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Palytoxin Induces K^+ Efflux from Yeast Cells Expressing the Mammalian Sodium Pump

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SUMMARY

Palytoxin causes potassium efflux and sodium influx in all investigated animal cells. Much evidence points to the sodium pump (Na^+/K^+-ATPase) as the target of the toxin. A heterologous expression system for mammalian Na^+/K^+-ATPase in the brewers yeast *Saccharomyces cerevisiae* has been used to test this hypothesis. Yeast cells do not contain endogenous sodium pumps but can be transformed with vectors coding for the α and β subunits of the mammalian sodium pump. We now show that transformed yeast cells expressing both α and β subunits of Na^+/K^+-ATPase are highly sensitive to the toxin, as measured by the loss of intracellular potassium. Palytoxin-induced potassium efflux is completely inhibited by 500 μM ouabain. In contrast, nontransformed yeast cells or cells expressing either the α or β subunits are insensitive to palytoxin. Thus, the α/β heterodimer of the sodium pump is required for the release of potassium induced by palytoxin. The results suggest that palytoxin converts the sodium pump into an open channel, allowing the passage of alkali ions.

Palytoxin is synthesized by corals of the family Palythoa (e.g., *Palythoa caribaeorum*) (1) and is the most potent animal toxin known. In rabbits, mice, and guinea pigs it has an LD_{50} of about 10–250 ng/kg of body weight (1, 2). For humans it is a food poison because it occurs, perhaps secondarily, also in crabs. Palytoxin is a rather unique and large molecule with the structural formula $C_{129}H_{223}N_3O_{54}$ (3, 4). It consists of three residues connected by peptide bonds, i.e., a large amino-terminal polyhydroxy ω-amino acid followed by a dehydro-β-alanine residue and an aminopropanol group (3, 4). The number of free hydroxyl groups is 42 (3, 4).

Palytoxin promotes Na^+ influx into and K^+ efflux from vertebrate cells, with both ions following their electrochemical gradients (5–10). Entry of sodium promotes Na^+/Ca^{2+} exchange and depolarization. The latter process opens voltage-dependent Ca^{2+} channels. The resulting increase in intracellular Ca^{2+} concentration triggers numerous secondary pharmacological actions of palytoxin on contractile and secretory cells (1). It is K^+ efflux, however, that can be measured easily in cell suspensions of erythrocytes.

Ouabain and other cardioactive steroids inhibit the action of palytoxin in erythrocytes and smooth muscle from many species. This observation has led to the conclusion that the sodium pump is involved in the binding of palytoxin (11, 12). In several tissues, however, ouabain does not affect the action of palytoxin (13, 14), and membrane constituents other than the sodium pump are thought to be involved as targets for the toxin (15).

Na^+/K^+-ATPase is an oligomeric membrane-spanning enzyme of animal cells. It consists of a catalytic α subunit of M_r 112,000 and a β subunit of M_r 56,000 (16, 17). The α subunit binds and hydrolyzes ATP, occludes Na^+ or K^+ ions, and recognizes cardioactive steroids, which inhibit the catalytic activity of the enzyme (16, 17). The role of the β subunit is largely unknown, but it apparently stabilizes the α subunit and is required for the transport of the α subunit from the endoplasmic reticulum through the Golgi apparatus to the extracellular membrane (18–20). Recent findings indicate that the β subunit, although it is not directly involved in ATP hydrolysis or ion transport, is absolutely required for Na^+/K^+-ATPase activity (18, 21).

