of total) upon centrifugation. Replacement of glass fibre by Millipore membrane filters led to an unacceptably high blank.

Unlabeled palytoxin might diminish binding of the tracer indirectly, i.e. by abolishing the $K^+$ gradient of the cells and/or increasing extracellular $K^+$ (see Fig. 9). Therefore we pretreated human erythrocytes with valinomycin ($10^{-4}$ M, final concentration) which made the cells (1% v/v) losing their total $K^+$. Palytoxin bound to, and was displaced from these suspensions as from intact cells.

*Potassium release from erythrocytes.* Human erythrocytes were suspended (1.5% v/v) in NHBC and kept on ice until used. Palytoxin and other agents were diluted in the same buffer containing 0.1% serum albumin. Aliquots of palytoxin dilutions (50 µl) were mixed with 3 ml red cell suspension and shaken for 30 min at 37°C. After centrifugation (5 min at $1000 \times g$) potassium was measured in the supernatant using a potassium-sensitive electrode (Orion, Lorch) connected to a three digit millivolt meter (PHM 84 Radiometer, Copenhagen). Calibration was performed in the same buffer.

*Thin-layer chromatography.* The radiochemical purity of the tracer was checked by TLC on silica gel plates (Riedel de Haen, Hannover, F.R.G.) with *n*-butanol/ pyridine/ acetic acid/ water (30 : 20 : 6 : 24, v/v) and on HPTLC plates silicagel $NH_2F_{254s}$ precoated, Merck, Darmstadt) with 1-pentanol/pyridine/water (7 : 7 : 6, v/v). Both yielded single spots for unlabeled and affinity-purified labeled palytoxin with the same $R_F$ values (0.44 for the first and 0.28 for the second system).

## Results

*1. Preparation and properties of iodinated palytoxin*

Under the conditions chosen, 17 ± 4% ($n = 7$) of the $^{125}I^-$ applied migrated with the palytoxin fraction in gel filtration, yielding a specific radioactivity of about 560 Ci/mmol. In two preparations a specific radioactivity of 1900 and 2300 Ci/mmol was obtained, indicating numerically an 1 : 1 iodination. The pharmacological activity, as measured by $K^+$ release, decreased by the iodinating process to 21 ± 5% ($n = 7$).

Gel filtration on Sephadex G-10 removed unreacted iodide and a faster-moving contaminant (Fig. 1). Although there were some indications for a slight retardation of $^{125}$I-palytoxin against the bulk of toxin, variation of gel filtration conditions and use of numerous ionic exchange and hydrophobic supports did not separate native from labeled palytoxin. Only 2% of the radioactivity obtained

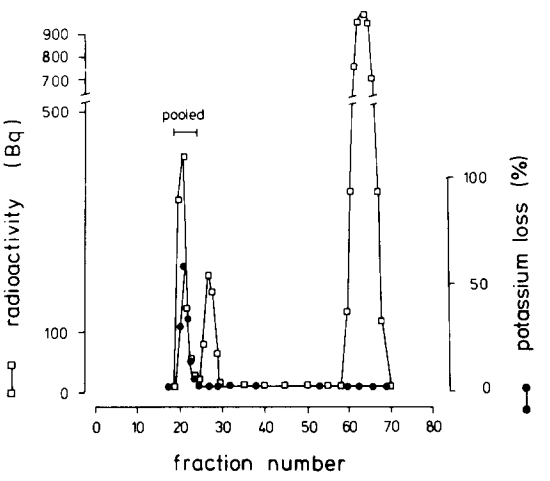


Fig. 1. Gelfiltration of $^{125}$I-palytoxin. The iodinated sample (see Substances) was run through a Sephadex G-10 fine column (40×1 cm) with 50 mM Tris-HCl (pH 7.4) containing 0.1% bovine serum albumin as eluant. Fractions of 0.55 ml were collected and dilutions (1 : 100, 5 µl) checked for radioactivity (□) and potassium loss from erythrocytes (●). Fractions 20–24 were pooled and subjected to affinity purification.

by gel filtration was bound to an excess of erythrocytes, whereas 17% of the initial radioactivity was recovered in this step. To purify further, we made use of the affinity of palytoxin to membranes [15,22], particularly those from kidneys. Recovery of biological activity and radioactivity in this step (see Methods) was 20% when referred to the combined fractions after gel filtration. Only a part of the radioactivity eluted from kidney membranes (about 20%) could be bound by an excess of erythrocytes. Repetition of affinity purification did not increase the percentage of binding. As shown by thin-layer chromatography on two systems (see Methods), the affinity purified radiolabeled palytoxin co-chromatographed with the native substance, and radioactive contaminants were nearly absent.

In order to measure the concentration of palytoxin in the final radiolabeled mixture and to calculate the specific radioactivity, two different techniques were used. Firstly we measured the pharmacological activity, i.e. $K^+$ release from erythrocytes, and compared it with a dilution series of authentic palytoxin. By that way, the pharmacological equivalents were determined (Fig. 2A). Secondly, we measured the equivalents in terms of binding by the self-displacement technique described by Roulston [23] (Fig. 2B). The values obtained by the two different approaches coincided. Thus the affinity-purified radiolabeled product behaved like original palytoxin with respect to the quotient between pharmacological potency and binding properties. The concentrations of $^{125}$I-palytoxin which are given throughout this communication are based on this combination of pharmacological and binding data. Since pharmacological and binding potency might have decreased in parallel by iodination, the data given on the concentrations of $^{125}$I-palytoxin must be regarded as relative until a separation of iodinated and native palytoxin has been achieved.

