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Palytoxin-Induced Na^+ Influx into Yeast Cells Expressing the Mammalian Sodium Pump Is Due to the Formation of a Channel within the Enzyme

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SUMMARY

Palytoxin forms ionic channels in animal cell membranes but does not have similar effects on bacteria or yeast cells. These channels appear to be associated with the sodium pump. Using a heterologous expression system for the mammalian sodium pump in the yeast *Saccharomyces cerevisiae*, we recently demonstrated palytoxin-induced K^+ efflux from yeast cells. Using the same system, we now show that the palytoxin-induced Na^+ influx measured by others in animal cells is also directly associated with the sodium pump. Under the influence of palytoxin, yeast cells that express the mammalian sodium pump exchange extracellular Na^+ ions for intracellular K^+ ions with a stoichiometry of ~1:1. Both fluxes can be inhibited by ouabain. K^+ efflux can also be observed when extracellular Na^+ is replaced by Li^+, Cs^+, or NH_4^+. These data suggest that all palytoxin-induced ion fluxes measured so far in various cell systems are directly associated with the sodium pump. Palytoxin-induced Na^+ influx or K^+ efflux does not occur with yeast cells that express a truncated form of the sodium pump that is missing 44 of the carboxyl-terminal amino acids of the $\alpha 1$ subunit. Scatchard analysis reveals only a slightly lower affinity of the truncated form for [^{3}H]ouabain compared with the affinity of the native enzyme. Yeast cells expressing the truncated enzyme can bind [^{3}H]ouabain, which can be displaced by palytoxin. Therefore, the inability of the truncated form to conduct ions under the influence of palytoxin is not due to the removal of the palytoxin binding site but rather to the removal of a part of the enzyme that participates in a direct or indirect way in the formation of the palytoxin-induced channel. Based on these findings, we conclude that palytoxin opens a channel within and not merely in the vicinity of the sodium pump. This might be the same channel that under normal conditions actively transports Na^+ and K^+ ions.

The Na^+/K^+-ATPase, or sodium pump, is an oligomeric enzyme composed of $\alpha 1$ and β subunits. Various isoforms of both subunits are known. The enzyme is integrated into all animal cell membranes and catalyzes the transport of Na^+ ions out of and the transport of K^+ ions into the cell (1–3). The transport process occurs against the electrochemical gradients of the ions and requires energy that is provided in the form of ATP. One molecule of ATP becomes hydrolyzed by the enzyme in each pumping cycle, and three intracellular Na^+ ions are exchanged for two extracellular K^+ ions (1, 3). The hydrolytic activity and the transport of ions are inhibited by various cardioactive steroids that are produced either by plants (cardenolides and bufadienolides) or by animals (bufadienolides) and are known to interact only with the sodium pump (1, 3, 4).

Recent findings strengthen earlier conclusions that in addition to the cardioactive steroids, palytoxin interacts specifically with the sodium pump (5, 6). Palytoxin, the most potent animal toxin known, is synthesized by corals of the genus *Palythoa*. The LD_{50} for rodents is ~10–250 ng/kg body weight (6, 7). In their chemical structure and in their action on the sodium pump, palytoxins are completely different from cardioactive steroids. Cardioactive steroids, like the widely used water-soluble cardenolide ouabain, inhibit flux of ions (4), whereas palytoxin appears to transform the sodium pump into an open channel that allows efflux of K^+ ions and influx of Na^+ ions in all animal cells investigated (5, 6, 8–13). First conclusions about the involvement of the sodium pump in the action of palytoxin arose from the observation that ouabain inhibits the action of palytoxin in erythrocytes and smooth muscle from many species (14, 15). In some tissues, however, ouabain does not inhibit the action of palytoxin (6, 16, 17). This could be one of the reasons for the postulation that the action of palytoxin might involve other membrane molecules, such as a H^+ channel, a H^+/Na^+ ex-

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ABBREVIATIONS: Na^+/K^+-ATPase, sodium- and potassium-activated ATP (EC 3.6.1.37); HEPES, 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid; SDS, sodium dodecyl sulfate; YNB, yeast nitrogen base.

changer, or a Na^+ channel, rather than the sodium pump (18–21). A cytolytic action of palytoxin on mouse neuroblastoma cells has also been discussed (22).

Using a heterologous expression system of the Na^+/K^+-ATPase in the yeast *Saccharomyces cerevisiae,* we recently provided direct evidence that palytoxin acts through the sodium pump (5). Yeast cells expressing both $\alpha 1$ and β subunits of the mammalian sodium pump lose intracellular K^+ when they are exposed to palytoxin. A similar effect was not observed with untransformed yeast cells or with cells expressing only the $\alpha 1$ or only the β subunit of the enzyme (5). Although these results show that Na^+/K^+-ATPase is involved in palytoxin-induced K^+ efflux, they do not verify whether other effects of palytoxin that have been observed for other cell systems can also occur when the sodium pump is expressed in the yeast. Because palytoxin is known to promote Na^+ influx in erythrocytes (6), we were interested in investigating whether a similar observation could be made with yeast cells expressing the sodium pump. We further investigated whether K^+ efflux can also be observed when the extracellular Na^+ is replaced by other monovalent cations. Several different cations are known to permeate the palytoxin-induced channel based on data from patch-clamp experiments with other cellular systems (23, 24). These cations are also known to be recognized as Na^+ surrogates by the sodium pump.

We also considered whether the palytoxin-induced channel originates within the sodium pump, as palytoxin keeps the α/β-heterodimer in a permanently open state, or, alternatively, whether the channel is formed between the palytoxin molecule and parts of the protein of either the $\alpha 1$ or β subunit. To determine which of the two possibilities might be correct, the palytoxin effect was measured with yeast cells expressing a truncated form of the $\alpha 1$ subunit of the sodium pump. The results that we present indicate that the palytoxin-induced ion fluxes occur through a channel that forms within the Na^+/K^+-ATPase protein. They also suggest that palytoxin transforms the sodium pump into a permanently open channel, which might be the same channel that under normal conditions actively transports Na^+ and K^+ ions.

