J. Membrane Biol. 192, 181–189 (2003)
DOI: 10.1007/s00232-002-1074-9

The Journal of
Membrane
Biology

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Effects of Palytoxin on Cation Occlusion and Phosphorylation of the (Na^+,K^+)-ATPase

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Abstract. Palytoxin (PTX) inhibits the $(Na^+ + K^+)$-driven pump and simultaneously opens channels that are equally permeable to Na^+ and K^+ in red cells and other cell membranes. In an effort to understand the mechanism by which PTX induces these fluxes, we have studied the effects of PTX on: 1) K^+ and Na^+ occlusion by the pump protein; 2) phosphorylation and dephosphorylation of the enzyme when a phosphoenzyme is formed from ATP and from P_i; and 3) *p*-nitro phenyl phosphatase (*p*-NPPase) activity associated with the (Na^+,K^+)-ATPase. We have found that palytoxin 1) increases the rate of deocclusion of $K^+(Rb^+)$ in a time- and concentration-dependent manner, whereas Na^+ occluded in the presence of oligomycin is unaffected by the toxin; 2) makes phosphorylation from P_i insensitive to K^+, and 3) stimulates the *p*-NPPase activity. The results are consistent with the notion that PTX produces a conformation of the Na^+,K^+-pump that resembles the one observed when ATP is bound to its low-affinity binding site. Further, they suggest that the channels that are formed by PTX might arise as a consequence of a perturbation in the ATPase structure, leading to the loss of control of the outside "gate" of the enzyme and hence to an uncoupling of the ion transport from the catalytic function of the ATPase.

Key words: Palytoxin — (Na^+,K^+)-ATPase — *p*-NPPase — Cation occlusion — Phosphorylation — ATPase channel

Introduction

Palytoxin (PTX) isolated from the marine soft coral (genus *Palythoa*) is one of the most potent nonproteinic animal toxins, with high cytotoxicity to mammalian cells in vitro as well as in vivo. Since its discovery by D. Malo in Hawaii (Malo, 1951), it has been found that it produces a plethora of pharmacological actions at the cellular, tissue and (full) organism level, described in several reviews (Moore & Scheuer, 1971; Ibrahim & Shier, 1987; Habermann, 1989; Tosteson, 2000). Of importance to the work we will present is the fact that palytoxin inhibits the active transport of Na^+ and K^+ across cell membranes and, most strikingly, alters the cellular cationic permeability, making cells equally permeable to Na^+ and K^+ (Habermann & Chhatwal, 1982; Tosteson et al., 1991; Tosteson, 2000). It has been shown by Scheiner-Bobis et al. (Scheiner-Bobis et al., 1994), that palytoxin binds to the Na^+-pump and turns the (Na^+,K^+)-ATPase into a channel, but the actual mechanism of that transformation is not understood. Work to establish the molecular mechanism of palytoxin's action has further shown that PTX alters the activity of the *p*-NPPase in such a way that in its presence the phosphatase can be activated by Na^+ as well as K^+, consistent with the notion that the PTX-(Na^+,K^+-ATPase) complex accepts Na^+ as well as K^+ at the K^+-activation site (Tosteson et al., 1997). We have shown in a preliminary report that in the toxin-enzyme complex, the K^+ occlusion site is altered as well, suggesting that the ionic permeability changes observed in the presence of palytoxin might be due to a disruption of the coupling of ion transport to ATP hydrolysis (Tosteson et al., 1997). If this is, indeed, the mode of action of palytoxin, studies of the properties of the cation-binding sites should help to further the understanding of the molecular mechanism of PTX's toxicity.

A partial account of the work presented in this communication was published in a preliminary report (Tosteson et al., 1997).

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Materials and Methods

Enzyme Preparation

Na,K-ATPase (EC 3.6.1.37) from pig and dog kidney were purified as previously described (Askari, Huang & McCormick, 1983; Esmann, 1994). The specific hydrolytic activity measured at 37°C and under optimal conditions (in mM: 150 NaCl, 20 KCl, 3 ATP and 3 $MgCl_2$, pH 7.4) was about 25 U/mg protein for both pig kidney and dog kidney enzyme. The protein content was determined as described by Jensen & Ottolenghi (1983), using bovine serum albumin as standard. We have used both enzymes throughout our experiments with qualitatively similar results.

Measurement of $^{86}Rb^+$ Occlusion

$^{86}Rb^+$ occlusion was determined as described by Hasenauer et al. (Hasenauer, Huang & Askari, 1993), in the presence and in the absence of palytoxin, at the concentrations indicated in the figures. Briefly, the enzyme (70 μg/ml) was added to an incubation medium containing 1 mM Tris-borate (pH 7.0), 200 mM sucrose, 12 mM histidine (pH 6.8) and incubated for 30 minutes at room temperature, in the absence and in the presence of PTX. The occlusion reaction was done at 4°C and started by adding ^{86}Rb. An initial sample (in duplicate) was withdrawn 4 s after addition of the isotope and passed through the cation-exchange column. The enzyme, containing bound ^{86}Rb, was collected in the eluate and counted using conventional procedures. Aliquots of 0.5 ml (in duplicate) were then removed at various time intervals and passed through the cation exchange column.