*2. General characteristics of $^{125}$I-palytoxin binding*

Association and dissociation of palytoxin is fast (Fig. 3). The association velocity constant was $9.2 \cdot 10^{-3}$ $min^{-1} \cdot pM^{-1}$. The dissociation velocity constant ($9.1 \cdot 10^{-2}$ $min^{-1}$) was the same whether $^{125}$I-palytoxin was 'chased' by an excess ($3 \cdot 10^{-8}$ M) of unlabeled palytoxin, or whether the sample

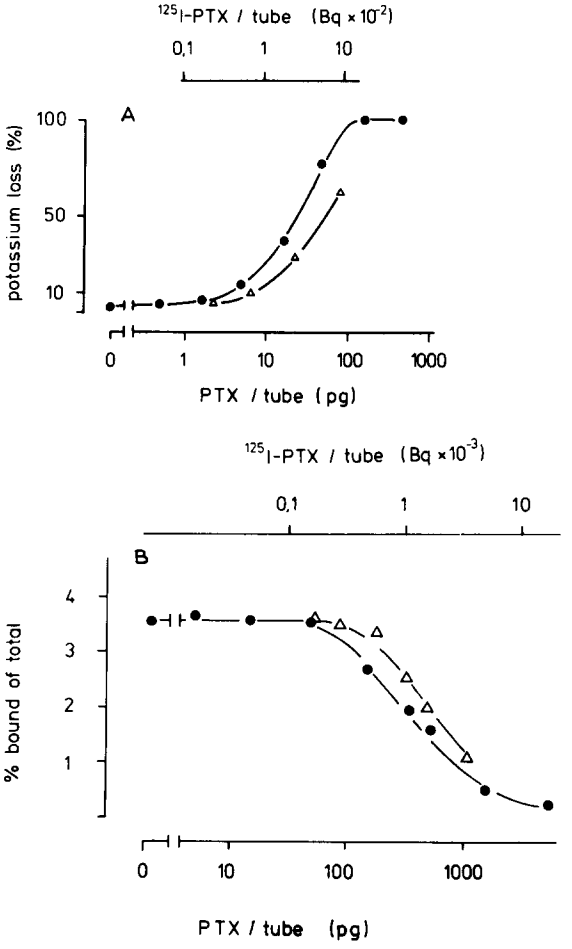


Fig. 2. Determination of the specific radioactivity of iodinated palytoxin. (A) Determination from potassium loss. The diagram shows the potassium loss from erythrocytes due to native palytoxin (●) and to $^{125}$I-palytoxin (20–1000 Bq per tube, △). For standard conditions of $K^+$ release see Methods. The specific activity was calculated by division of the $IC_{50}$-values obtained from the respective plots, yielding 530 Ci/mmol. (B) Determination from self displacement on erythrocytes. One plot (●) illustrates the competitive inhibition of $^{125}$I-palytoxin binding (200 Bq per tube) by increasing amounts of unlabeled palytoxin. The other (△) illustrates the saturation of binding with increasing amounts of $^{125}$I-palytoxin (200–3500 Bq per tube). The experiments were performed under standard conditions for binding (see Methods). Calculation of the specific activity was done as in (A). From the data a specific activity of 485 Ci/mmol was calculated.

was diluted 50-fold with buffer. $K_d$ was calculated from the velocity measurements as $10^{-11}$ M.

The influence of temperature was measured by two ways. In the first, palytoxin was incubated

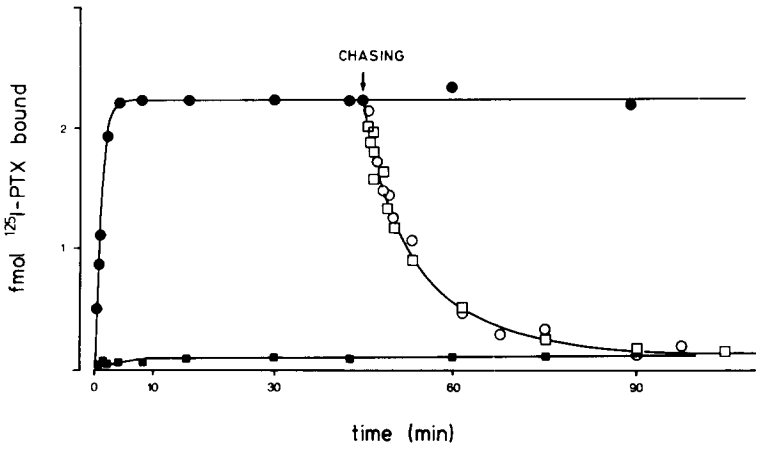


Fig. 3. Velocity of $^{125}$I-palytoxin binding to human erythrocytes. Erythrocytes (final concn. 0.67% v/v) were incubated with $^{125}$I-palytoxin (29 fmol) in a total volume of 0.3 ml at 37°C. Total (●) and nonspecific (■, in the presence of $3 \cdot 10^{-8}$ M palytoxin) binding were determined. At the indicated times duplicate samples were filtered as described. After 45 min the dissociation was induced either by 50-fold dilution (○) with NHBC buffer or by addition of an excess ($3 \cdot 10^{-8}$ M final concentration) of palytoxin (□).

with red cells for 30 min as usual, however at varying temperatures. The maximum of binding was at 30°C, with slight decreases at 23 and 37°C. When the cells were first preincubated at variable temperatures, and then the assay was run at 37°C as usual, the decreases at 37°C and lower temperatures disappeared, and the loss of binding at 45°C became more prominent (not shown). The pH-optimum was broad between 6.0 and 7.5 (not shown). Binding was in equilibrium after a few min (see Fig. 3).