Comparison of the effects of palytoxin on cells expressing Na^+/K^+-ATPase with its effects on cells lacking this enzyme would be an appropriate approach to test the hypothesis of the involvement of the sodium pump in the action of palytoxin. Because animal cells lacking the sodium pump are unknown, we used a heterologous expression system of mammalian Na^+/K^+-ATPase in the yeast *Saccharomyces cerevisiae* to address the aforementioned issue. Yeast cells do not contain an endogenous sodium pump whose presence could interfere with the intended measurements. Both α and β subunits of the mam-

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ABBREVIATIONS: Na^+/K^+-ATPase, sodium- and potassium-activated adenosine 5′-triphosphatase (EC 3.6.1.37); EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ECL, enhanced chemiluminescence.

malian sodium pump can, however, be expressed in yeast (18, 21). They assemble to form a functional enzyme that, like the native mammalian sodium pump, hydrolyzes ATP, transports K^+ and Na^+ ions, and is inhibited by ouabain (18, 21). The major questions of the study presented here are whether nontransformed yeast cells are insensitive to palytoxin, whether expression of either α or β subunits or the complete α/β heterodimeric enzyme renders the cells sensitive to the toxin, and whether the effects in transformed yeast cells are similar to those observed in erythrocytes or other mammalian tissues.

Materials and Methods

Vectors and cells. Three different vectors were used to transform yeast, i.e., pCGY1406$\alpha\beta$, YEpα3, and pCGY1406β. Vector pCGY1406$\alpha\beta$ codes for sheep kidney α1 subunit and dog kidney β subunit. YEpα3 is a derivative of YEp1PT and codes for rat α3 subunit. The construction of these vectors has already been reported (18). To prepare vector pCGY1406β, pCGY1406$\alpha\beta$ was digested with *Kpn*I to remove the α1 cDNA and was ligated to a circular plasmid with T4 DNA ligase. All three vectors are shuttle vectors that provide ampicillin resistance to bacteria and tryptophan auxotrophy to yeast cells. For transformations according to the lithium acetate method (22) the trp^- *S. cerevisiae* strain 20B12 (18) was used. Transformed yeast cells were grown in selective minimal medium, and nontransformed cells were grown in minimal medium supplemented with tryptophan.

Western blot of expressed α and β subunits of Na^+/K^+-ATPase. Yeast cells transformed with either pCGY1406$\alpha\beta$, pCGY1406β, or YEpα3 were grown for 30 hr in selective minimal medium (to A^{600} = 5–8). Cells were collected by centrifugation at 1500 × *g* and were washed twice with ice-cold water. The cells were then suspended at 1 g/ml in 200 mM Tris·HCl, pH 7.4, 10% glycerol, 2 mM dithiothreitol, 1 mM EDTA, 0.5 mg/ml pepstatin, 0.5 mg/ml leupeptin, 0.2 mg/ml phenylmethylsulfonyl fluoride. Cells were broken by rigorous stirring of 5 ml of cold cell suspension with 5 ml of glass beads (0.2-mm diameter) in a 50-ml tube for 3 min. Homogenates were centrifuged for 10 min at 7000 × *g* to remove cell debris. The 7000 × *g* supernatant was centrifuged for 1 hr at 100,000 × *g* to isolate the microsomal fraction.

A total of 40 μg of the microsomal protein was fractionated on sodium dodecyl sulfate-polyacrylamide gels containing 8% polyacrylamide and 0.3% *N,N'*-methylenebisacrylamide (23). Proteins of known molecular mass were run in parallel as molecular weight markers. After electrophoresis the proteins were electroblotted to a nitrocellulose membrane. Antibodies raised in rabbits against pig α1 or dog β were used as primary antibodies. The commercially available ECL Western blotting system was used to detect the expressed α or β subunits.

Interaction with palytoxin of Na^+/K^+-ATPase expressed in yeast. Yeast cells expressing either α, β, or α plus β subunits of Na^+/K^+-ATPase were diluted to 10^8 cells/ml in GHBC buffer (300 mM glucose, 0.5 mM boric acid, 1 mM $CaCl_2$, 10 mM HEPES, pH 7.4) and incubated with increasing concentrations of palytoxin for various times at 30°. After centrifugation (10 min at 8000 × *g*), the supernatant K^+ concentration was determined by flame photometry. The total K^+ content of the suspension was between 200 and 300 μM, as determined after lysis with 0.02% sodium dodecyl sulfate for 10 min at 95°. All experiments were repeated at least twice and produced the same result. Because the standard error of parallel samples was very small, the data points given represent single measurements.

