Channel induction by palytoxin in yeast cells expressing Na^+,K^+-ATPase or its chimera with sarco/endoplasmic reticulum Ca^{2+}-ATPase

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Abstract **Palytoxin (PTX) induces a cation channel through interaction with Na^+,K^+-ATPase. It is unclear how this action relates to the enzyme catalytic activity. We examined whether the action of PTX depends on the catalytic domain specific for Na^+,K^+-ATPase. Wild-type Na^+,K^+-ATPase α-subunit (NNN) or its chimera (NCN), in which the catalytic domain was replaced with that of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase, was co-expressed with β-subunit in the yeast *Saccharomyces cerevisiae*. PTX (0.1–100 nM) increased K^+ efflux in NNN- or NCN-transfected cells to a similar degree but not in non-transfected cells. When ouabain-resistant NNN and NCN were expressed, PTX also increased K^+ efflux. Ouabain inhibited the effect of PTX in NNN or NCN cells but not in ouabain-resistant cells. These data suggest that the channel-forming action of PTX does not depend on the catalytic domain species.**

Key words: Palytoxin; Na^+,K^+-ATPase; Channel; Catalytic domain; Yeast; Chimera

1. Introduction

Palytoxin (PTX), isolated from *Palythoa* spp., is the most potent marine toxin known. We have reported that PTX induces a cation channel, which is permeable to monovalent cations, in cardiac ventricular or vascular smooth muscle cells [1–3]. Its lethal action is ascribed to an increase in cytosolic Na^+ concentration and the subsequent increase in cytosolic Ca^{2+} [4]. Cardiac glycosides such as ouabain antagonize the action of PTX and many reports suggest that the site of action of PTX is the Na^+,K^+-ATPase (for review, [5]). This hypothesis was proved by the work of Scheiner-Bobis's group, in which they showed that PTX increased K^+ permeability in yeast cells transfected with the Na^+,K^+-ATPase but not in non-transfected cells [6]. It is supposed that the Na^+,K^+-ATPase includes a channel, which permits passage of Na^+ and K^+ [7,8]. Therefore, the PTX-sensitive channel is thought to inherently exist in the enzyme and to be responsible for transport of Na^+ and K^+. However, it has not been determined where the PTX-sensitive channel is located in the enzyme and whether the action of PTX to open the channel depends on the catalytic activity.

In order to determine whether the PTX-sensitive channel activity depends on the catalytic domain species of the enzyme, we co-expressed a chimeric α-subunit composed of the Na^+,K^+-ATPase and the catalytic domain of the sarcoplasmic/endoplasmic Ca^{2+}-ATPase (SERCA) with the β-subunit in the yeast *Saccharomyces cerevisiae*, which does not contain endogenous Na^+,K^+-ATPase [9], and observed the effect of PTX to increase K^+ efflux from the cells as an index of channel-forming action.

2. Materials and methods

2.1. cDNA construction

We wanted to construct a chimeric ATPase (NCN), in which the large cytoplasmic loop between the two putative membrane-spanning regions M4 and M5 was exchanged between the chicken Na^+,K^+-ATPase α1-subunit (NNN) [10] and the chicken SERCA1 (CCC) [11]. This was done by recombination between the encoding cDNAs. The 3′- and 5′-ends of the exchanged fragments were generated by the restriction enzymes for the unique endogenous *Eco*NI site [12] and the unique introduced *Kpn*I site [13], respectively. The endogenous *Eco*NI site corresponds to Lys^{724} of NNN or Lys^{712} of CCC. The unique *Kpn*I site at the position encoding Gly^{377} of NNN or Gly^{354} of CCC was created by oligonucleotide-directed mutagenesis without amino acid mutation.

For the construction of ouabain-resistant NNN (ORNNN) or NCN (ORNCN), the cDNA sequences corresponding to Thr^{116} and Asn^{127} in NNN or NCN were mutated to Arg and Asp, respectively, using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). Mutation at these sites renders the enzyme resistant to ouabain [14,15].