Based on these experiments and on the standard conditions previously found for $K^+$ release [13], we have selected the incubation times (30 min), pH (7.4) and temperature (37°C) for further binding studies.

Binding of $^{125}$I-palytoxin to human erythrocytes is saturable (Fig. 4). Variation of the erythrocyte concentration shows that specific binding runs through a maximum between 1 and 3% of red cells (Fig. 5). Its decrease with higher cell concentrations may be due to inhibitory $K^+$ con-

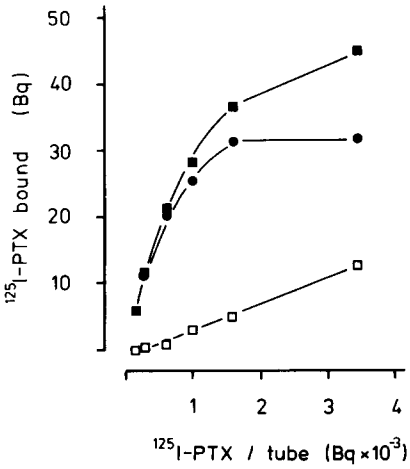


Fig. 4. Binding of $^{125}$I-palytoxin to human erythrocytes is saturable. Erythrocytes (final concn. 0.2% v/v) were incubated with increasing amounts of $^{125}$I-palytoxin in the 0.3 ml standard binding assay. Total (■), nonspecifically (□) and specifically (●) bound radioactivity are shown in dependence of the radioactivity applied (abscissa).

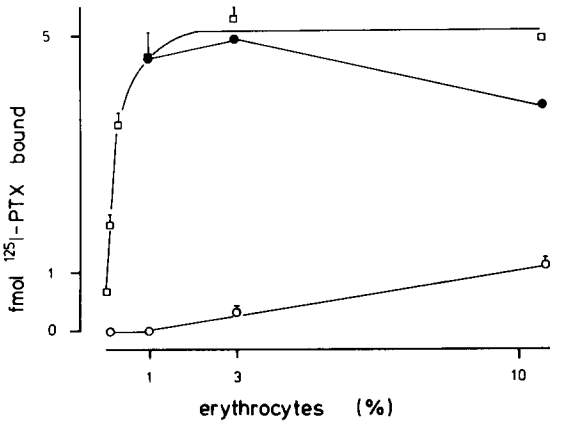


Fig. 5. Binding of $^{125}$I-palytoxin to different concentrations of erythrocytes. Erythrocytes were suspended in NHBC to give the final percentage (v/v, abscissa). The suspensions were incubated with 32 fmol $^{125}$I-palytoxin in the absence and presence of $3 \cdot 10^{-8}$ M palytoxin for 30 min at 37°C. The final volume was 0.3 ml. Total (□), nonspecific (○) and specific (●) binding are shown.

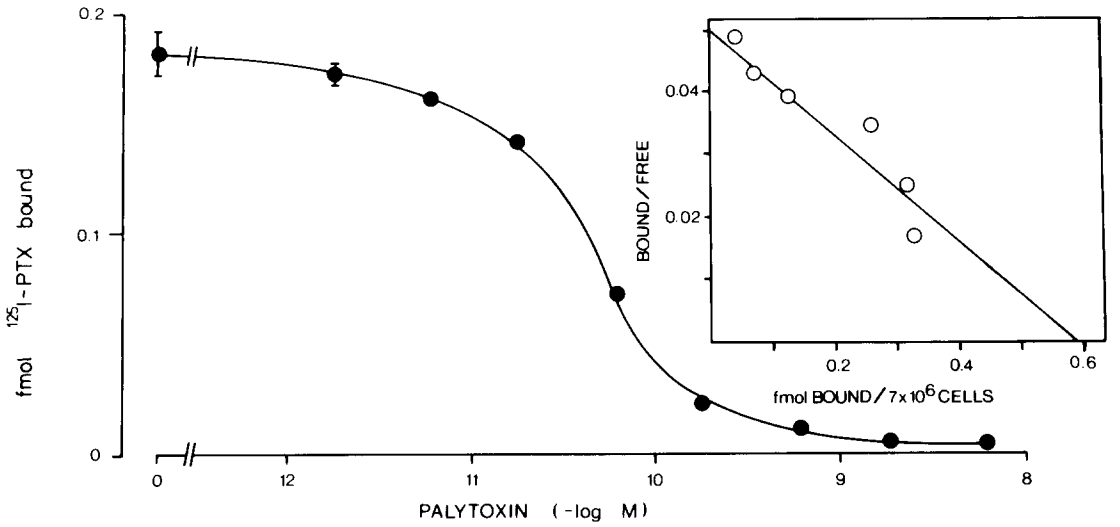


Fig. 6. Competition of $^{125}$I-palytoxin with native palytoxin for binding sites on human erythrocytes. Red cells (final concn. 0.22% v/v) were incubated with 2.4 fmol $^{125}$I-palytoxin and native palytoxin (abscissa) in a final volume of 0.2 ml for 30 min at 37°C. All data are the means of triplicate determinations. Scatchard analysis (inset) of this experiment gave a $K_d$ of 25 pM and 370 binding sites per erythrocyte.

centrations in the assay (see Fig. 9). Scatchard plots were obtained under standard conditions using a mixture of unlabeled and labeled palytoxin (Fig. 6). Taking into account that 20% of $^{125}$I-palytoxin added can be bound, the $K_d$ was calculated as $(2.2 \pm 1.5) \cdot 10^{-11}$ M, and $B_{max}$ as $202 \pm 96$ binding sites per red cell ($n = 6$). For comparison, the data from Fig. 10 for ouabain binding were evaluated according to Scatchard (not shown). $B_{max}$ was found in the same range (255 sites/cell).