Materials and Methods

Vectors and cells. For expression of $\alpha 1$ and β subunits of the Na^+/K^+-ATPase in *S. cerevisiae,* the shuttle vector pCGY1406$\alpha\beta$ was used (25). Information about the yeast strain 20B12, vector, transformation, and growth conditions of the transformed yeast cells have been described (5, 25, 26).

Production of truncated $\alpha 1$ subunits. For the insertion of a stop codon within the *Eco*81I site of the $\alpha 1$ subunit, the following strategy was used. Because the vector pCGY1406$\alpha\beta$ contains two *Eco*81I sites, subcloning was required to insert the stop codon at the right place within the $\alpha 1$ subunit. An *Xho*I/*Apa*I fragment of the vector pCGY1406$\alpha\beta$ that also contains the *Eco*81I site of the $\alpha 1$ subunit was ligated into equivalent sites of the commercially available bacterial plasmid pBluescript II SK^+. The new plasmid contains a unique *Eco*81I site within the subcloned fragment of the $\alpha 1$ subunit. After digestion with *Eco*81I and purification from a 1% agarose gel, an unphosphorylated oligonucleotide with the sequence 5′-TGACTAGATAGTACTATCTAG-3′ was ligated into the *Eco*81I site of the linearized plasmid. This oligonucleotide contains stop codons in all three coding frames. It also contains a *Sca*I site that allows fast detection of the correct clones. Identified correct clones were digested with *Xho*I and *Apa*I. The *Xho*I/*Apa*I fragment of the $\alpha 1$ subunit that now contained the inserted oligonucleotide was ligated back into the *Xho*I/*Apa*I sites of the pCGY1406$\alpha\beta$. The new plasmid is designated pCGY1406$\alpha 1$-Δct44β and codes for an $\alpha 1$ subunit lacking 44 carboxyl-terminal amino acids. This form of the truncated $\alpha 1$ subunit is designated $\alpha 1$-Δct44. The expressed enzyme is designated $\alpha 1$-Δct44β.

Isolation of yeast microsomes enriched in Na^+/K^+-ATPase. Single yeast colonies either untransformed or transformed with either pCGY1406$\alpha\beta$ or pCGY1406$\alpha 1$-Δct44β were grown overnight in 5 ml of YNB medium (1.5g YNB without amino acids and 2.5g $(NH_4)_2SO_4$ per liter) to an A_{600} of ~1. Thereafter, cells were transferred to 500 ml of YNB medium and were incubated for an additional 24 hr. These cells were used to isolate microsomal membranes as previously described (26, 27). Yeast microsomes enriched in Na^+/K^+-ATPase were prepared by a method similar to the one used for the isolation of the enzyme from kidney microsomes (28) according to modifications previously described (25, 26). The method involves treatment of the microsomes by SDS followed by centrifugation through a discontinuous sucrose gradient. The partially purified Na^+/K^+-ATPase that results from this method was used for measuring the overall activity and the Na^+-ATPase activity of Na^+/K^+-ATPase.

Determination of the overall Na^+/K^+-ATPase activity and the Na^+-ATPase partial activity in yeast microsomes. The overall activity of Na^+/K^+-ATPase was determined in aliquots of 50 μg of microsomal protein in the presence or absence of 100 μM ouabain with the use of a coupled spectrophotometric assay (29). The formation of ADP by the Na^+/K^+-ATPase is coupled via phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, and NAD^+ to the formation of NADH plus H^+. The formation of NADH plus H^+ can be continuously measured at 366 nm in a spectrophotometer (ϵ = 3300 liters/mol/cm). The Na^+-ATPase activity was measured by incubating 50 μg of microsomal protein with or without 100 μM ouabain under the conditions of the coupled spectrophotometric assay that is used for the determination of the overall activity, except that K^+ or its surrogates were omitted from the medium.

Immunodetection of native and truncated $\alpha 1$ subunits. A total of 25 μg of protein (30) from each microsomal membrane preparation enriched in Na^+/K^+-ATPase was separated on SDS-polyacrylamide gels prepared according to Laemmli (31). Gels contained 5% acrylamide and 0.25% *N,N′*-methylenebisacrylamide. Molecular weight markers were run in parallel. One SDS-polyacrylamide gel was stained with Coomassie Brilliant Blue, and a second one was used in a Western transfer of the proteins onto nitrocellulose. Immunodetection was carried out using a polyclonal antibody against the $\alpha 1$ subunit and the commercially available enhanced chemiluminescence system.

Interaction of palytoxin with yeast cells expressing native or truncated Na^+/K^+-ATPase. Single yeast colonies transformed with pCGY1406$\alpha\beta$ or pCGY1406$\alpha 1$-Δct44β were incubated in 5 ml YNB medium for 20–24 hr at 30° with continual shaking. Thereafter, cell suspensions were transferred to flasks containing 150–200 ml of YNB medium and were incubated under the same conditions for an additional 20–24 hr. Cells were then centrifuged at 3000 × *g* for 5 min, washed twice with cold water at 4°, and suspended in buffer A consisting of 10 mM HEPES, pH 7.5 (adjusted with Tris base), 0.5 mM borate, 1 mM $CaCl_2$, and 400 mM of NaCl to give a concentration of 15 × 10^6 cells/ml. In some cases, NaCl was replaced by equimolar concentrations of NH_4Cl, CsCl, LiCl, guanidinium hydrochloride, or urea.

A total of 1 ml of the cell suspension was incubated with various concentrations of palytoxin as described in the appropriate figure legends. After various times of incubation at room temperature with continuous shaking, cell suspensions were centrifuged in a microfuge for 2 min. Supernatants were collected for flame-photometric K^+ detection. The pellets were washed twice with 1 ml of water at 4° and were then dissolved in 1 ml of 0.1% lithium dodecyl sulfate by a 10-min incubation at 80°. Debris was removed by centrifugation, and

Na^+ was determined in the supernatant by flame photometry. Nonspecifically entrapped Na^+ was determined under the same conditions but in the absence of palytoxin and was subtracted from the other samples.