Materials. Growth media were obtained from Merck (Darmstadt, Germany) or Difco (Detroit, MI). Palytoxin from *P. caribaeorum* was purchased from Dr. L. Béress (Christian-Albrechts-Universität, Kiel, Germany). The ECL system is a product of Amersham (Little Chalfont, UK). Acrylamide solutions were from Roth (Karlsruhe, Germany) and molecular weight markers were from Sigma Chemical Co. (St. Louis, MO). All other chemicals and biochemicals were of the highest purity available.

Results and Discussion

Yeast cells were transformed with the vector pCGY1406$\alpha\beta$, which codes for sheep α1 and dog β subunits (18). Other cells were transformed with the vector YEpα3, carrying the cDNA for the rat α3 subunit, or with the vector pCGY1406β, which codes for the dog β subunit. YEpα3 is known to produce the α3 subunit (24) and was therefore preferred over the new construction of a vector carrying the cDNA for α1. Nontransformed cells served as controls. Yeast cells transformed with the vectors mentioned above expressed the expected proteins, as verified in a Western blot using a polyclonal antibody raised against pig kidney α subunit and a polyclonal antibody raised against dog kidney β subunit (Fig. 1). The antibody raised against the α1 subunit from pig kidney recognized sheep α1 and rat α3. Sheep α1, rat α3, and pig α1 subunits share about 80% homology in their primary structures.

Potassium release from yeast cells served as an indicator of palytoxin action. In a physiological environment, palytoxin probably causes first the depolarizing movement of Na^+ into excitable cells and then the outflow of K^+ from the depolarized cells (25). Patch-clamp experiments in ventricular cells demonstrated palytoxin channels to be equally permeable to Na^+ and K^+ (25). For that reason, and because yeast cells display very active, outwardly directed, Na^+ transport (26) that might interfere with the accumulation of Na^+ (26), measurement of K^+ release was preferred.

Both nontransformed and transformed cells retained K^+ in the absence of the toxin for the time assessed. Nontransformed yeast cells and cells expressing either the α3 subunit or the β subunit alone were insensitive to palytoxin (Fig. 2). In contrast, cells expressing both α and β subunits of the sodium pump lost

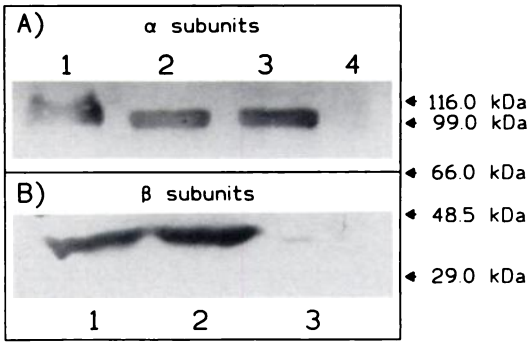

Fig. 1. Western blot of expressed α and β subunits of Na^+/K^+-ATPase. The molecular weight markers are β-galactosidase (*M,* 116,000), phosphorylase *b* (*M,* 97,000), bovine serum albumin (*M,* 66,000), fumarase (*M,* 48,500), and carbonic anhydrase (*M,* 29,000). A, Identification of expressed α subunits. The α1 subunit from pig kidney and the sheep α1 or the rat α3 from yeast were detected in a Western blot using the commercially available ECL system. The primary polyclonal antibody was raised in rabbits against α1 from pig kidney. The antibody recognizes kidney α1 (*lane 1*), α1 from yeast transformed with pCGY1406$\alpha\beta$ (*lane 2*), and α3 from yeast transformed with YEpα3 (*lane 3*). All α subunits migrate with a molecular weight of 100,000. Proteins from nontransformed yeast (*lane 4*) do not interact with the antibody. B, Detection of expressed β subunits. The primary polyclonal antibody was raised in rabbits against dog β subunit from kidney microsomes. Yeast cells transformed with pCGY1405$\alpha\beta$ (*lane 1*) or with pCGY1406β (*lane 2*) express the β subunit with *M,* 42,000. This indicates that the β subunit of *M,* 35,000 becomes core-glycosylated in the yeast cells. Fully glycosylated β subunit from dog or pig kidneys runs at approximately *M,* 56,000 (data not shown). Nontransformed yeast cells (*lane 3*) do not express a protein at *M,* 42,000 that can be recognized by the antibody. The results conform to earlier reports (18).