Human and dog erythrocytes were compared with respect to $^{125}$I-palytoxin binding, because dog red cells are resistant to palytoxin [12] and deficient in $(Na^+ + K^+)$-ATPase. On human erythrocytes (0.67% v/v, final concentration) total binding of $^{125}$I-palytoxin (3 fmol/0.3 ml) was 0.20 fmol and nonspecific binding was 0.01 fmol under standard conditions (see Methods). The respective numbers for dog erythrocytes were only 0.01 fmol and 0.006 fmol. Thus binding to intact canine cells was negligible.

Despite of their ATPase deficiency, membranes from dog erythrocytes can bind native palytoxin [22]. Therefore we have assayed $^{125}$I-palytoxin binding on membranes prepared according to Dodge et al. [21] from both man and dog red cells. Whereas total binding was the same in both sam-

TABLE I

BINDING OF $^{125}$I-PALYTOXIN TO RED CELL MEMBRANES FROM MAN AND DOG

Membranes (0.1 mg protein) from human and dog erythrocytes according to Dodge et al. [21] were incubated with 21 fmol $^{125}$I-palytoxin in the presence or absence of 1 mM ATP and/or $3 \cdot 10^{-8}$ M palytoxin at 37°C for 30 min in a final volume of 0.3 ml and further treated as described for erythrocytes. All data are the means of triplicate determinations.

| | $^{125}$I-Palytoxin (fmol bound) | | | |
|---|---|---|---|---|
| | man | | dog | |
| | +ATP | −ATP | +ATP | −ATP |
| Total binding | 2.30±0.13 | 0.89±0.0 | 2.29±0.07 | 2.29±0.18 |
| Nonspecific binding | 0.85±0.05 | 0.83±0.02 | 2.28±0.13 | 2.30±0.09 |

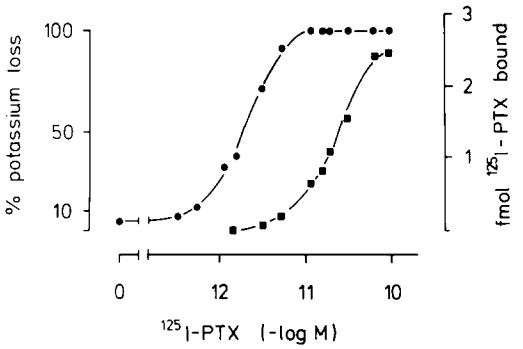


Fig. 7. Action and binding of $^{125}$I-palytoxin on human erythrocytes. Erythrocytes (1% v/v) were incubated with $^{125}$I-palytoxin (abscissa) in a final volume of 3.05 ml for 30 min at 37 °C. After centrifugation for 5 min at 1000 × *g* the potassium content in the supernatant was determined with a potassium-sensitive electrode. The radioactivity in the pellet was measured after fast resuspension in ice-cold buffer and immediate filtration through glass fibre filters. The loss of potassium (●) is shifted to the left as compared with the binding (■) to the erythrocytes.

ples, specific binding was absent in dog membranes but amounted to 63% of total in human membranes. The experiment also shows that presence of ATP is a precondition for specific binding of the tracer (Table I), which will be dealt with later.

Palytoxin releases $K^+$ in concentrations as low as a few pmol/l. The number of palytoxin molecules required per cell (about 20) for potassium release [13] is 10-times smaller than $B_{max}$ calculated according to Scatchard (see Fig. 6). In order to look for additional signs of receptor excess, we have compared binding and action of palytoxin in the same assay. The concentration-effect curve was found to be shifted by a factor of about 10 to the left of the binding diagram. Thus only a small fraction of the molecules bound is required for action (Fig. 7).

### *3. Promoters and inhibitors of $^{125}$I-palytoxin binding*

Borate not only enhances the pharmacological effects of palytoxin [13] but also promotes its binding to red cells (Fig. 8A). Half-maximal effect is achieved with borate concentrations about $2 \cdot 10^{-5}$ M, and about $10^{-4}$ M is required for full activation, when the concentration of $Ca^{2+}$ was kept optimal at 1 mM.

Earth alkali ions represent another group of activating agents, as previously shown for the $K^+$ release from erythrocytes [13,32]. To promote binding of $^{125}$I-palytoxin, 3 mM $Ca^{2+}$ was optimal (Fig. 8B). Addition of EGTA further decreased the small residual binding in the absence of added $Ca^{2+}$. We presume that EGTA removes traces of $Ca^{2+}$ present in the incubation mixture, for instance on the surface of red cells. The other earth alkali ions can be arranged, according to promotion of binding, as $Ca^{2+} > Sr^{2+} > Ba^{2+}$ (Table II), whereas $Mg^{2+}$ did not activate (not shown).

Binding of [$^3$H]ouabain was not influenced by borate or $Ca^{2+}$ (not shown).