Measurements of $^{86}Rb^+$ Deocclusion

$^{86}Rb^+$ deocclusion was determined using an enzyme labeled with $^{86}Rb^+$ in the borate-containing sucrose-histidine buffer. Palytoxin (concentration as indicated) was added at time zero and duplicate aliquots (0.5 ml) of the sample were removed at the times indicated. The samples were passed through the cation-exchange column and counted as described above. Whenever indicated, the dilution medium contained RbCl at the final concentration indicated.

Determination of $^{22}Na^+$ Occlusion

$^{22}Na^+$ occlusion was determined following the protocol described by Esmann and Skou (1985). In brief, the pig kidney enzyme (or the dog kidney enzyme) was incubated with $^{22}Na^+$ (1 mM total) in a medium containing 20 mM Histidine, 20 μg/ml oligomycin, (a concentration that provides full occlusion in the absence and presence of borate) and in the absence and presence of 50 μM PTX. The blank control was obtained in the absence of oligomycin. The amount of $^{22}Na^+$ bound to the enzyme was determined from the eluate of the cation exchange column, as well as the amount of protein present in the sample.

p-NPPase Activity

p-NPPase activity was determined using the colorimetric assay as described by Skou (1974). For all enzyme preparations tested, we determined the optimum concentrations of $MgCl_2$ and of *p*-nitro phenyl phosphate in the absence of palytoxin. The assays in the presence of palytoxin were conducted without changing the [$MgCl_2$] or the [*p*-NPP].

Determination of Phosphorylation of the (Na^+, K^+)-ATPase

The measurements of phosphorylation of the enzyme either from [γ-^{32}P]ATP or from $^{32}P_i$ were performed according to Cornelius (1995). The phosphorylation from [γ-^{32}P]ATP (10 μM) was performed in a medium containing 4 mM $MgCl_2$, 1 mM borate, 30 mM imidazole (pH 7.4) and either 16 or 100 mM NaCl, in the absence or in the presence of palytoxin, as indicated in the figures. Phosphorylation from $^{32}P_i$ (0.5 mM) was carried out in the presence of 4 mM $MgCl_2$, 1 mM borate, 10 mM HEPES/10 mM MES, pH 7.5, in the presence or absence of palytoxin and/or K^+. The reactions were terminated by adding an acid stop-solution containing 10% trichloracetic acid (TCA), 100 mM phosphoric acid and 20% glycerol (0°C). Protein concentration and radioactivity were determined after resuspension in 1 M NaOH at 55°C.

Dephosphorylation of the Phosphoenzyme

These measurements were started with an enzyme phosphorylated with 0.5 mM $^{32}P_i$, by chasing the labeled, bound $^{32}P_i$ with 50 mM unlabeled P_i (in the absence or in the presence of PTX). If the phosphoenzyme had been made in the presence of K^+, $^{32}P_i$ was chased with 50 mM unlabeled P_i and 1 mM KCl (±PTX as indicated). In both cases, the chase was followed by addition of the stop solution containing 10% TCA and 2 mM sodium pyrophosphate.

Materials

[γ-^{32}P]ATP and $^{32}P_i$ were from Amersham. *N*-Methyl-D-glucamine, *N*-2-Hydroxyethylpiperazine-*N*′-ethane sulfonic acid (HEPES) and 2-*N*-morpholino ethane sulfonic acid (MES), were purchased from Sigma. Palytoxin was purchased from Hawaii Biotechnology Group Inc. with the help of Dr. Gary Bignami. All chemicals were reagent grade.

Results

Effects of Palytoxin on Cation Binding and Release in the Absence of Phosphorylation

Potassium Binding

The aim of the experiments described in this section was to characterize the effects of PTX on the cation binding sites of the enzyme. To this end, we measured the extent of cation bound to the isolated enzyme, as well as the extent of release of the cation under various experimental conditions.

The results of the determination of Rb^+ binding in the absence and presence of palytoxin are shown in Fig. 1. The maximum extent of Rb^+ occlusion in the absence of PTX is comparable to values previously reported (*see* legend Fig. 1) (Forbush III, 1987; Hasenauer et al., 1993). In the presence of PTX, the initial rate of Rb^+ binding is not changed appreciably at all concentrations of PTX tested (5–300 μM) (*see* inset Fig. 1*A*). Nonetheless, as shown in Fig. 1*A*, in the presence of 10 μM palytoxin, the apparent equi-