For the measurement of the palytoxin-induced K^+ efflux in a flow ionometer, yeast cells were suspended at a concentration of 15×10^6 cells/ml in buffer A containing 400 mM of NaCl, LiCl, or NH_4Cl. Reactions were started by the addition of various concentrations of palytoxin under continuous stirring. The total volume of each sample was 1 ml. The cell suspension was continuously forwarded via a peristaltic pump to the K^+-sensitive electrode. The time elapsed between the addition of palytoxin until signal detection was 20 sec. K^+ efflux (extracellular K^+ concentration) was monitored every 1 sec in a bead volume of 10 μl. The electric signals were digitized and analyzed with a computer program.

Inhibition of the palytoxin-induced Na^+ influx by ouabain was determined by incubating cells under the above conditions with 50 nM palytoxin and various concentrations of ouabain. The reaction was initiated by pipetting the cell suspension into tubes that contained palytoxin and ouabain.

The total cytosolic volume of the cells was determined by centrifugation for 10 min at 7000 $\times$ *g* of cell suspensions of 60×10^6 or 120×10^6 cells/ml in hematocrit tubes. From the total cellular volume determined, 26% was subtracted for the intercellular space and 12% for the space between cell wall and plasma membrane (32).

Scatchard analysis of [^{3}H]ouabain binding to microsomal membranes prepared from yeast cells. A total of 1 mg of microsomal protein isolated from yeast cells expressing either the native or the truncated Na^+/K^+-ATPase was incubated in a buffer containing 50 mM Tris·HCl, pH 7.5, 3 mM H_3PO_4, 5 mM $MgCl_2$, and increasing concentrations of [^{3}H]ouabain (0.5–300 nM). The total volume of each sample was 1 ml. For the determination of nonspecific binding, some samples also contained 1 mM unlabeled ouabain (25). In each sample, radioactivity was kept constant at 12 pCi. Samples were incubated to equilibrium at 37° for 30 min and then transferred to ice for 5 min. Membranes were collected by centrifugation at 13,000 $\times$ *g* for 10 min. The supernatant was removed by aspiration, and the amount of [^{3}H]ouabain bound to the sedimented protein was determined by scintillation counting.

Binding of [^{3}H]ouabain to whole yeast cells expressing native or truncated Na^+/K^+-ATPase. Yeast cells at a concentration of 10×10^6 cells/ml were suspended in buffer A and incubated in the presence or absence of 200 nM palytoxin with 120 nM [^{3}H]ouabain for 20 min at room temperature. Nontransformed yeast cells were used as a control. Thereafter, cell suspensions were filtered through sterilization filters (pore size 0.2 μm) using a vacuum filtration apparatus. The cells on the filters were washed with 4 ml of water at 4°. Filters were transferred into scintillation vials, and radioactivity was determined by scintillation counting.

Materials. Growth media were obtained from Difco (Detroit, MI). Palytoxin from *Palythoa caribaeorum* was purchased from Dr. L. Bèress (Christian-Albrechts-Universität, Kiel, Germany). Electrophoresis chemicals were obtained from Roth (Karlsruhe, Germany), and molecular weight markers were obtained from Sigma Chemical Co. (St. Louis, MO). The enhanced chemiluminescence Western blot analysis system and [^{3}H]ouabain (28 Ci/mmol) were from Amersham (Little Chalfont, England). Oligonucleotides were synthesized by Roth (Karlsruhe, Germany). Restriction enzymes were obtained from United States Biochemicals (Cleveland, OH) or from MBI Fermentas (Vilnius, Lithuania). The plasmid pBluescript II SK$^+$ is a product of Stratagene (La Jolla, CA). All other chemicals and biochemicals were of the highest purity available. The flow ionometer was from ZABS GmbH (Marburg/Lahn, Germany).

Results and Discussion

Yeast cells expressing both α1 and β subunits of the mammalian sodium pump are sensitive to palytoxin. Ion fluxes for both Na^+ and K^+ ions can be observed under these conditions (Fig. 1). The stoichiometry of the inward-directed Na^+ flux and outward-directed K^+ flux is almost 1:1. In most of the experiments, a slightly higher concentration of cytosolic Na^+ was observed compared with the concentration of K^+ that had left the cytosol. The reason for this observation might be that 400 mM NaCl was present extracellularly, whereas the intracellular concentration of K^+ in our experiments was determined to be ~150 ± 5 mM. This value is in good agreement with the value of 150–200 mM for intracellular [K^+] in yeast cells at low extracellular pH (33). It is possible that Na^+ ions can still flow into the cell even when no K^+ can be exchanged, probably over a Na^+/H^+ exchanger or by a Na^+/X^- cotransporter. A palytoxin-induced Na^+ influx or K^+ efflux, however, could only be detected with yeast cells that expressed the mammalian sodium pump. Nontransformed cells under similar incubation conditions were completely insensitive to concentrations of palytoxin up to 1 μM (not shown). This and the fact that ouabain inhibits Na^+ influx into and K^+ efflux out of yeast cells that express the Na^+/K^+-ATPase (Fig. 2) verify that the observed ion movements are due to the interaction of the palytoxin molecule with the expressed sodium pump. The involvement of a Na^+/H^+ exchanger or a Na^+ channel in palytoxin-induced Na^+ influx postulated earlier does not appear to be required to explain the observed ion fluxes or their inhibition by ouabain (18–22). The IC_{50} values for ouabain inhibition of the palytoxin-