Potassium ions are known to inhibit the palytoxin effects on erythrocytes [13]. They prevent $^{125}$I-palytoxin binding too. The $IC_{50}$ of $K^+$ is approximately the same whether $^{125}$I-palytoxin ($IC_{50}$ 2.2 mM) or [$^3$H]ouabain ($IC_{50}$ 1.5 mM) is used as a ligand (Fig. 9A). The cation also reverses $^{125}$I-palytoxin binding (Fig. 9B).

Pretreatment of the erythrocytes with ouabain

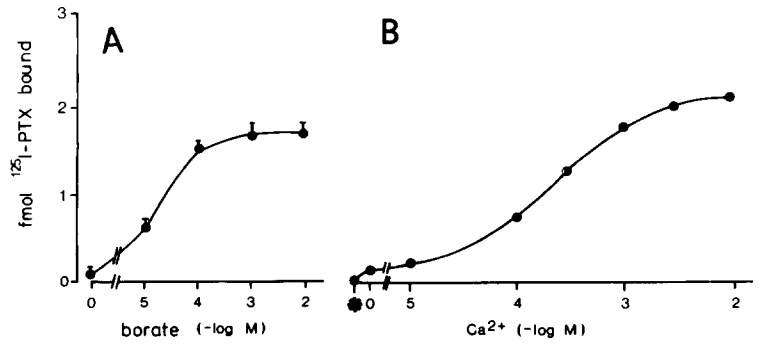


Fig. 8. Specific binding of $^{125}$I-palytoxin to human erythrocytes is promoted by borate (A) and calcium (B). $^{125}$I-Palytoxin (30 fmol) were incubated with erythrocytes (final concn. 0.65% v/v) and the given concentrations of borate or calcium in a total volume of 0.32 ml as described in Methods. In (A) the borate concentration was varied at a constant $Ca^{2+}$-concentration (1 mM) (B) EGTA (1 mM final concentration, *) or $Ca^{2+}$ (abscissa) was added at a fixed borate concentration of 0.5 mM. The ordinate shows the specific binding.

TABLE II

PROMOTION OF $^{125}$I-PALYTOXIN BINDING BY DIVALENT CATIONS

Human erythrocytes (final concn. 0.65%) were incubated with the given cations, EGTA (final concentration 1 mM for all additives) or buffer alone (NHB: NaCl 150 mM/Hepes 10 mM/borate 0.5 mM, pH 7.4) for 30 min at 37°C. Nonspecific binding was determined in the presence of $3 \cdot 10^{-8}$ M palytoxin. Total volume was 0.31 ml, containing 13 fmol $^{125}$I-palytoxin.

| Cation added | $^{125}$I-Palytoxin (fmol bound) | |
|---|---|---|
| | total binding | nonspecific binding |
| none | $0.09 \pm 0.01$ | $0.06 \pm 0.01$ |
| EGTA | $0.05 \pm 0.01$ | $0.04 \pm 0.02$ |
| $Ca^{2+}$ | $0.91 \pm 0.02$ | $0.10 \pm 0.01$ |
| $Sr^{2+}$ | $0.73 \pm 0.03$ | $0.08 \pm 0.02$ |
| $Ba^{2+}$ | $0.23 \pm 0.01$ | $0.04 \pm 0.01$ |
| $Mn^{2+}$ | $0.09 \pm 0$ | $0.04 \pm 0$ |

inhibits the action of palytoxin [12]. Cardiac glycosides and their aglycons were compared with unlabeled palytoxin with respect to competition with $^{125}$I-palytoxin binding. All agents were inferior against palytoxin whose $K_d$ was $2 \cdot 10^{-11}$ M. Ouabain and digitoxin inhibited $^{125}$I-palytoxin

TABLE III

ATP DEPLETION DIMINISHES SPECIFIC BINDING OF $^{125}$I-PALYTOXIN AND [$^3$H]OUABAIN

Red cells were pretreated according to Ref. 20, washed and resuspended to 10% (for ouabain binding) and 1% (for palytoxin binding), respectively. Under standard conditions (see Methods), the cells were exposed to 210 fmol [$^3$H]ouabain or 16 fmol $^{125}$I-palytoxin, with or without excess ($10^{-4}$ M for ouabain and $3 \cdot 10^{-8}$ M for palytoxin) of unlabeled drug, for 4.5 h at 37°C. In the controls 8.5 fmol [$^3$H]ouabain and 0.53 fmol $^{125}$I-palytoxin were specifically bound, which was set as 100%. Given are the residual specific binding values after the two pretreatments.

| Preincubation | % specifically bound | |
|---|---|---|
| | $^{125}$I-palytoxin | [$^3$H]ouabain |
| None | 100 | 100 |
| With glucose, but without depleting agents | 77 | 81 |
| Without glucose, but with inosine and iodoacetamide | 1 | 17 |