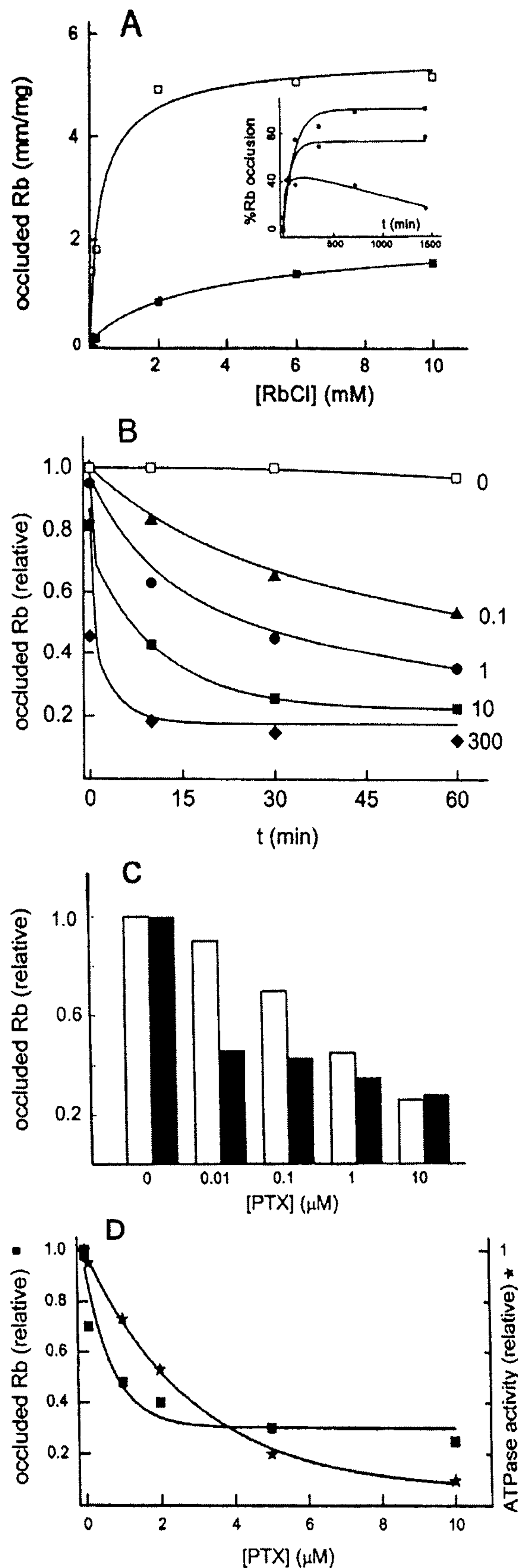

Fig. 1. Effect of palytoxin on the occlusion of $^{86}Rb^+$ by the dog kidney Na,K-ATPase. All experiments were performed at 4°C. Occlusion was terminated after 24 hours of incubation in media indicated below. Spontaneous deocclusion was allowed to proceed for the times indicated below. Each sample was processed as described in Materials and Methods, all assays were done in triplicate, and the mean of three values is presented. The experiments were repeated at least two times on separate days. (*A*) Binding of Rb^+ to the enzyme as a function of [RbCl]. The incubations were performed in the presence of varying [RbCl] (50 μM to 10 mM) in the absence (□) and in the presence (■) of PTX. The individual values differed from the mean by less than 5% for control experiments, and by less than 15% for experiments with palytoxin. [Palytoxin] = 10 μM. Hyperbolic fitting of the curves yielded $K_{0.5} \approx 0.3 \pm 0.1$ mM, $Binding_{max} \approx 5.4 \pm 0.4$ nmol/mg for control (□) and $K_{0.5} \approx 2.7 \pm 0.8$ mM, and $Binding_{max} \approx 1.9 \pm 0.5$ nmol/mg in the presence of PTX (■). *Inset:* Time course of Rb^+ occlusion in the absence (■) or in the presence of 5 μM (■) or 300 μM (●) PTX; [RbCl] = 200 μM (for clarity, not all the experimental points are shown). The ordinate depicts Rb^+ occlusion relative to the maximum value obtained in the absence of PTX. (*B*) Time course of Rb^+ release. Rb^+ was occluded for 24 hr and its release followed in the absence (□) or in the presence of 0.1 μM (▲), 1 μM (●), 10 μM (■) or 300 μM (◆) PTX. The first time point shown was taken 4 s after dilution of radioactive Rb^+. Rb^+ that remained bound was normalized to the value of Rb^+ occluded measured just before addition of PTX. The lines are double-exponential fits to the data points, shown to aid in visualization. (*C*) Effect of [PTX] on Rb^+ release. Rb^+ (200 μM) was occluded for 24 hrs in the absence of PTX. The radioactive samples were then diluted in media containing the [PTX] indicated in the abscissa and either 0 mM RbCl (*white bars*) or 10 mM RbCl (*black bars*). The amount of Rb^+ bound to the enzyme 60 s after dilution is plotted relative to the value that obtains in the absence of Rb. (*D*) Comparison of the effect of [PTX] on Rb^+ release and on (Na^+, K^+)-ATPase activity. Rb^+ bound to the enzyme (relative to the amount remaining in the absence of PTX; left hand scale) 30 min after dilution in a medium containing the [PTX] indicated (■). The enzymatic activity was measured in separate samples, at the same [PTX]. The ATPase activity is shown relative to the activity in the absence of PTX (*) (right hand scale). Enzyme source: dog kidney.