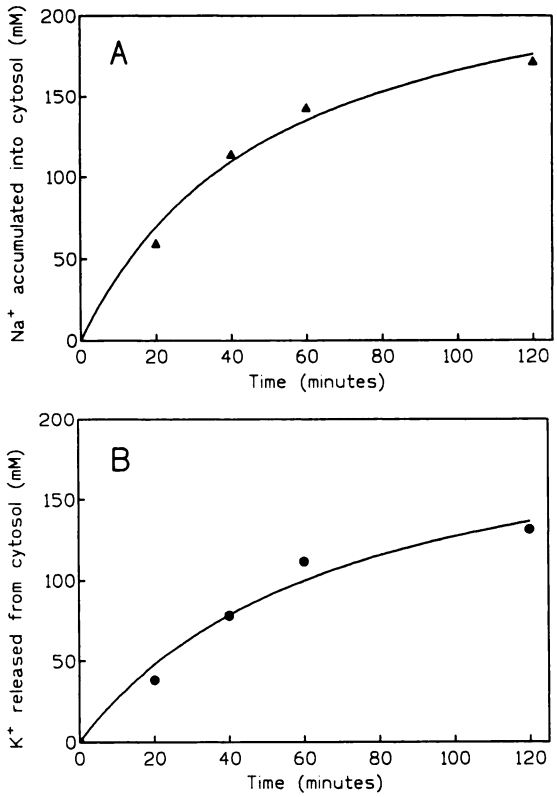

Fig. 1. Time course of the action of palytoxin on yeast cells expressing α1 and β subunits of Na^+/K^+-ATPase. Transformed yeast cells were grown for 2 days, diluted to 15×10^6 cells/ml in buffer A with 400 mM NaCl, and incubated with 50 nM palytoxin for various times. Na^+ influx (A) and K^+ efflux (B) were determined as described under Materials and Methods. The results are expressed as changes in the cytosolic concentration of the ions. Each point represents the mean of three measurements. Standard error was <5% of each mean value.

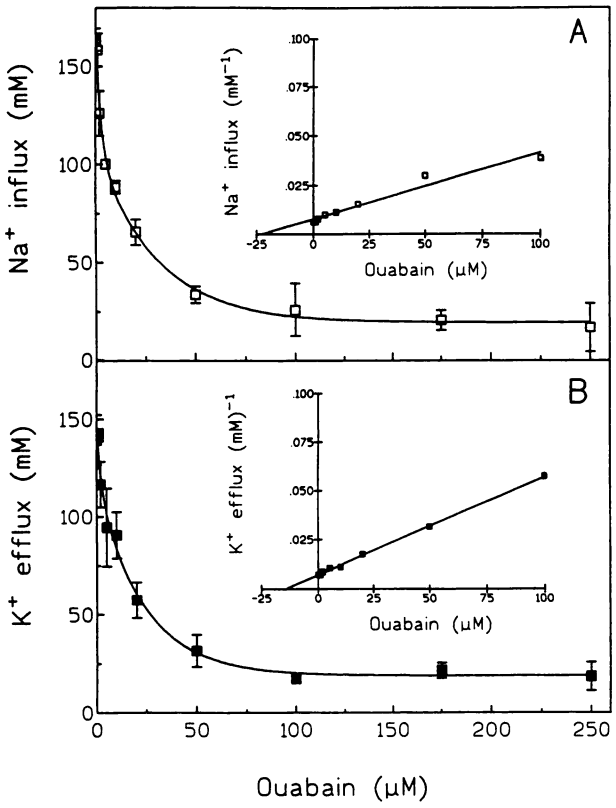

Fig. 2. Inhibition of palytoxin-induced Na^+ influx and K^+ efflux by ouabain. Each sample contained a total of 15×10^6 yeast cells/ml in buffer A with 400 mM NaCl, 50 nM palytoxin, and the given concentration of ouabain. Incubation was performed at room temperature for 2 hr, after which intracellular Na^+ and extracellular K^+ were determined. Values are mean of triplicate measurements (± standard error). A, Ouabain inhibition of Na^+ influx into yeast cells. *Inset*, IC_{50} is calculated to be 20.2 μM. B, Ouabain inhibition of K^+ efflux. *Inset*, IC_{50} is 14.6 μM.

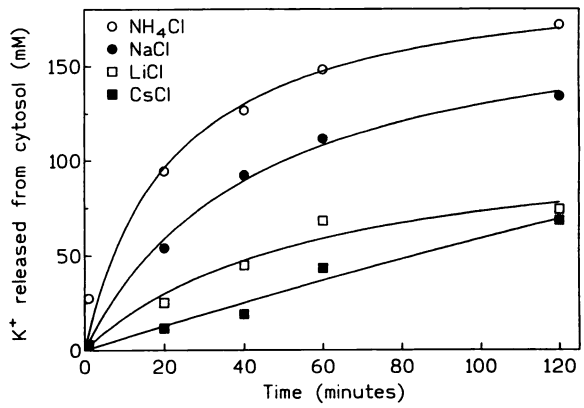

Fig. 3. Time course of palytoxin-induced K^+ efflux from yeast cells in the presence of various extracellular cations. Yeast cells were incubated in buffer A with 400 mM of either NaCl, LiCl, CsCl, NH_4Cl, guanidinium hydrochloride, or urea and 50 nM palytoxin. Experimental conditions were as described for Fig. 1. Each point represents the mean of three measurements, where standard error was <5% of the mean. K^+ efflux was not observed when buffer A contained guanidinium hydrochloride or urea.

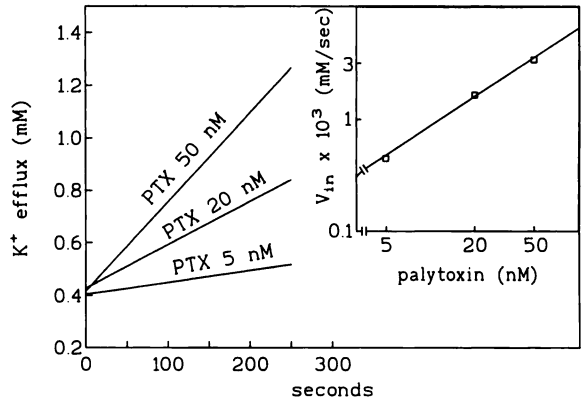

Fig. 4. Determination of the order of the reaction for the palytoxin (*PTX*)-induced K^+ efflux. Palytoxin-induced K^+ efflux from yeast cells expressing the sodium pump was measured with a flow ionometer in the presence of 400 mM extracellular NaCl. For the first 4 min, K^+ efflux, here determined as the extracellular K^+ concentration, was linear with time. The plot of the log of the initial velocity (V_{in}) versus the log of the palytoxin concentration (A) gives a straight line with the slope $n = 0.89$, thus indicating a first order reaction (*inset*). From the formula $V_{in} = k \cdot A^n$, k_{Na^+} is calculated to be 68×10^{-6} sec^{-1}.

induced Na^+ influx and K^+ efflux as determined from Dixon plots (Fig. 2, *insets*) are comparable (20.2 μM and 16.4 μM, respectively).