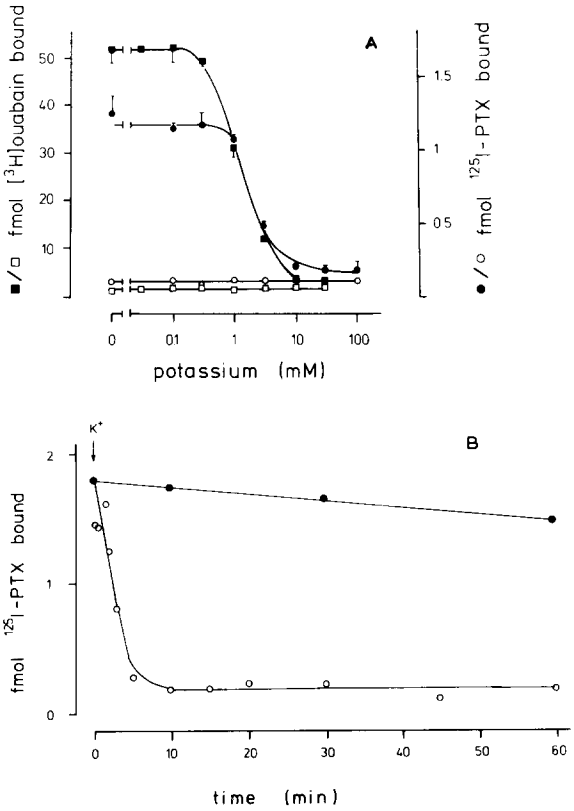


Fig. 9. Influence of potassium ions. (A) Prevention of $^{125}$I-palytoxin and [$^3$H]ouabain binding. $^{125}$I-palytoxin (20 fmol, circles) was incubated with erythrocytes (final concentration 0.67% v/v) at 37°C for 30 min with the given concentrations of potassium chloride. Total volume was 0.3 ml. For ouabain binding erythrocytes (0.7% v/v in a final volume of 3.1 ml) were incubated with [$^3$H]ouabain (3.65 pmol, squares) at 37°C for 6 h with the given concentrations of potassium chloride. Nonspecific binding was determined in the presence of $3 \cdot 10^{-8}$ M palytoxin (○) or $10^{-4}$ M ouabain (□). (B) Reversion of $^{125}$I-palytoxin binding. The cell suspension (0.3 ml, final concentration 0.65% v/v) was preincubated for 45 min at 37°C with 28 fmol $^{125}$I-palytoxin. Then potassium chloride (10 µl, 1 M) was added. Triplicates were diluted and filtered at the times given on the abscissa. ● represents $K^+$-free controls.

binding with $K_i = 3 \cdot 10^{-9}$ M, and digitoxigenin with $K_i = 8.5 \cdot 10^{-8}$ M, ouabagenin ($K_i = 6 \cdot 10^{-6}$ M) being the least affine competitor. Conversely, binding of radiolabeled ouabain was inhibited not only by ouabain ($K_d$ $2.6 \cdot 10^{-9}$ M) and (about 400-times less) by ouabagenin ($K_i$ $1.1 \cdot 10^{-6}$ M) but also with particularly high affinity by palytoxin, whose $K_i$ was as low as $2.6 \cdot 10^{-11}$ M (Fig. 10). Comparison between these data shows that the

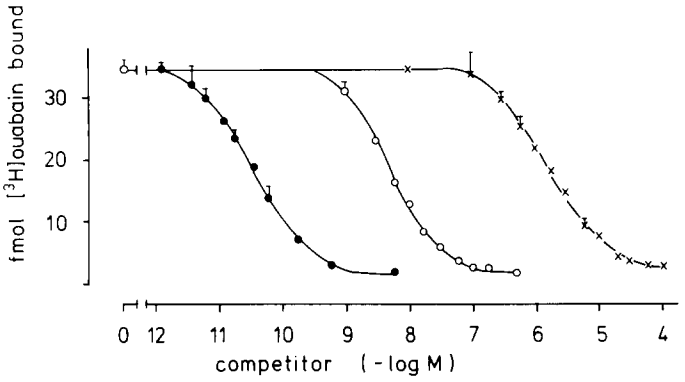


Fig. 10. (left) Competition of [$^3$H]ouabain with palytoxin (●), ouabain (○) and ouabagenin (×) for binding sites on human erythrocytes. [$^3$H]Ouabain (3.65 pmol) and erythrocytes (3 ml, 0.7% v/v) were incubated with the respective agent in a final volume of 3.1 ml for 6 h at 37°C.

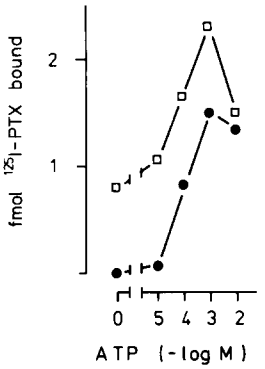


Fig. 11. (right) ATP promotes binding of $^{125}$I-palytoxin to membranes from human erythrocytes. The membranes (0.1 mg in 0.2 ml NHBC) were preincubated with ATP for 15 min at 25°C. Then the binding assay was started by their addition to the tracer (24 fmol $^{125}$I-palytoxin) in the presence and absence of $3 \cdot 10^{-8}$ M palytoxin. The final volume was 0.3 ml, and the other conditions were described for binding on erythrocytes. Total (□) and specific (●) binding is shown at various final concentrations of ATP (abscissa).

individual unlabeled ligands exert approximately the same affinities for the binding sites of [$^3$H]ouabain and $^{125}$I-palytoxin.

In order to assess the role of intracellular ATP for palytoxin binding, cells were depleted by treatment with metabolic inhibitors in the absence of glucose. The procedure lowers the ATP concentration below 0.5 $\mu$M [20]. Such cells had largely lost their ability to bind $^{125}$I-palytoxin and also [$^3$H]ouabain, whereas non-poisoned control cells largely retained binding of both ligands (Table III).