2.2. Expression of cDNAs in yeast cells

Synthetic complete and drop-out media were prepared in a manner similar to that described elsewhere [16]. The cDNAs encoding NNN, NCN, ORNNN and ORNCN were cloned into a yeast expression vector, pYES2 (Invitrogen Japan, Tokyo, Japan) and then introduced into yeast *S. cerevisiae* strain BJ3505 cells (*Mata pep4::HIS3 prb1-D1.6R HIS3 lys2-208 trp1-D101 ura3-52 gal2 can*) [17] together with the chicken Na^+,K^+-ATPase β-subunit cDNA cloned into a NMV4 expression vector (β-NMV4, *TRP1*, *CEN4*) [18] using the lithium

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Abbreviations: PTX, palytoxin; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; NNN, wild-type α-subunit; NCN, chimeric α-subunit containing the catalytic domain of SERCA Ca^{2+}-ATPase; ORNNN, ouabain-resistant NNN; ORNCN, ouabain-resistant NCN

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acetate method. Transfected cells were grown for 2–4 days on a synthetic selection plate lacking uracil and tryptophan at 30°C until colony formation. Then, the colony was inoculated into a test tube containing liquid selective medium and incubated for 24 h.

The α- and β-subunits were co-expressed under control of the GAL1 promoter in the selective medium containing 2% galactose. Four to eight hours later, the cells were collected, centrifuged at 3000×*g* for 5 min at 4°C and subjected to experimentation.

2.3. K^+ efflux from transfected yeast cells

The cells were suspended at a concentration of 1×10^7 cells/ml in NHBC buffer (in mM: 200 NaCl, 10 HEPES, 0.5 borate, 1 $CaCl_2$, pH 7.5), rinsed twice with NHBC buffer to remove extracellular K^+, then resuspended in the buffer.

PTX at each concentration was added to the cell suspension in a test tube, incubated for an appropriate time and then centrifuged at 12 000×*g* for 1 min at 4°C. Potassium in the supernatant was measured as K^+ efflux from the cells by flame photometry. To measure potassium remaining in the cells, lysis buffer (0.02% sodium dodecyl sulfate, 200 mM NaCl, 10 mM HEPES, 0.5 mM borate, 1 mM $CaCl_2$, pH 7.5) was added to the pellet. The cells were destroyed by boiling for 10 min at 95°C and centrifuged at 12 000×*g* for 1 min at 4°C. Potassium concentration in the supernatant was measured to estimate residual potassium in the cells. The intracellular volume of yeast cells was calculated as described previously [19].

3. Results

3.1. PTX-induced K^+ efflux in cells transfected with wild-type Na^+,K^+-ATPase and chimeric ATPase

First, we measured the PTX-induced K^+ efflux from yeast cells transfected with NNN or NCN. PTX (0.1–100 nM) dose-dependently increased K^+ efflux in NNN- or NCN-transfected cells, whereas PTX at the highest concentration (100 nM) did not stimulate K^+ efflux in non-transfected cells (Fig. 1). The amount of K^+ effluxed from the cells over 2 h was similar between NNN- and NCN-transfected cells at each PTX concentration and the concentration–response curves overlapped. PTX (100 nM)-induced K^+ efflux 2 h after application was 57.4 ± 7.3% ($n=6$) in NNN cells and 65.4 ± 11.1% ($n=6$) in NCN cells of the total cellular K^+. However, the time-course of K^+ efflux induced by 100 nM PTX in NCN cells was slow compared to that in NNN cells (Fig. 2). When

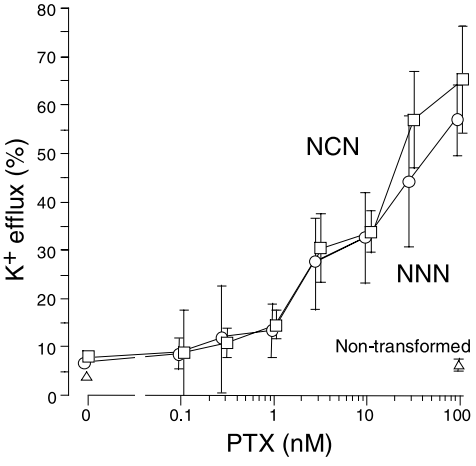

Fig. 1. Concentration-dependent effect of PTX on K^+ efflux from yeast cells transfected with NNN or NCN. K^+ that flowed out from NNN- (circles), NCN- (squares) or non-transfected (triangles) cells over 2 h after application of PTX is expressed as percentage of total cellular K. Each point represents mean ± S.E.M. of six determinations.