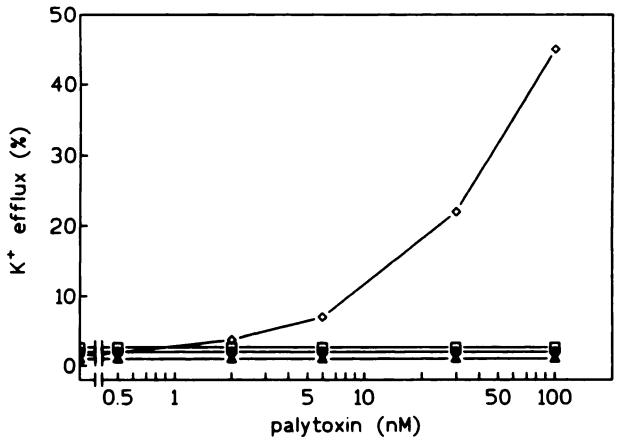

Fig. 2. Requirement of α and β subunits for the interaction of palytoxin with Na^+/K^+-ATPase. Yeast cells expressing either α (●), β (▲), or α and β (◇) subunits of Na^+/K^+-ATPase were grown for 2 days, then diluted to 10^8 cells/ml in GHBC buffer, and incubated with various concentrations of palytoxin for 60 min at 30°. Then, K^+ was determined in the supernatant (*ordinate*). Nontransformed yeast cells served as control (□). Total K^+ content of the suspension was between 200 μM and 300 μM.

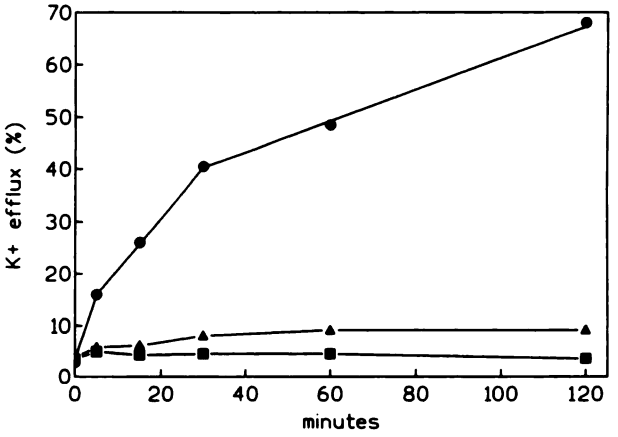

Fig. 3. Time course of the action of palytoxin on yeast expressing the sodium pump. Yeast cells expressing α and β subunits of Na^+/K^+-ATPase were incubated with 50 nM (●) or 1.6 nM (▲) palytoxin for various times. Experimental conditions were as described for Fig. 2. Nonspecific K^+ release was monitored with transformed cells in the absence of palytoxin (■).

about 50% of their K^+ under the same conditions, with effects being observed at palytoxin concentrations as low as 6 nM. Hence, both α and β subunits are required for palytoxin action, but this does not necessarily indicate that palytoxin interacts with α and β subunits simultaneously. Alternatively, both subunits might cooperate to obtain correct folding of the two separate proteins and of the palytoxin binding site within one of the two subunits.