librium level of occluded Rb^+ at all concentrations of Rb^+ tested is significantly lower than in its absence. The overall effect of palytoxin seems to be an apparent reduction in the affinity of the enzyme for Rb^+. The $K_{0.5} \approx 0.3 \pm 0.1$ mM in the absence of PTX increases to a $K_{0.5} \approx 2.7 \pm 0.8$ mM when PTX (10 μM) is present (fitted using a hyperbolic saturation curve, *see* Fig. 1*A*). A possible explanation for the lower apparent equilibrium level of occluded Rb^+ in the presence of PTX is that toxin binding leads to an increased rate of release of the Rb^+ bound to the enzyme. This hypothesis was confirmed by the results of the experiments shown in Fig. 1*B*, namely the release of Rb^+ as a function of time in the dilution media, in the absence and in the presence of different concentrations of PTX. The time course of this release in the presence of PTX is biphasic, with an initial drop in the bound Rb^+ within the first 4 s, which is almost over by the first minute after the sample is put in the dilution media, followed by a

slower release, the rate of which is higher than the rate in the absence of PTX (Fig. 1*B*). Of interest is that when [PTX] ≥ 10 μM, the release of Rb^+ seems to reach a plateau so that even after 1 hour in the release media, there remain 10 to 30% of Rb^+ bound even at the highest PTX concentration tested (300 μM) (*see* Fig. 1*B*).

In the absence of PTX, Rb^+ in the dilution medium does not affect the release of occluded Rb^+ (Glynn, Hara & Richards, 1984; Liu & Askari, 1997). The data shown in Fig. 1*C* reveal that this is not the case in the presence of PTX. Palytoxin accelerates deocclusion of Rb^+ and this effect is markedly enhanced by 10 mM RbCl in the dilution medium so that ≥40% of the occluded Rb^+ is released within the first minute, and this level remains unchanged after two hours in the deocclusion medium (*data not shown*).

To test if the effect of Rb^+ on the spontaneous deocclusion of Rb^+ in the presence of PTX is due to an incomplete inhibition of the Na^+ pump, we measured the effect of varying concentrations of PTX on the hydrolytic activity of the protein. As shown in Fig. 1*D*, even though the effects of PTX on the release of Rb^+ and on the inhibition of the enzyme activity are highly correlated, the catalytic activity of the enzyme is inhibited by more than 95% when [PTX] ≈8 μM, but approximately 30% of Rb^+ remains occluded even at PTX concentrations 30 times as high (*see* Fig. 1*B*). Thus, we think that the effect of Rb^+ on the kinetics of Rb^+ deocclusion might reflect the presence of two pockets of occluded Rb^+ (K^+) with different sensitivities to palytoxin, similar to what Liu and Askari (1997) have described for the sensitivity of Rb^+ occlusion to ATP.

Sodium Binding

To determine if Na^+ binding is also modified by PTX, we tested the ability of PTX to release Na^+ that had been occluded in the presence of oligomycin. We found that the amount of Na^+ bound was practically the same in the absence (2.7 ± 0.5 nmol/mg) as in the presence (2.8 ± 0.7 nmol/mg) of 50 μM palytoxin, indicating that palytoxin does not affect the oligomycin-induced occlusion of Na^+ (dog kidney as well as pig kidney ATPase). An obvious explanation for this result would be that PTX cannot bind to the enzyme in the presence of oligomycin. However, we and others have found that PTX increases the cationic permeability of cells to the same extent in the absence and in the presence of oligomycin (Ozaki, Nagase & Urakawa, 1985; Tosteson, 2000), indicating that PTX binds under these experimental conditions and promotes the formation of the nonselective cationic channel. Thus, our results on the effect of PTX on cation occlusion are consistent with the idea that formation of the PTX-induced channels modifies

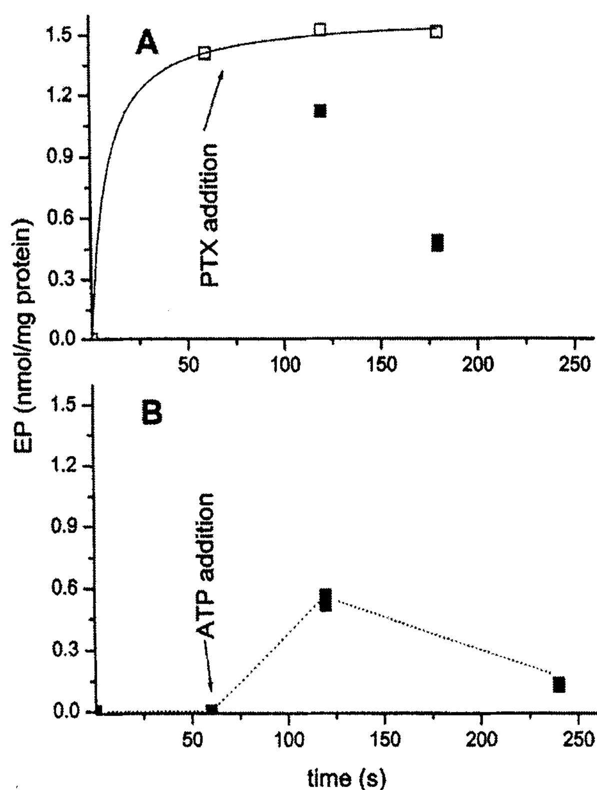

Fig. 2. Effect of PTX on the steady-state level of ATP phosphorylation. (*A*) Enzyme dephosphorylation by PTX. The enzyme was phosphorylated in the absence (□) of PTX. At the arrow, PTX was added to a final concentration of 5 μM leading to a loss of bound phosphate (■). The line was drawn according to a single-exponential fit; not all experimental points are shown. (*B*) Effect of PTX on phosphorylation. The enzyme was preincubated with PTX for 1 min and [γ^{32}P]ATP was added as indicated. The broken line joins the experimental points to help visualization. Enzyme source: pig kidney.

the K^+-but not the Na^+-occlusion sites in the (Na^+,K^+)-ATPase. Reinforcing this hypothesis, Lingrel et al. (1996) have found that mutations in a serine residue located on the putative 5th transmembrane segment yields an ATPase with an affinity reduced for K^+ but unchanged for Na^+.