Provided intracellular ATP is required for the interaction between palytoxin and the outside membrane surface, the nucleotide should also promote fixation of the ligand to the stroma preparation according to Dodge et al. [21]. It is evident from Fig. 11 that ATP concentrations at $10^{-4}$ M and above promote specific binding. ATP is most prominent in this respect, whereas ADP and GTP are of minor potency, and the non-hydrolysable analogs and AMP are inactive (Table IV). As far as investigated, the same is true for [$^3$H]ouabain binding. But unlike $^{125}$I-palytoxin, binding of [$^3$H]ouabain is considerably promoted by $Mg^{2+}$ and $P_i$, which indicates a different mode of interaction of the two radioactive ligands with ($Na^+ + K^+$)-ATPase (Table IV).

TABLE IV

INFLUENCE OF NUCLEOTIDES, AND Mg WITH $P_i$, ON SPECIFIC BINDING OF $^{125}$I-PALYTOXIN AND [$^3$H]OUABAIN TO HUMAN RED CELL MEMBRANES

Membranes (0.1 mg protein for $^{125}$I-palytoxin and 0.5 mg protein for [$^3$H]ouabain in 0.2 ml NHBC) were incubated with the tracers (16 fmol for $^{125}$I-palytoxin and 210 fmol for [$^3$H]ouabain) in the presence and absence of unlabeled substance ($3 \cdot 10^{-8}$ M palytoxin or $10^{-4}$ M ouabain) at 37°C for 4.5 h in NHBC (final volume 0.3 ml) and further processed as described in Methods. The final concentration of the nucleotides was 1 mM. Binding in the presence of Mg and $P_i$ (3 mM each) was assayed in 50 mM Tris-HCl buffer (pH 7.4) with 1 mM borate. Binding of the tracers was in the same range in both buffer systems. All data are the means of triplicate determinations. n.d., not determined.

| Substance added | Specific binding (fmol) | |
|---|---|---|
| | $^{125}$I-palytoxin | [$^3$H]ouabain |
| None | 0.01 | 0.18 |
| ATP | 0.24 | 2.36 |
| ADP | 0.11 | 0.62 |
| AMP | < 0.01 | n.d. |
| [S]pppA | < 0.01 | n.d. |
| p[$CH_2$]ppA | < 0.01 | n.d. |
| GTP | 0.06 | 0.36 |
| $Mg^{2+}$, $P_i$ | < 0.01 | 2.12 |

## Discussion

*1. Properties of radiolabeled palytoxin*

Iodinated palytoxin of high specific radioactivity was required because the toxin releases $K^+$ from red cells in concentrations of less than a femtomole per assay [12]. However, tyrosine and histidine are absent from the toxin molecule, and its free ω-amino group is indispensable for the pharmacological actions (Refs. 24, 25, and own unpublished data), which forbids the introduction of $^{125}I$ according to Bolton and Hunter [26]. We have taken advantage of the known interaction of iodine with unsaturated carbon bonds. Thereby arachidonic acid yields iodinated products [27,28]. Presumably some of the eight double bonds in palytoxin are iodinated like those in fatty acids.

The native palytoxin appeared homogeneous in all tests for purity, which does not exclude the coexistence of isomers differing by their relative pharmacological potencies and/or by susceptibility to radiolabeling. Palytoxin has 64 centers of optical asymmetry. Even if native palytoxin is homogeneous in terms of stereochemistry, iodination may alter potency and/or binding behaviour. Because there is so far no way to separate labeled from native palytoxin, the crude radioiodinated product is a mixture of native and iodinated, among them partially inactivated compounds. Further bio-specific purification was mandatory.

We have subjected the tracer to an adsorption-desorption step on membranes rich in $(Na^+ + K^+)$-ATPase. The enzyme is known to bind palytoxin [15,16]. A similar procedure has been used for parathormone on kidney membranes [29]. Final radiochemical homogeneity was ascertained in two systems of thin-layer chromatography. The purified material was characterized by its ability to release $K^+$ from erythrocytes and to undergo self-displacement. The two independent tests were found to correlate in quantitative terms which indicates parallelism between binding and effect.

*2. Binding on erythrocytes*

Without exception, specific binding of $^{125}I$-palytoxin to intact red cells mirrors the pharmacological effects of palytoxin described here and before on this substrate, their activation and inhibition [12–14,22]. Conversely, the strict correlation may be taken as proof for the utility of our tracer and argues against an iodination artefact.

$^{125}I$-Palytoxin affinity is high ($K_d$ in the range of $10^{-11}$ M), as expected from the unusual potency of the toxin. Although $B_{max}$ is very low (about 200 sites per cell), it is still above calculations [13] that about 20 molecules per cell are statistically sufficient to deprive erythrocytes from their $K^+$ by 50%. The present binding data indicate that partial receptor occupation is sufficient to exert the full effect. We have now found a difference by a factor of 10 between $IC_{50}$ and $K_d$, both measured on erythrocytes in the same assay, which fits to the quotient between $B_{max}$ and the pharmacological data mentioned. $B_{max}$ is in the order of magnitude of red cell $(Na^+ + K^+)$-ATPase, which has been reported to contribute between 200 and 1200 molecules per cell [30]. $^{125}I$-Palytoxin binding is reversible, and so is the membrane damage by small palytoxin concentrations [13].

Influence of activators and inhibitors is also congruent for binding and action. For instance, borate is known to promote all effects of palytoxin studied [12,15,31]. It also enhances binding of unlabeled palytoxin to brain matter [22] and, as communicated here, of $^{125}I$-palytoxin to erythrocytes. Borate reacts with polyol structures like those in palytoxin, and may coerce the toxin into a conformation advantageous for binding.