Palytoxin-induced K^+ efflux from yeast expressing both subunits of the sodium pump was dependent on time and palytoxin concentration (Fig. 3). Palytoxin at 50 nM caused a continuous outflow of up to 70% of total cellular K^+ within 2 hr. A low concentration of palytoxin (1.6 nM), however, increased the amount of K^+ in the incubation medium just above background levels (Fig. 3). The action of palytoxin on yeast cells was slower than its action on erythrocytes or on muscle cells, where complete K^+ outflow was observed within a few minutes with 10 nM toxin (27). Moreover, yeast cells differed from erythrocytes by a very gradual concentration versus effect ratio. K^+ loss from yeast cells was always incomplete, even when 1 μM palytoxin was used (see also Fig. 4). The reason for this atypical behavior is at present not clear. Probably the heterologous ATPase may assume a set of conformations in the yeast cell membrane that vary in their affinity for palytoxin. Alternatively, or additionally, intracellular K^+ may exist in subcompartments, for instance vacuoles, of different palytoxin sensitivity. A similar concentration versus effect ratio had been observed in dog erythrocytes, which are known for their low content of both K^+ ions and Na^+/K^+-ATPase activity (10).

The role of Na^+/K^+-ATPase in the action of palytoxin is emphasized by the inhibitory effect of ouabain. Ouabain and other cardioactive steroids do not bind to yeast cells or to membrane preparations of yeast cells unless the sodium pump is expressed (18, 21). As observed with erythrocytes (11, 12, 28), ouabain at 5 μM markedly inhibited the K^+ outflow induced by high concentrations (1 μM) of palytoxin (Fig. 4). The K^+ efflux was completely abolished by 500 μM ouabain at palytoxin concentrations below 0.1 μM. The same concentration of ouabain reduced K^+ efflux to 85% when 1 μM palytoxin was used (Fig. 4). This is indicative of the interaction of palytoxin with the sodium pump (1, 11, 12, 28), which is the only known receptor for cardioactive steroids (29, 30). Rat erythrocytes express a ouabain-insensitive isoform of the α1 subunit of Na^+/K^+-ATPase (31) but share high palytoxin sensitivity with human erythrocytes (1). Thus, it may be that the binding sites for palytoxin and ouabain are overlapping but not identical (1).

Borate ions potentiated the palytoxin-induced K^+ efflux from yeast cells expressing the sodium pump (Fig. 5A), as previously observed for erythrocytes or neurosynaptosomes (1). In the absence of palytoxin, borate did not induce efflux of K^+ from erythrocytes (1) or from yeast cells (Fig. 5). It is possible that borate interacts with some of the 41 hydroxyl groups of the palytoxin molecule, similarly to the way it interacts with carbohydrates. Complex formation might favor a particular shape of the palytoxin molecule that can be better recognized by the sodium pump or it might link palytoxin to the carbohydrates of the β subunit.

As in erythrocytes, Ca^{2+} enhanced the action of palytoxin in yeast cells expressing the sodium pump (Fig. 5B). Ca^{2+} is known

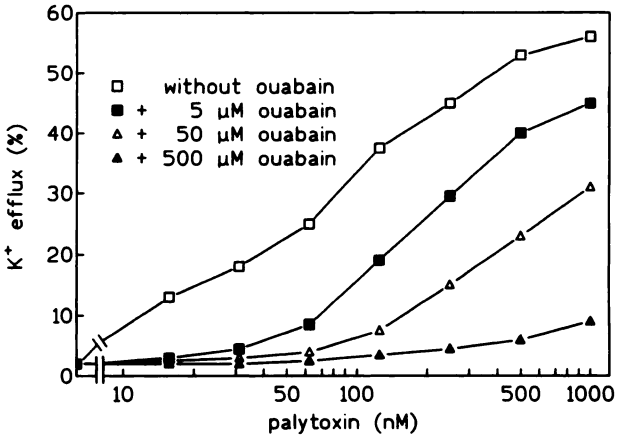

Fig. 4. Ouabain inhibition of K^+ efflux induced by palytoxin. The cells were preincubated with various concentrations of ouabain in GHBC buffer (see Fig. 2) for 30 min at 30° before palytoxin was added. One hour later, K^+ content was determined in the supernatant.