Effects of Palytoxin on Phosphorylation

[γ-^{32}P]ATP

The experiments presented thus far have dealt with the effects of palytoxin binding to the enzyme in the nonphosphorylated state and the subsequent effects on the binding of cations. Since phosphorylation can lead to binding or release of cations, we decided next to determine if the enzyme can be phosphorylated in the presence of palytoxin. Previous work from our laboratories has shown that ATP is not hydrolyzed in human red cells exposed to palytoxin (Tosteson et al., 1991). To determine if this is due to PTX displacing bound ATP or to a displacement of P_i, we first

Table 1. Effect of 5 μM palytoxin on the phosphorylation of Na, K-ATPase from P_i and dephosphorylation properties of the phosphoenzyme

Phosphorylation conditions	[PHOSPHOENZYME] (nmol/mg ± SD)		
	Equilibrium level of phosphoenzyme	Residual phosphoenzyme (2s) spontaneous	K^+
PTX 0	2.00 ± 0.15	1.70 ± 0.13	1.78 ± 0.15
PTX 5 μM	2.72 ± 0.10	2.47 ± 0.15	2.33 ± 0.1
PTX 0; KCl 1 mM	0.53 ± 0.15		0.11 ± 0.13
PTX 5 μM; KCl 1 mM	2.12 ± 0.10		2.0 ± 0.15

Phosphorylation from $^{32}P_i$ (0.5 mM) was carried out in the presence of 4 mM $MgCl_2$, 1 mM borate, 10 mM HEPES/10 mM MES, pH 7.5. Other conditions as indicated in the Table. Phosphorylation was stopped by addition of a cold acid solution. Dephosphorylation of the phosphoenzyme was determined by chasing with 50 mM unlabeled P_i and stopping the reaction 2 s after the chase by addition of cold acid. The chase solution was of the same composition as that used for phosphorylation and indicated in the Table, except that it also contained 50 mM unlabeled P_i. The data are mean values of three different determinations.

measured the steady-state level of ATP phosphorylation under two different experimental conditions, as summarized in Fig. 2. Panel *A* of the figure shows the results of experiments in which the ATPase was incubated in the presence of radioactively labeled ATP and continued to be incubated with [γ-^{32}P]ATP while simultaneously being exposed to 5 μM PTX. We found that there is a fairly rapid displacement of ^{32}P from the enzyme under these conditions. Furthermore, as shown by the results depicted in panel *B*, when PTX is present at the time of labeling, the amount of the phosphorylated form of the enzyme is greatly reduced, suggesting that binding of palytoxin either hinders binding of ATP or greatly stimulates release of bound P_i or both. These results prompted us to determine if phosphate could be bound at all in the presence of palytoxin, by studying the phosphorylated enzyme formed from $^{32}P_i$.

$^{32}P_i$ Phosphorylation in the Absence of Monovalent Cations

Table 1 shows that the equilibrium level of phosphorylation from 0.5 mM $^{32}P_i$, in the absence of monovalent cations and ouabain, is about 2 nmol/mg protein, in agreement with previous reports (Post, Taniguchi & Toda, 1974; Cornelius, Fedosova & Klodos, 1998). Under identical experimental conditions, palytoxin increases the equilibrium level of phosphorylation by 30% and the rate of spontaneous dephosphorylation of the phosphoenzyme was found to be equal to that in the absence of PTX (*data not shown*), suggesting that the dephosphorylation process, measured in the absence of cations, is not affected by palytoxin (*see* Table 1). Addition of 1 mM KCl to the dephosphorylation media does not increase the rate of dephosphorylation of the E_2P phosphoenzyme formed either in the absence or in the presence of palytoxin (*not shown*). These results indicate that palytoxin increases the level of phosphorylation, suggesting a stimulation of the formation of an acid-stable phosphoform by the toxin. To try to determine if this conformation is one already identified, we took advantage of the fact that when the fluorescent dye RH421 binds to the enzyme, the fluorescence response increases upon phosphorylation of the enzyme either by addition of P_i or by addition of ATP (Fedosova, Cornelius & Klodos, 1995). Thus, we tested the action of PTX on the phosphorylation of the RH421 enzyme and found that PTX completely abolishes the response of the system (*data not shown*), suggesting that the conformation of the phosphoform obtained in the presence of PTX is different from the one found in the absence of PTX.