Efflux of K^+ ions can also be observed when extracellular Na^+ is replaced by either alkali metal cations, like Cs^+ or Li^+, or by NH_4^+ (Fig. 3). This is in good agreement with results showing that the effect of palytoxin on the calculated membrane potential of erythrocytes or on the induction of single-channel current in guinea pig ventricular myocytes can also be observed when extracellular Na^+ is replaced by these ions (23, 24).

The efflux rates of K^+ as measured by a flow ionometer, however, are affected by the nature of the extracellularly applied cation. To determine the order of the reaction for the K^+ efflux, yeast cells were incubated with 50, 20, or 5 nM palytoxin in a buffer containing 400 mM NaCl, and the K^+ efflux was measured for 4 min in a flow ionometer. During this time, the initial velocity (V_{in}) of the K^+ efflux is linear and can be described by the formula $V_{in} = k \cdot A^n$, where k is the rate constant, A is the concentration of palytoxin, and n is the order of the reaction. A plot of the $\log V_{in}$ against the $\log[A]$ gives a straight line with the slope = n (Fig. 4). The slope of the straight line is 0.89 and indicates a first order reaction. Therefore, $k = V_{in} \cdot A^{-1}$. In this experiment, the rate constant for K^+ efflux in the presence of extracellular Na^+ is $k_{Na^+} = 68 \times 10^{-6}$ sec^{-1}. From similar studies, the rates for K^+ efflux in the presence of extracellular NH_4^+ or Li^+ were determined as $k_{NH_4^+} = 72 \times 10^{-6}$ sec^{-1} and $k_{Li^+} = 21.4 \times 10^{-6}$ sec^{-1}, respectively. The efflux rate of K^+ in the presence of extracellular Cs^+ could not be determined because of the strong interaction of Cs^+ ions with the K^+ electrode. The differences in the efflux rates could account for the results presented in Fig. 3. Maximum efflux of K^+ in the presence of different extracellular cations after 3 hr of incubation with palytoxin, however, is not the same in the various samples (not shown). A measurement beyond that point is not reliable because yeast cells start to become unstable. It might be that maximal K^+ efflux can never be reached with every extracellular cation. The reason could be that yeast cells are able to reabsorb K^+ from the medium (33), thus reaching a steady state condition between K^+ efflux and K^+ influx at different times.

Although the influx of NH_4^+, Li^+, or Cs^+ was not measured directly in our experiments, we assume that these ions

are exchanged for intracellular K^+. This assumption is supported by the fact that K^+ efflux could not be observed when the extracellular cations were replaced by 400 mM urea (not shown). Obviously, K^+ ions can leave the cell only when a counter cation can enter the cell in exchange. Positively charged guanidinium hydrochloride, however, could not substitute for extracellular alkali metal cations or NH_4^+ (not shown), thus indicating that the entering cation is possibly discriminated not only by its charge but also by its size and the size of the pore.

Size and charge, however, must not be the only significant parameters for the K^+ fluxes observed in our experiments and elsewhere. A direct comparison of the radii of the various cations as determined from standard crystal lattices reveals that Li^+ (r = 0.68 Å) < Na^+ (r = 0.97 Å) < NH_4^+ (r = 1.43 Å) < Cs^+ (r = 1.67 Å). One would therefore expect that Li^+ would be more effective and NH_4^+ less effective than Na^+ in inducing K^+ efflux, but the opposite is the case. The effective radii of the ions, however, depend on their degree of hydration. A comparison of the sizes of hydrated ions reveals that Cs^+ (r = 2.28 Å) < NH_4^+ (r = 2.32 Å) < Na^+ (r = 2.76 Å) < Li^+ (r = 3.4 Å). The fact that the efflux rate of K^+ is indirectly proportional to the radii of the hydrated ions for NH_4^+, Na^+, and Li^+ appears to indicate that ions are hydrated during flux, but this assumption is contradicted by the fact that in our experiments Cs^+ was less effective than Na^+. It is not known, however, whether ions pass through the palytoxin-induced ion channel (or the Na^+/K^+-ATPase) in a hydrated form and how many water molecules are bound to an ion during its passage.

Our observation that the efflux rate of K^+ is reduced when extracellular Na^+ is replaced by Li^+ or Cs^+ (Fig. 3) is consistent with earlier results demonstrating that these cations were less effective than Na^+ in the induction of single-channel currents in palytoxin-treated ventricular myocytes (23, 24). In the same experiments, however, the bulkier NH_4^+ was more effective than Na^+ (24). This is also in good agreement with our observation that the extracellular NH_4^+ accelerates K^+ efflux compared with extracellular Na^+.

The results verify that Na^+ and K^+ ion fluxes are caused by the interaction of palytoxin with the mammalian sodium pump that is expressed in yeast cells. Because extracellular Na^+ can be replaced by NH_4^+, Li^+, or Cs^+, these data further indicate that the ion fluxes observed in our experiments and elsewhere are probably also due to the interaction of palytoxin with the sodium pump. Thus, palytoxin apparently induces the opening of a channel within or in the vicinity of the sodium pump that allows the extracellular monovalent cations NH_4^+, Na^+, Cs^+, or Li^+ to flow into the cells down their electrochemical gradient in exchange for intracellular K^+. It is not yet clear whether the outward-directed K^+ fluxes and inward-directed fluxes of the other ions occur simultaneously or consecutively. These ion movements, however, are sufficient to explain the depolarization induced by palytoxin in other cellular systems.

The nature of the palytoxin-induced channel is unknown. After palytoxin binds to the protein of the sodium pump, one might imagine that the channel is formed between the palytoxin molecule and parts of the Na^+/K^+-ATPase (Fig. 5A). Alternatively, palytoxin might bind to the protein and induce the formation of the channel within the sodium pump, possibly by arresting a permanently open state of the physiological channel of the enzyme (Fig. 5B). This structure of the enzyme is apparently formed by transmembrane segments of the α subunit (34, 35).