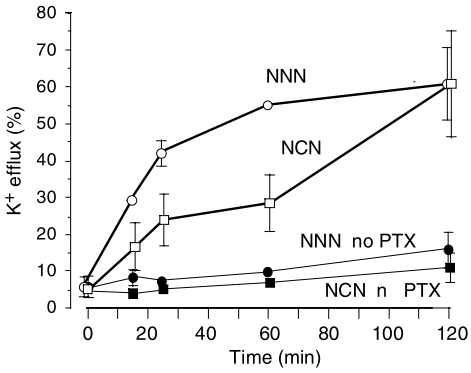

Fig. 2. Time-course of PTX-induced K^+ efflux from yeast cells transfected with NNN or NCN. PTX (100 nM) was applied at time 0 to NNN- (open circles) or NCN- (open squares) transfected cells. K^+ efflux is expressed as percentage of total cellular K^+. Closed circles and closed squares are control K^+ efflux in the absence of PTX. Each point represents mean ± S.E.M. of six determinations.

borate was absent in the external medium, the amount of K^+ that effluxed from the cells decreased to about half of that in the presence of 1 mM borate (31.1 ± 1.1% vs. 64.3 ± 0.7% in the absence or presence of borate, respectively ($n=6$), 2 h after application of PTX in NNN cells), consistent with the data that borate promoted the action of PTX [20,21].

3.2. Effects of ATPase inhibitors

Ouabain (10–100 μM) inhibited the PTX-induced K^+ efflux. The inhibition by ouabain was slightly greater in NNN cells than in NCN cells (Fig. 3). On the other hand, thapsigargin (1 μM), a SERCA inhibitor, did not affect the efflux in either NNN or NCN cells (data not shown). Likewise, amiloride (30 μM), which inhibits Na^+-H^+ exchange, Na^+-Ca^{2+} exchange and epithelial Na^+ channels, had no effect on the PTX-induced K^+ efflux (data not shown).

When sodium orthovanadate (50–200 μM), which inhibits a P-type ATPase by competing with ATP at the catalytic site, was applied 2 h before the addition of PTX (100 nM), the action of PTX to cause K^+ efflux was inhibited to a similar degree in NNN- and NCN-transfected cells (Fig. 4). In other experiments, sodium azide (0.5 or 1 mM), which inhibits ATP production, was applied to cells 2 h before PTX. Sodium azide also depressed the action of 100 nM PTX (Fig. 4).

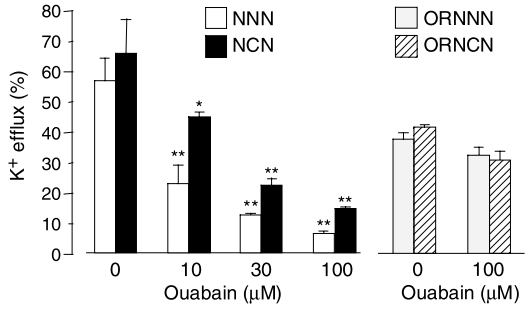

Fig. 3. Effect of ouabain on the PTX-induced K^+ efflux in NNN- or NCN-transfected cells (left panel) and in ORNNN- or ORNCN-transfected cells (right panel). Cells were pretreated with ouabain 30 min prior to PTX. K^+ efflux is expressed as percentage of total cellular K^+. Each point represents mean ± S.E.M. of six determinations. $^*P<0.05$, $^{**}P<0.01$ (vs. in the absence of ouabain, Dunnett's post-hoc test following ANOVA).

3.3. Action of PTX in ouabain-resistant cells

Next, we tested the effect of PTX on ORNNN- or ORNCN-transfected cells. PTX (100 nM) increased K^+ efflux from these cells (Fig. 5). The maximum effect was similar between ORNNN or ORNCN cells. Ouabain at 100 μM did not affect the PTX-induced K^+ efflux (Fig. 3).

Sodium orthovanadate (50–200 μM) inhibited the PTX-induced K^+ efflux in ORNNN- or ORNCN-transfected cells, although the inhibition was smaller than the case in NNN or NCN cells. Two hours pretreatment with sodium azide (0.5 or 1 mM) also inhibited the K^+ efflux in these cells. In this case, too, the inhibitory effect of sodium azide was less compared to the effect on ouabain-sensitive cells.

4. Discussion

Habermann's group first speculated that the site of action of PTX was within the Na^+,K^+-ATPase or in its vicinity [21,22]. Later studies demonstrated that PTX interacts with the Na^+,K^+-ATPase since PTX exerted an effect when the Na^+,K^+-ATPase was expressed in yeast cells [6], which do not have the endogenous enzyme, or when the Na^+,K^+-ATPase protein was incorporated into a liposome [23] or a planar lipid bilayer [24,25]. The present study employed yeast expression system as used by Scheiner-Bobis's group [6] and our data were in agreement with the above findings since PTX increased K^+ efflux in yeast cells that were transfected with cDNA encoding the Na^+,K^+-ATPase but not in non-transfected cells.