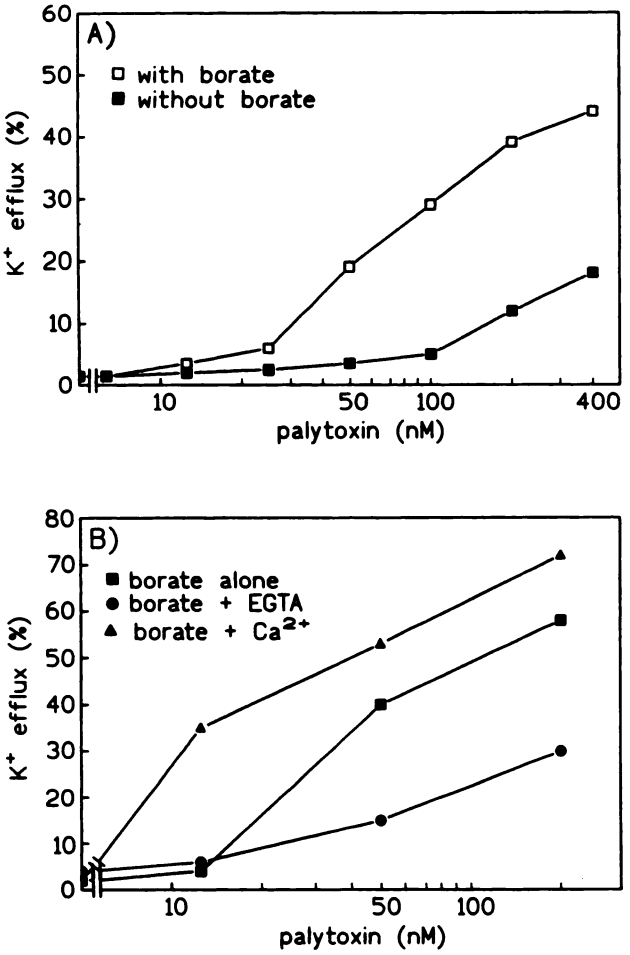

Fig. 5. Activation by borate and calcium of the palytoxin effect on transformed yeast. A, Borate. Activation by 0.5 mM borate was determined in a buffer solution composed of 300 mM glucose, 1 mM $CaCl_2$, 10 mM HEPES, pH 7.4. B, Calcium. The activation of the palytoxin effect by 1 mM Ca^{2+} (▲) and its inhibition by 1 mM EGTA (●) were measured in 300 mM glucose, 0.5 mM borate, 10 mM HEPES, pH 7.4. The inhibition by EGTA (●) caused in cells without additionally applied Ca^{2+} (■) indicates the presence of residual Ca^{2+} in the cell suspension.

to promote palytoxin binding (13). All experiments clearly show that yeast cells must express both α and β subunits of the mammalian sodium pump to respond to palytoxin. These observations, together with the inhibition by ouabain, strongly suggest that the toxin binds to and acts via the sodium pump.

Interaction of palytoxin with Na^+/K^+-ATPase probably leads to the formation of a channel that allows outflow of K^+ from the yeast cells. Channel formation due to the action of palytoxin has been detected in several cell types by the patch-clamp technique (25, 32, 33). This channel may originate from the sodium pump itself, because palytoxin keeps the heterodimer in a permanently open state, or it may be located in the membrane in close proximity to the pump. Expression of isoforms, mutants, and chimeras of the Na^+/K^+-ATPase in yeast will be an essential tool to address this question.

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