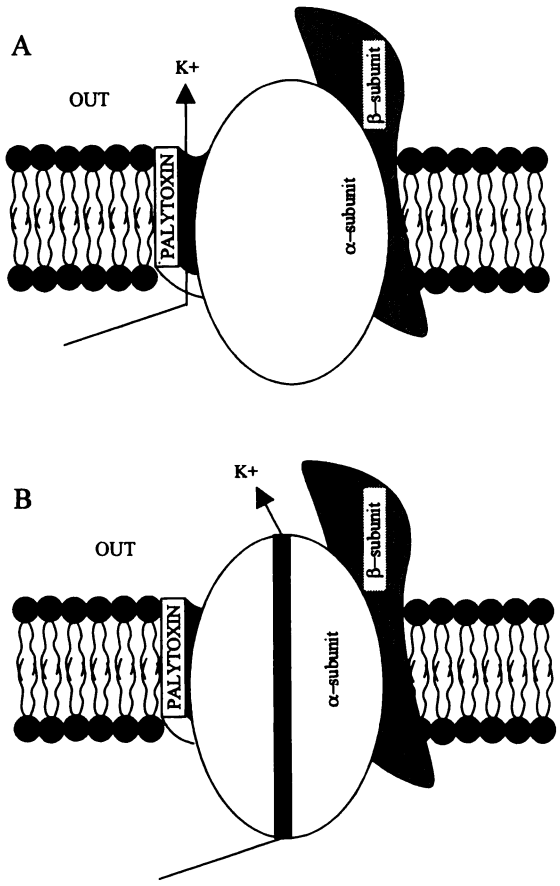

Fig. 5. Possible interactions of the palytoxin molecule with Na^+/K^+-ATPase. A, Ion channel is formed between the palytoxin molecule and parts of the Na^+/K^+-ATPase. B, Binding of palytoxin to the molecule induces the opening of a channel within the enzyme that might be the physiological ion-conducting channel.

Tryptic digestion of Na^+/K^+-ATPase leads to the removal of the hydrophilic parts of the enzyme that account for ~50% of the total protein (34–36). The remaining membrane-embedded parts of the enzyme, however, are still able to occlude Na^+ and Rb^+ ions in a 3:2 stoichiometry (36). Rb^+ is a K^+ surrogate (1, 3, 37). The total amount of occluded Na^+ or Rb^+ within these so-called 19-kDa membranes is approximately twice that occluded by native enzyme (34, 36). This preparation of the sodium pump lacks ATP-dependent functions (34, 36). It contains a 19-kDa fragment that is derived from the carboxyl-terminal end of the α subunit. When reconstituted into phospholipid vesicles, membranes containing the 19-kDa fragment can exhibit a Rb^+/Rb^+ exchange (34). This fragment is absolutely essential for the occlusion of Na^+ or Rb^+ ions and must therefore be either directly or indirectly involved in the formation of the ion-occluding structure of the sodium pump (34–36, 38). Labeling experiments using dicyclohexyl-carbodiimide suggest a direct involvement of the 19-kDa structure in ion occlusion and in the formation of the channel of the Na^+/K^+-ATPase (35, 38).

If the physiological channel of the sodium pump is required to obtain the effects of palytoxin, then removal of parts of the 19-kDa structure should considerably affect palytoxin-in-

duced K^+ efflux from yeast cells expressing this truncated form of the sodium pump. Therefore, we examined the interaction of palytoxin with yeast cells expressing a truncated enzyme missing 44 of the carboxyl-terminal amino acids of the α subunit. The truncated enzyme was produced by inserting a stop codon into the *Eco*81I restriction site of the cDNA of the α1 subunit.

Yeast cells express the truncated enzyme, as verified by Western blot (Fig. 6). The small difference between the relative molecular masses of the α1 (110,000) and α1-Δct44 (105,500) subunits is not detectable under these conditions. Binding of [^{3}H]ouabain to the two forms of the enzyme is not essentially affected by the truncation. Scatchard analysis of [^{3}H]ouabain binding to the microsomal fractions from yeast cells expressing either the native or the truncated form of the enzyme in the presence of inorganic phosphate and Mg^{2+} reveals a K_D = 3.8 nM for the native $\alpha1\beta$/ouabain complex and a K_D = 8.8 nM for the α1-Δct44β/ouabain complex (Fig. 7). Thus, the affinity of the truncated enzyme for ouabain is slightly reduced but can still be characterized as high affinity binding (4). The ability of the truncated form of the sodium pump to bind ouabain is not unexpected. Ouabain binding was demonstrated with chimeric enzymes constructed between the amino-terminal part of the α subunit of the sodium pump and the carboxyl-terminal part of the Ca^{2+}-ATPase (39–43). Because ouabain does not bind to the Ca^{2+}-ATPase, these experiments clearly show that an intact α subunit is not required for ouabain binding and also demonstrate that ouabain binds to the amino-terminal portion of the enzyme. Stable Na^+/K^+-ATPase/ouabain complexes can form only when the enzyme is phosphorylated. Therefore, one should assume that the truncated enzyme, like the unmodified Na^+/K^+-ATPase, can become phosphorylated by inorganic phosphate. In our experiments, the formation of a phosphoenzyme was not measured directly because the membrane preparations used also contain the endogenous yeast H^+-ATPase that also becomes phosphorylated by ATP (44). Phosphorylation, however, of a naturally occurring truncated form of the α1 subunit (referred to as α-1-T) has been reported (45). The α-1-T form of the α1 subunit of the Na^+/K^+-ATPase is missing a large part of its carboxyl-terminal end that accounts for ~40 kDa (46, 47). The truncated form of the enzyme used in our experiments is lacking only 4.5 kDa of the carboxyl-terminal end of its α1 subunit. Therefore, it is likely that this truncated form can still become phosphorylated by inorganic phosphate.