Na^+,K^+-ATPase and SERCA are P-type ATPases, which undergo a cyclic change of phosphorylated and dephosphorylated conformations and have about 30% identity in amino acid sequences. The domain responsible for ATP hydrolysis is a central part of the ATPase located in the cytoplasmic loop between the fourth and fifth membrane-spanning regions. In the chimera NCN, the catalytic domain of the Na^+,K^+-ATPase α-subunit was replaced with that of SERCA. An important finding in this study is that PTX induced channel activity not only in cells expressing NNN but also in cells expressing NCN. Although the development of K^+ efflux after treatment with PTX in NCN cells was slow compared to that in NNN cells, the maximum effect was similar between two cell types and the concentration–response curve for the NCN cells overlapped that for the wild-type expressing cells. This strongly suggests that the induction of a channel by PTX does not depend on the catalytic domain species. The PTX-sensitive channel and the PTX binding site were clearly preserved after the catalytic domain was replaced with that of SERCA. The overlapping of concentration–effect curves for NNN and NCN implies that replacement of the catalytic domain did not affect the affinity of PTX to the α-subunit. Nevertheless, the possibility remains that a conformational change after insertion of the domain from SERCA may make the induction of a channel by PTX less easy because the development of PTX-induced K^+ efflux was slowed in NCN cells.

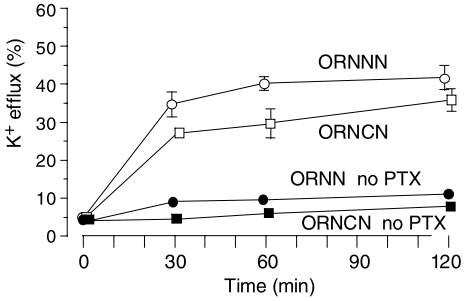

Fig. 5. Time-course of PTX-induced K^+ efflux from yeast cells transfected with ORNNN or ORNCN. PTX (100 nM) was applied at time 0 to ORNNN- (open circles) or ORNCN- (open squares) transfected cells. K^+ efflux is expressed as percentage of total cellular K. Closed circles and closed squares are control K^+ efflux in the absence of PTX. Each point represents mean ± S.E.M. of six determinations.

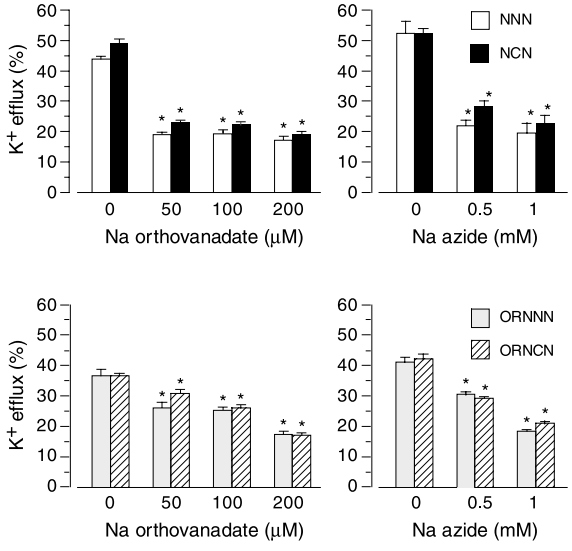

Fig. 4. Effect of sodium orthovanadate and sodium azide on the PTX-induced K^+ efflux in NNN- or NCN-transfected cells (upper panels) and in ORNNN- or ORNCN-transfected cells (lower panels). Cells were pretreated with sodium orthovanadate or sodium azide 30 min or 2 h prior to PTX, respectively. K^+ efflux is expressed as percentage of total cellular K. Each point represents mean ± S.E.M. of six determinations. $^*P < 0.01$ (vs. in the absence of ouabain, Dunnett's post-hoc test following ANOVA).