$^{32}P_i$ Phosphorylation in the Presence of KCl

The presence of 1 mM KCl (and absence of PTX) leads to a reduction in the equilibrium level of the acid-stable phosphoenzyme to about 26% of the level in the absence of alkali cation (Table 1), as has been previously described (Post, Hegyvary & Kume, 1972). Under these experimental conditions, the presence of palytoxin increases significantly the level of acid-stable phosphoenzyme to the phosphorylation level in the absence of palytoxin and K^+. Furthermore, the phosphoenzymes obtained in the absence and presence of PTX were found to be also different in their sensitivity to the *presence* of K^+ in the dephosphorylation medium. Thus, as has been found previously, the data in Table 1 show that the phosphoenzyme formed in the absence of PTX dephosphorylates faster when KCl is present in the chase solution, since all the label is lost in 2 s. In contrast, the dephosphorylation of the phosphoenzyme formed in the presence of palytoxin (and K^+) is not affected by K^+ in the medium since the level of dephosphorylated enzyme is approximately the same without and with

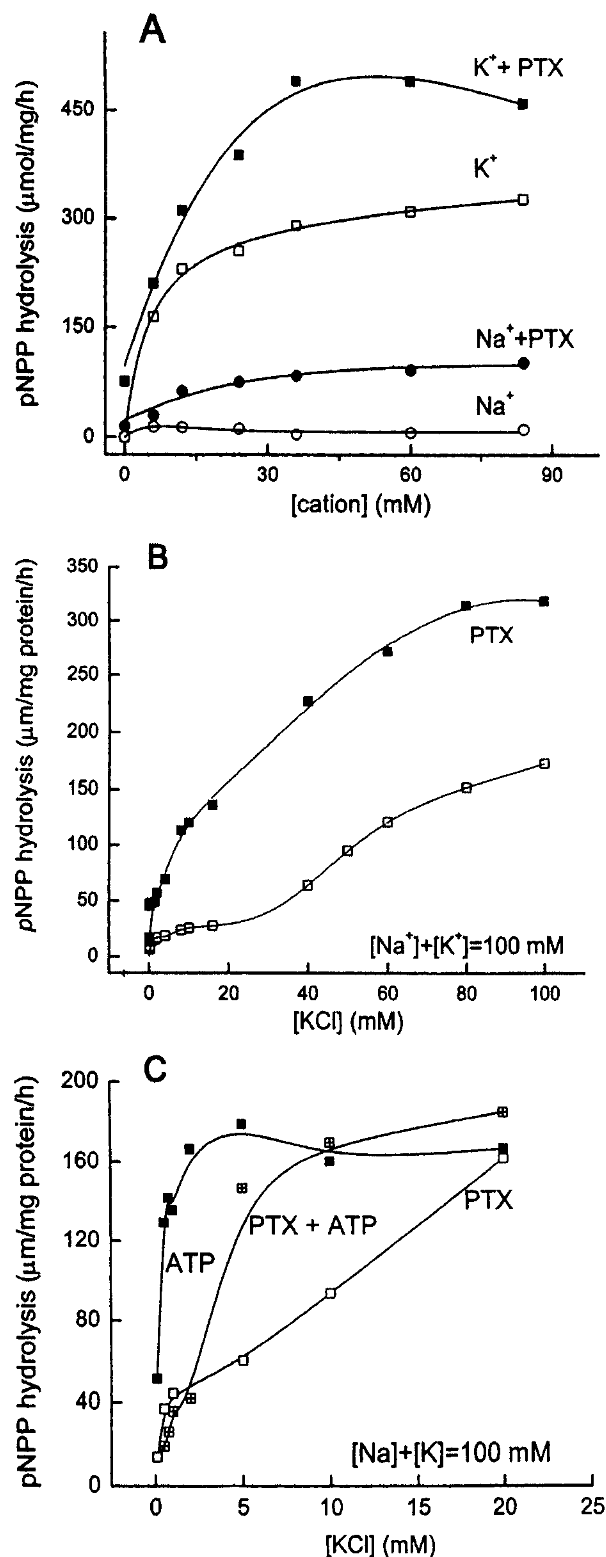

Fig. 3. Effect of palytoxin on the hydrolysis of the K^+-dependent *p*-NPPase. Enzyme was suspended in assay medium that contained 1 mM borate, 0.2 mM EGTA, Mg^{2+} and *p*-NPP (concentrations indicated below) in addition to NaCl or KCl (*A*) or NaCl + KCl (*B* and *C*). [PTX] = 10 μM when present. The enzymatic reaction was started after obtaining a steady baseline, by addition of an aliquot of concentrated $MgCl_2$ to obtain the desired final concentration. (*A*) Effect of cation concentration on the hydrolysis of *p*-NPP in the presence and absence of PTX. Cations: (○, ●) Na^+; (□, ■) K^+. The open symbols indicate the absence of PTX. 10 mM $MgCl_2$, 20 mM *p*-NPP. (*B*) Effect of varying cation concentration on the K^+ activation of the *p*-NPPase: 20 mM $MgCl_2$; 20 mM *p*-NPP; ([NaCl] + [KCl]) = 100 mM. (*C*) Effect of ATP and PTX on the *p*-NPPase activity: 10 mM $MgCl_2$; 20 mM *p*-NPP; ([NaCl] + [KCl]) = 100 mM; 0.1 mM ATP. All experiments shown were performed with each of the enzyme preparations.