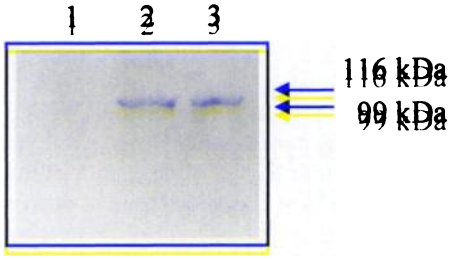

Fig. 6. Immunodetection of native α1 subunit and truncated α1-Δct44 subunit of the sodium pump. The isolation of microsomal protein and the conditions for electrophoresis and immunoblotting are described in Materials and Methods. *Lane 2*, the polyclonal antibody raised in rabbit against the α1 subunit of the pig kidney Na^+/K^+-ATPase recognizes the α1 subunit from yeast cells transformed with pCGY1406$\alpha\beta$. *Lane 3*, truncated α1-Δct44 subunit from yeast cells transformed with pCGY1406α1-Δct44β is visualized using the same antibody. The small difference between the molecular masses of α1 and α1-Δct44 (110,000 and 105,500, respectively) is not apparent. There is no protein, however, in the microsomal fraction from untransformed yeast cells that can be recognized by the antiserum (*lane 1*). In all three lanes, the interaction of the primary antibody with the native or truncated α1 subunits was visualized by a peroxidase-conjugated antibody against rabbit IgG.

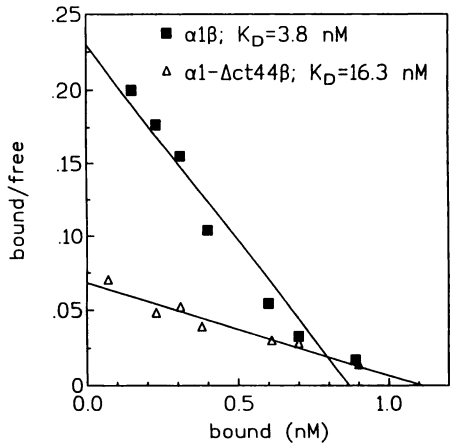

Fig. 7. Scatchard analysis of [^{3}H]ouabain binding to microsomal membranes from yeast cells expressing native or truncated Na^+/K^+-ATPase. A total of 1 mg of microsomal protein was incubated for 30 min with increasing concentrations of [^{3}H]ouabain in 10 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$, and 3 mM Tris-PO_4, pH 7.5. The amount of radioactive ouabain in each sample was kept constant.

Although the truncated form of the enzyme binds [^{3}H]ouabain, yeast cells that express this enzyme are insensitive to palytoxin (Fig. 8). Thus, the removal of portions of the carboxyl-terminal 19-kDa structure affects the palytoxin-induced K^+ efflux. Because this structure is essential for the formation of the ion channel that occludes Na^+ or Rb^+ ions, our result supports the idea that the physiological channel of the sodium pump is required to obtain palytoxin-induced ion fluxes.

Alternatively, the inability of palytoxin to induce a channel in yeast cells expressing the truncated form of the enzyme might be due to the removal of the palytoxin binding site, or the truncated enzyme might not reach the plasma membranes. This latter possibility was investigated by measuring

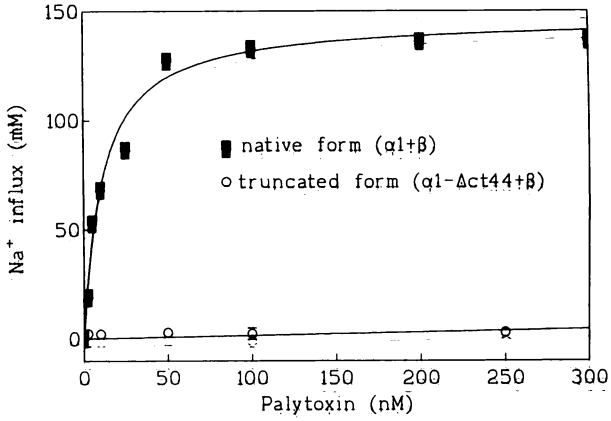

Fig. 8. Palytoxin concentration dependence of Na^+ influx in yeast cells expressing native or truncated Na^+/K^+-ATPase. Yeast cells expressing the indicated forms of the α^1 subunits were incubated for 60 min with various concentrations of palytoxin, and Na^+ influx was determined as described in Materials and Methods. Each point represents the mean of three measurements, where standard error was <5% of the mean.

the binding of [^{3}H]ouabain to whole yeast cells expressing either the native or the truncated form of the enzyme. [^{3}H]Ouabain binds to cells expressing the truncated form of the enzyme (Fig. 9), demonstrating that the truncated form of the sodium pump is expressed and present at the cell surface. Total ouabain binding, however, is reduced in the truncated form compared with the native enzyme. Because [^{3}H]ouabain was used for equilibrium binding at a concentration that is ~13-fold greater than its K_D, the decreased affinity of the truncated enzyme for the radioligand (Fig. 7) does not appear to be the main reason for its reduced incorporation. Furthermore, the results of the Western blot do not indicate a reduced expression level of the truncated form (Fig. 6). A reduced abundance of the truncated enzyme in the yeast plasma membrane could be the reason for reduced ouabain binding. Alternatively, the truncated enzyme *in vivo* might not be able to undergo the conformational changes required for ouabain binding (i.e., phosphorylation by ATP; Fig. 10) that the native enzyme undergoes. This difference is not as apparent under the *in vitro* conditions of the Scatchard analysis of ouabain binding, where phosphorylation of the native or truncated enzymes occurs by inorganic phosphate.