Similarly, $Ca^{2+}$ enhances the $K^+$ release from red cells by palytoxin, but not by related cytolysins [13]. It favours binding of unlabeled palytoxin to brain membranes [22]. Now we have found that $Ca^{2+}$ also promotes binding of $^{125}I$-palytoxin to red cells. We have shown that $Sr^{2+}$ and, slightly, $Ba^{2+}$ can partially replace $Ca^{2+}$ in the binding assay, as they do with respect to $K^+$ release from erythrocytes [32]. Promotion of binding is sufficient to explain the enhancement of action. It is no longer necessary to dispute [14] a possible Gardos effect.

$K^+$ in the millimolar range inhibits and reverses $^{125}I$-palytoxin binding. In the same concentration range, it inhibits [$^3H$]ouabain binding. Likewise it blocks $^{86}Rb$ release and haemolysis by unlabeled palytoxin [13]. $K^+$ sensitivity indicates that palytoxin is a ligand of ATPase.

*3. $^{125}$I-Palytoxin binding and ($Na^+ + K^+$)-ATPase*

Many previous data have indicated close similarities, but not identity between the binding sites for ouabain and palytoxin. Palytoxin, with kinetics distinct from ouabain, can displace [$^3$H]ouabain [12]. Like ouabain, the toxin inhibits ($Na^+ + K^+$)-ATPase [15,16], however, in a range far above its $IC_{50}$ on erythrocytes. The palytoxin effects on human erythrocytes [12,14], on smooth muscle [4–6,33], and on *Torpedo californica* electric tissue [34] all are susceptible to inhibition by ouabain.

The binding data prove that the antagonism occurs at the receptor level. Palytoxin competes not only with $^{125}$I-palytoxin but also with [$^3$H]ouabain, both in the concentration range of about $10^{-11}$ M. Conversely, ouabain displaces $^{125}$I-palytoxin, again in the same $10^{-9}$ M concentration range as it does with [$^3$H]ouabain, and $10^{-6}$ M concentrations of ouabagenin are required for displacing either ligand.

Receptor occupation by ouabain depends on the ionic environment and on the ATP content of the cell. It is inhibited by external $K^+$ [30]. It has already been mentioned that $K^+$ suppresses binding of both $^{125}$I-palytoxin and [$^3$H]ouabain in the same millimolar concentration range. As to ATP it has been shown that depletion of energy-rich phosphates much diminishes the potency of palytoxin [14]. By the same procedure, binding of both $^{125}$I-palytoxin and [$^3$H]ouabain are depressed. Conversely specific binding of $^{125}$I-palytoxin is nearly missing on red cell membranes. Addition of ATP restores it, again according with [$^3$H]ouabain binding. This finding indicates that both ligands preferentially interact with the $E_2P$ state of ($Na^+ + K^+$)-ATPase, provided $K^+$ is low.

On the background of these close similarities, four major differences between ouabain and palytoxin are noteworthy.

First, and most importantly, palytoxin increases fast and strongly the $K^+$ permeability, which does not occur with ouabain. In terms of pharmacology, palytoxin is to be regarded as an agonist, and ouabain as its antagonist.

Inhibition of ($Na^+ + K^+$)-ATPase is not the cause of the palytoxin effects, although it may accompany cytolysis. Otherwise ouabain should also release $K^+$.

Second, displacement by ouabain and its genin of $^{125}$I-palytoxin runs parallel with their potency as palytoxin antagonists. Ouabain is about 1000-times more potent than ouabagenin in inhibiting palytoxin actions [35] and in displacing $^{125}$I-palytoxin whereas both steroids are about equipotent as inhibitors of brain ($Na^+ + K^+$)-ATPase [35,36]. The data, like pharmacological observations by others [36] seem to indicate that inhibition of ($Na^+ + K^+$)-ATPase and inhibition of palytoxin actions by cardenolides are not strictly coincident, provided the erythrocyte enzyme behaves like the reference enzyme from brain. However, as long as the activity of the red cell ATPase has not yet been measured in situ, the pharmacological actions of palytoxin and ouabain cannot be compared quantitatively at their putative target.

Third, presence of $Mg^{2+}$ and $P_i$ favours the binding of [$^3$H]ouabain, but not of $^{125}$I-palytoxin. This difference can so far not yet be explained. $Mg^{2+}$ and $P_i$ probably promote the shift from the $E_1$ to the $E_2$ state of ($Na^+ + K^+$)-ATPase [37,38].

Fourth, rat erythrocytes are particularly sensitive to palytoxin [12] and bind $^{125}$I-palytoxin. However, ouabain inhibits binding of $^{125}$I-palytoxin (own observations) and potassium release by palytoxin [12,36] only marginally on these cells. Rat ATPase is known to be particularly insensitive to ouabain.

Taken altogether, our binding data strongly support our previously [14] given scheme on the interactions between palytoxin, ouabain and human red cell ($Na^+ + K^+$)-ATPase, which had been delineated from pharmacological studies. In the presence of the toxin, the enzyme or its close vicinity are converted into a small pore which allows the movement of small ions. Ouabain serves as a competitive antagonist. Future studies will be devoted to the question whether this mode of action of palytoxin is restricted to the erythrocyte or extends to membranes of other intact cells. The pharmacological role of so-called 'nonspecific' binding deserves further attention, because it may hide still another mode of action of palytoxin.

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