K^+. In other words, the effects of K^+ on the level of phosphorylation and its ability to induce dephosphorylation are abolished by palytoxin, suggesting that the PTX-containing enzyme is capable of binding P_i in a K^+-insensitive manner, presumably due to the rapid deocclusion of the cation induced by the toxin.

Effect of Palytoxin on the *p*-NPPase Activity

It has been proposed that the K^+-occluded form of the dephosphoenzyme is responsible for the phosphatase activity that is associated with the (Na^+,K^+)-ATPase (Post et al., 1972). Thus, if the effect of palytoxin binding to the enzyme results in the rapid deocclusion of K^+, then we would expect that the phosphatase activity will be impaired in the presence of the toxin. However, as shown in Fig. 3, we have found that PTX increases the *p*-NPPase activity, not only when Na^+ and K^+ are present but also in the presence of each of these cations alone. Figure 3*A* shows that in the presence of PTX either K^+ or Na^+ present in the medium as the sole major cation increases the hydrolysis of the *p*-NPPase, underscoring the loss of the cationic selectivity of the pathway. Panel *B* of Fig. 3 shows that PTX increases the hydrolytic activity of the *p*-NPPase as the concentration of K^+ is increased, (at constant [Na^+ + K^+]). The most salient feature in this graph is the activating effect of high [Na^+] on the activity of the *p*-NPPase, reminiscent of the activating effect of ATP, in the absence of PTX (Skou, 1974). Thus, as shown in Fig. 3*B*, at low [K^+]/[Na^+], the catalytic activity is increased and the apparent affinity for K^+ seems to be increased as well, although since the enzyme loses its ability to distinguish between Na^+ and K^+, the increased enzymatic activity of the *p*-NPPase (at low [K^+]/[Na^+]) could be due in part to an activation by Na^+. Figure 3*C* shows the effect of ATP on the hydrolysis of the *p*-NPPase in the presence and absence of PTX. With no toxin present and with ATP in the medium, the *p*-NPPase activation curve as a function of increasing [K^+] is very steep at the low K^+/Na^+ concentration ratio, as was shown previously (Skou, 1974). In the presence of PTX and ATP, the region with a low [K^+]/[Na^+] is not so steep as with ATP alone, and not so steep (at very low [K^+]/[Na^+]) as with PTX alone, under-

scoring the partial loss of the activation induced by ATP as well as the loss of selectivity for K^+ over Na^+.

Discussion

Work in several laboratories as well as in our own has established that the main effects of palytoxin are: 1) the opening of a very large and poorly selective cationic permeability that can be prevented or reversed by ouabain (Tosteson et al., 1991); and 2) the inhibition of the catalysis of the K^+ and Na^+-dependent hydrolysis of ATP and activation of the *p*-NPPase (Tosteson et al., 1997; Tosteson, 2000) and references therein).

Three other compounds have been shown to have the dual role of activators of the *p*-NPPase and inhibitors of the (Na^+, K^+)-ATPase: phlorizin (Robinson, 1969), ouabain at low concentrations (Pitts & Askari, 1971) and thimerosal (Henderson & Askari, 1977). However, there are several differences in the modes of action of these compounds and PTX. In particular and most importantly, only PTX increases the cationic conductance of cell membranes and produces a loss of the discrimination of the pump between Na^+ and K^+. Ouabain, at low concentrations and in the absence of K^+, stimulates the Mg^{++}-dependent *p*-NPPase activity; in the presence of K^+, ouabain inhibits this activity. In contrast, PTX activates the K^+-phosphatase in a [K^+]-dependent manner, at concentrations that inhibit the ATPase $>80\%$ (*see* Figs. 1*D* and 3). Thimerosal affects the conformational transitions between the nonphosphorylated forms of the enzyme without affecting those between the phosphorylated states (Kaplan & Mone, 1985). In contrast, PTX modifies both the dephospho- and the phosphorylated conformations of the enzyme, as described above (*see* Results). Phlorizin seems to increase the K^+ affinity and the stability of occluded Rb^+ (Post et al., 1972), whereas PTX increases the rate of the spontaneous deocclusion and seems to decrease the affinity of the enzyme for K^+ (*see* Fig. 1).

The results reported in this paper have further shown that the PTX-E complex retains some of the catalytic activities of the (Na^+, K^+)-ATPase. In particular, we have found the following:

1) The complex of the pump protein with palytoxin retains the capacity of the native enzyme to occlude both Na^+ and K^+ (Rb^+). We found that the extent of occlusion for Na^+ binding was the same in E and PTX-E, whereas palytoxin reduces the apparent affinity of the occlusion site for K^+, since it accelerates the deocclusion in a [PTX]- and [K^+]-dependent manner, without substantially altering the rate of occlusion (*see* Fig. 1). It is striking that in the presence of PTX, both K^+ and Na^+ high-affinity binding can occur, despite the presence of channels that permit high fluxes of these cations.