It has been previously shown that binding of ouabain and palytoxin is mutually exclusive (6). As with the native enzyme, bound [^{3}H]ouabain can also be displaced from the truncated enzyme by 200 nM palytoxin (Fig. 9). Based on this finding, it is unlikely that the truncation resulted in the removal of the palytoxin binding sites. Thus, the inability of palytoxin to induce K^+ efflux or Na^+ influx in yeast cells that express the truncated form of the Na^+/K^+-ATPase is probably due to the removal of portions of the enzyme that participate in the formation of the palytoxin-induced channel. Depending on which of the various models for the secondary structure of the α1 subunit of the sodium pump is used, the amino acids removed account for either a carboxyl-terminal cytosolic fragment or a single transmembrane segment (35, 48, 49). This portion of the α1 subunit, therefore, might

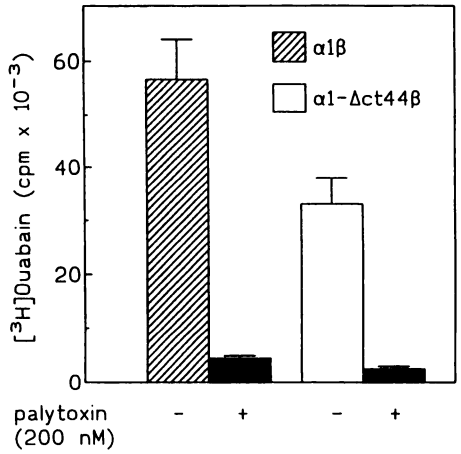

Fig. 9. Binding of [^{3}H]ouabain to whole yeast cells expressing native or the truncated α1 subunit and its displacement by palytoxin. Specific binding of [^{3}H]ouabain with all cells expressing either the native or the truncated form of the sodium pump was obtained by incubating 10 × 10^6 cells/ml with 120 nM [^{3}H]ouabain for 20 min at room temperature. Simultaneous presence of 200 nM palytoxin caused a significant reduction in the bound [^{3}H]ouabain. Background binding of [^{3}H]ouabain to nontransformed yeast cells was 980 ± 550 cpm (not shown). *Bars*, mean ± standard error of triplicate measurements.

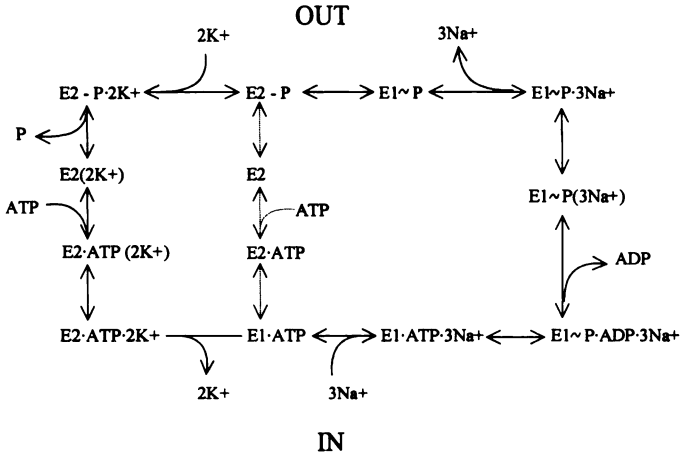

Fig. 10. Reactions of Na^+/K^+-ATPase (*solid lines*) in the presence of Na^+ and K^+ ions and of Na^+-ATPase (*dotted lines*) in the presence of Na^+ ions with K^+ omitted (1). The reactions from E1·ATP to E2-P are common for both activities (*right*). E1 and E2 are distinct conformational states of the enzyme. E1~P indicates a high energy phosphoenzyme, and E2-P indicates a low energy phosphoenzyme. *Parentheses*, occluded states of the ions.

participate in the formation of the palytoxin-induced channel, either as a part of the ion channel or by being necessary for conformational changes that lead to the formation of the ion channel.

Because palytoxin induces the flux of ions other than Na^+ that are also recognized and transported by the sodium pump, it is possible that the truncated parts of the α1 subunit are also involved in the exchange of Na^+ and K^+ ions under physiological conditions. This possibility, as well as whether the palytoxin-induced channel is the permanently opened form of the physiological, ion-conducting part of the sodium pump, remain to be investigated. The fact that the removed 44 carboxyl-terminal amino acids are a part of the 19-kDa structure that was shown earlier to be essential for occlusion of Na^+ or Rb^+ supports this idea.

Although binding of ouabain and palytoxin is not fundamentally affected by the truncation, a continuous ATP hydrolysis was not detectable in membrane preparations from yeast cells expressing the truncated form of the enzyme. Membranes from yeast cells expressing the unmodified enzyme exhibit a Na^+/K^+-ATPase activity of 33.8 ± 3.5 munits/mg of protein. The Na^+-ATPase activity measured in the absence of K^+ or its surrogates was 6.4 ± 1.9 munits/mg in the same membrane preparations. Recent experiments with Ca^{2+}-ATPase that is missing 33 carboxyl-terminal amino acids have demonstrated that this portion of the enzyme is essential for activity (50). Because Na^+/K^+-ATPase and Ca^{2+}-ATPase are members of the same enzyme family with some homology in functional domains, the carboxyl-terminal sequence of the α1 subunit of the sodium pump also appears to be essential for activity.

Under the conditions of the coupled spectrophotometric assay, ATP hydrolysis can be detected only if it happens continuously. Thus, our results do not verify whether the truncated form binds and hydrolyzes ATP only once at the outset of the reaction. If this does occur, however, ATP hydrolysis probably is not followed by a transport step for the ions. For both Na^+/K^+-ATPase or Na^+-ATPase activities, the first step of the reaction is phosphorylation of the enzyme by

ATP and subsequent occlusion of three Na^+ ions with simultaneous dissociation of ADP from the enzyme (Fig. 10). Because the removal of the 44 carboxyl-terminal amino acids disrupts the 19-kDa structure that is essential for occlusion of ions, the Na^+ occlusion step that is required for the continuation of the reaction does not occur. Therefore, it is not surprising that the truncated enzyme does not display the Na^+/K^+-ATPase and Na^+-ATPase activities that are associated with occlusion and deocclusion of ions.

The results are consistent with the palytoxin-induced ion fluxes occurring through the ion channel of the sodium pump that under physiological conditions maintains the transport of Na^+ and K^+ ions through the plasma membrane. This conclusion could be further investigated by the introduction of site-directed mutations in the predicted transmembrane domains of the α subunit of the sodium pump with subsequent expression of the mutants in yeast and investigation of their interactions with palytoxin; such research is in progress.

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