Scheiner-Bobis and Schneider [26] reported that yeast cells expressing a mutant Na^+,K^+-ATPase, in which the catalytic site was modified to resist phosphorylation by replacement of Asp^{369} with Ala, were sensitive to PTX just as the wild-type. Their data coincide with the present finding that the catalytic domain species is not essential for the PTX-induced opening of the channel and suggest that ATP hydrolysis by the catalytic domain is not directly linked to induction of a channel by PTX. Therefore, it seems that sustained opening of channel in the enzyme induced by PTX is independent of phosphorylation at this site.

In contrast to Scheiner-Bobis and Schneider's report [26] showing that phosphorylation in the catalytic domain is not required for channel induction by PTX, several reports showed that ATP promoted the action of PTX [24,27,28]. Habermann's group attributed the enhancement of the PTX effect by ATP to promotion of the PTX binding to membranes [27]. In our study, sodium azide and orthovanadate decreased the PTX-induced K^+ efflux in NNN- and NCN-transfected cells. Sodium azide inhibits ATP production through inhibition of oxidative phosphorylation in the mitochondria so that the inhibition of the action of PTX by sodium azide could be ascribed to a decrease in intracellular

ATP. Thus, ATP has a promoting effect on the action of PTX in transfected yeast cells. On the other hand, vanadate reacts with the E2 state (extracellular K^+ accessible conformation) of Na^+,K^+-ATPase to form a stable intermediate that inhibits the enzyme [29]. Inhibition of the action of PTX by vanadate was also observed in erythrocytes [24,30] or the distal colon [31]. Similarly to vanadate, ouabain preferentially binds to the Na^+,K^+-ATPase in the E2P state and inhibits the enzyme activity [32]. Based on the data on antagonism of the PTX action by ouabain or vanadate and acceleration of the action by ATP, the relationship between the PTX binding and a special state of the Na^+,K^+-ATPase has been inferred. However, the interpretations are contradictory, e.g. some groups argued that PTX binds to the E1 state (cytoplasmic Na^+-accessible conformation) [23,26] while others claimed that it binds to the E2 state [24,30]. Hence, more concrete evidence is needed to determine what state PTX prefers for its binding. Recently, Artigas and Gadsby [28] showed that MgATP, MgADP and poorly hydrolyzable MgAMPPNP increased the open probability of PTX-induced channels and suggested that this facilitation occurred through low affinity binding of ATP (namely at the E2 state). Their data tempt us to conjecture another possibility that some conformational change following binding of adenine nucleotides to the enzyme helps PTX induce the channel, as the case that the nucleotides modulate ATP-sensitive K^+ channels with no involvement of phosphorylation or ATP hydrolysis [33].

It was shown that PTX and ouabain compete for binding with each other [27]. In this study, ouabain did not affect the PTX-induced K^+ efflux in ORNNN- or ORNCN-transfected cells. After point mutations in the extracellular loop between the first and second transmembrane segments ouabain cannot bind to its high affinity site but may be able to bind to low affinity sites [34]. The present data showing that PTX exerted its effect in cells where similar mutations were introduced suggest that the binding site for PTX is not completely identical to the high affinity binding site for ouabain. Since ouabain could not affect the PTX-induced K^+ efflux at even 100 μM in ouabain-resistant cells, it is unlikely that the antagonism of the PTX action by ouabain in NNN or NCN cells is through the binding of ouabain to the low affinity site. Therefore, the high affinity binding site for ouabain could be partly included in the binding site for PTX.

The inherent presence of a channel in the Na^+,K^+-ATPase, which conveys Na^+ and K^+, has been predicted for two decades [7,8,35]. This channel could be uncovered when the Na^+,K^+-ATPase protein was incorporated in artificial membranes [36,37]. The similar sensitivity to ouabain, vanadate or ATP of the channel in reconstituted systems and the PTX-sensitive channel observed in living cells [28,30,38] suggests that the channel observed in Na^+,K^+-ATPase-incorporated membranes is identical to that induced by PTX. Therefore, it is very likely that PTX discloses a channel latent in the Na^+,K^+ pump. A problem is why PTX alters the ion transport through the enzyme from uphill movement to dissipative movement. An important issue in connection with this problem is whether an ion occlusion mechanism is modified by PTX. Recent work by Wu et al. [39] suggested that the N-terminus of the α-subunit behaves as an inactivation gate of the PTX-sensitive channel and the gate may be involved in the E1–E2 conformational change. Therefore, it is possible that the inactivation gate is responsible for ion occlusion. Analysis of PTX action in cells expressing the gene-manipulated Na^+,K^+-ATPase will lead to clarification of site and function of a latent channel in the pump.

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