2) The binding of PTX to the enzyme allows for phosphorylation and dephosporylation, but with profound modifications. In the presence of toxin, the extent of phosphorylation from ATP is greatly reduced by a PTX-promoted rapid dephosphorylation, in the presence of labeled ATP (*see* Fig. 2*A*) or by inhibition of ATP binding (*see* Fig. 2*B*). This effect is not observed when the enzyme is phosphorylated from P_i. In this case, however, PTX turns the phosphoenzyme into a K^+-insensitive form (*see* Table 1). These results reinforce the notion that the phosphorylated intermediates formed from ATP are different than those formed from P_i, as has been suggested previously (Cantley, Cantley & Josephson, 1978; Fedosova, Cornelius & Klodos, 1998).

3) Interaction of PTX with the enzyme produces an increase in the hydrolytic activity of the *p*-NPPase by Na^+ (in the absence of K^+) (Fig. 3). This result shows that the loss of cationic selectivity by the (Na^+, K^+)-ATPase is also seen in the associated phosphatase activity. Our results further suggest that the conformation of the ATPase responsible for the K^+-dependent phosphatase activity is a deoccluded E_2 form, E_2 K, as suggested by Berberián and Beaugé (1985). In the presence of PTX there is an effect of ATP on the *p*-NPPase activity both at low and at high [K^+]/[Na^+], as shown by Skou (1974), but PTX seems to inhibit some of the effect, as if there was a competition for a binding region (*see* Fig. 2*C*).

Some of the characteristics described for the PTX-E can be found in studies in which the (Na^+, K^+)-ATPase has been subjected to controlled proteolysis. Thus, Hasenauer et al. (1993) and Liu and Askari (1997) have shown that ATP added to the proteolysis-modified enzyme accelerates the deocclusion of K^+ without modifying the initial rate of occlusion of K^+, and that Rb^+ (K^+) in the medium stimulates the ATP-induced deocclusion even further. This was described in a preparation in which Lys^{766}, one of the nine residues that have previously been suggested to be involved in high-affinity ATP binding, was the only one remaining in the digested enzyme. Based on the fact that the presence of 2 mM ATP affects the kinetics of Rb^+ occlusion-deocclusion, as does the occupancy of the low-affinity ATP binding site in the unmodified enzyme, the authors speculate that this residue could be part of the low-affinity ATP binding site, and that the high-affinity binding site(s) and the low-affinity binding site(s) are different. Taking as a reference the model in which the pump protein traverses the bilayer ten times, this residue is part of the M5-M6 intramembrane fragment which Argüello and Kaplan

(Argüello & Kaplan, 1991, 1994) have found to be involved in Rb^+ occlusion. Lutsenko, Anderko and Kaplan (1995) have further suggested that the M5-M6 segment might be involved in phosphorylation/deocclusion as well. Jørgensen et al. (1999) have also shown an involvement of the M4-M5 as well as M6 and M8 intramembrane regions of the (Na^+, K^+)-ATPase, in high-affinity cation binding, through studies of protein mutations and expression into yeast. These authors conclude that both Na^+ and K^+ bind to the same region in the pump protein and that structural changes could account for the changes in affinity for the ions. On the other hand, Lingrel et al. (1996) have found that mutations in a serine residue located on the putative 5th transmembrane segment yields an ATPase with an affinity reduced for K^+ and unchanged for Na^+. These authors have further suggested that mutations of the two aspartic-acid residues on the putative transmembrane domain might be involved in the high-affinity K^+ binding. Taking all these results into account, and on the basis of our results showing that in the presence of PTX, K^+ occlusion is impaired by an increased rate of deocclusion, whereas Na^+ occlusion (in the presence of oligomycin) is not, we would argue that in the presence of palytoxin either the conformational change that it induces does not alter the Na^+ binding site or that Na^+ and K^+ do not share a common region for tight binding. This hypothesis is further reinforced by the results of Kaplan et al. (1996) who found that K^+ but not Na^+ could protect the M5-M6 from loss from the membrane upon extensive proteolysis. In recent communications, Guennoun and Horisberger (2000, 2002) have presented evidence that supports the involvement of residues in the M5 and M6 transmembrane domains in the palytoxin-induced cationic channel.

It is of interest to note that even though palytoxin binds exclusively to the external surface of the pump protein (Habermann, 1989), this binding translates to fundamental changes of the catalytic steps in the pump cycle that occur through ligand binding to cytoplasmic sites. A clear explanation of these several effects of PTX on the properties of the (Na^+, K^+)-pump must await determination of the high-resolution, three-dimensional structure of the PTX-enzyme complex.

The authors acknowledge the help provided by Dr. Amir Askari in facilitating the experiments on Rb^+ occlusion with the kind assistance of Dr. Liqin Liu. We also acknowledge Dr. Patricio Garrahan in whose laboratory Drs. S.B. Kaufman and R.M. González-Lebrero performed the experiments on the equilibrium level of occluded Rb^+ (pig kidney enzyme). We are indebted to Dr. N. Fedosova for performing the phosphorylation experiments in her laboratories (pig kidney enzyme). We are also grateful to her for her insight, support and extremely useful discussions. Finally, we acknowledge the helpful comments to the manuscript by Dr. Joseph F. Hoffman. This work was supported in part by a grant from the Dana Foundation to MTT.

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