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Functional role of the N-terminus of Na^+,K^+-ATPase α-subunit as an inactivation gate of palytoxin-induced pump channel

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Abstract

The N-terminus of the Na^+,K^+-ATPase α-subunit shows some homology to that of *Shaker*-B K^+ channels; the latter has been shown to mediate the N-type channel inactivation in a ball-and-chain mechanism. When the *Torpedo* Na^+,K^+-ATPase is expressed in *Xenopus* oocytes and the pump is transformed into an ion channel with palytoxin (PTX), the channel exhibits a time-dependent inactivation gating at positive potentials. The inactivation gating is eliminated when the N-terminus is truncated by deleting the first 35 amino acids after the initial methionine. The inactivation gating is restored when a synthetic N-terminal peptide is applied to the truncated pumps at the intracellular surface. Truncated pumps generate no electrogenic current and exhibit an altered stoichiometry for active transport. Thus, the N-terminus of the α-subunit appears to act like an inactivation gate and performs a critical step in the Na^+,K^+-ATPase pumping function.

Keywords: Na^+,K^+-ATPase; N-terminus; N-type inactivation; Pump channel; Palytoxin; *Shaker* K^+ channel

1. Introduction

The sodium pump (Na^+,K^+-ATPase) is a P-type ATPase that plays a central role in helping animal cells maintain basic cellular functions such as volume regulation, membrane excitability, and secondary solute transport (for reviews see Refs. [1,2]). The pump converts the energy released from ATP hydrolysis into electrochemical gradients for Na^+ and K^+ ions across the cell membrane. However, the exact mechanism of ion pumping action is not well understood. Recently, it has been shown that it is possible to transform the Na^+,K^+-ATPase into an ion channel using pharmacological agents. Palytoxin (PTX), produced by soft coral of the genus *Palythoa*, binds selectively to the Na^+,K^+-ATPase with a K_d of 20 pM [3] and transforms the pump into a channel permeable to monovalent cations with a single-channel conductance of 10 pS [4–7], although the presence of external Na^+ seems to be essential for channel activation [8]. This unique action of PTX allows us to study the structure inside the pump like regular ion channels.

The cytoplasmic N-terminus of the Na^+,K^+-ATPase α-subunit shows some homology to that of *Shaker*-B K^+ channels. In Fig. 1, the first 42 amino acids of *Torpedo* α-subunit are aligned with the first 33 of *Shaker*-B K^+ channels. Seven positively charged amino acids, five of which form a lysine cluster (underlined in Fig. 1), occupy analogous positions as those of *Shaker* channels. The first 19 amino acids of the *Shaker* channels have been shown to

Abbreviations: EGTA, ethylene glycol-bis-(β-aminoethyl ether)-*N,N*′-tetraacetic acid; EDTA, (ethylenedinitrilo)-*N,N*′-tetraacetic acid; DTT, DL-dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TEVC, two-electrode voltage clamp; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; TEA, tetraethylammonium; TMA, tetramethylammonium; PTX, palytoxin; I–V, current-voltage

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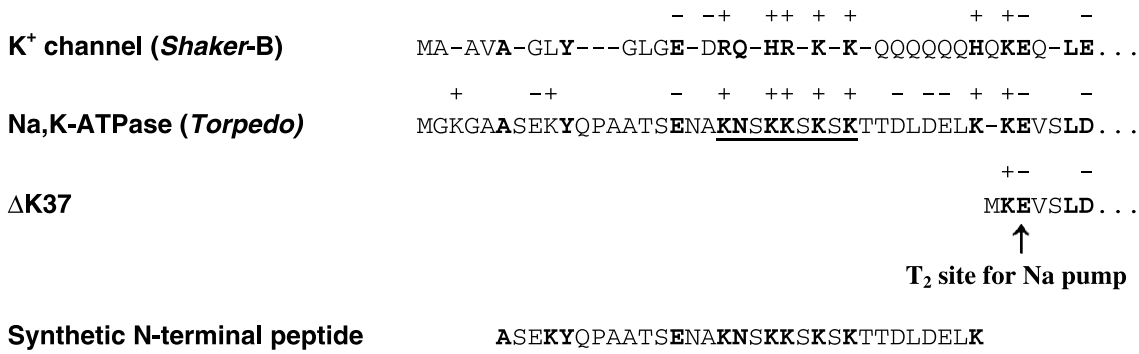

Fig. 1. Alignment of the amino acid sequences of the N-termini of the α-subunit of K^+ channel (*Shaker*-B), Na^+,K^+-ATPase (*Torpedo*), truncated Na^+,K^+-ATPase ΔK37, and the synthetic 31-mer N-terminal peptide. The + and − signs indicate the positively and negatively charged residues, the underline shows the lysine cluster, and the arrow points to the T_2 trypsin digestion site for the sodium pump. Identical or similar residues are represented in bold letters.

constitute the positively charged "ball" for a ball-and-chain mechanism of inactivation gating [9,10]. We have previously reported that truncation of the N-terminus of Na^+,K^+-ATPase α-subunit affects cation interaction with the pump [11,12] and truncation at the trypsin-digestion site, known as the T_2 site [13] (see Fig. 1), abolishes normal pump activity [11]. Therefore, we performed experiments to test the hypothesis that a ball-and-chain mechanism similar to the K^+ channel N-type inactivation may be involved in the pumping action.

The N-terminus of the α-subunit of the *Torpedo* Na^+,K^+-ATPase was truncated by deleting the first 35 amino acid residues after the initial methionine (ΔK37 mutant, Fig. 1). The wild-type and truncated pumps were expressed in *Xenopus* oocytes, and the expressed pumps were compared in terms of the inactivation kinetics of PTX-induced channel currents as well as functional properties such as ATPase enzymatic activities, Rb^+ and Na^+ fluxes and electrogenic pump currents. The main findings of this study are as follows. When the wild-type pump is transformed into an ion channel with PTX, the channel exhibits a time-dependent inactivation gating at positive potentials. The truncated pumps similarly treated with PTX show that the inactivation gating has been removed. Application of a synthetic N-terminal peptide (Fig. 1) partially restores the inactivation gating. The truncated pumps generate no electrogenic current and exhibit an altered stoichiometry for active transport. Thus, the N-terminus of the α-subunit appears to act similarly to the inactivation gate of *Shaker*-B K^+ channels and performs a critical step in the Na^+,K^+-ATPase pumping function. Some of the preliminary results of this study have been reported [14].

2. Materials and methods

2.1. Construction of deletion mutant ΔK37

The plasmids pSPT(α) and pSPT(β) containing the cDNA encoding the α- and β-subunits, respectively, of the *Torpedo* Na^+,K^+-ATPase were constructed as described previously [11]. Deletion mutant pSPT(ΔK37) was constructed from pSPT(α) using the same methods as described in Ref. [15].

2.2. Expression of Na^+,K^+-ATPase in Xenopus oocytes

Full-grown prophase-arrested oocytes were isolated from female frogs of *Xenopus laevis* and treated with 1.5 or 3 mg/ml collagenase for 2–4 h or overnight, respectively [11]. *Torpedo* α-subunits, either wild-type or the ΔK37 mutant, were expressed along with *Torpedo* β-subunits in oocytes by injection of the corresponding cRNAs (30 and 15 ng/oocyte for the α- and β-subunits, respectively) [11].

2.3. Voltage clamp and patch–clamp experiments

Electrophysiological experiments were performed either on intact oocytes with the conventional two-electrode voltage clamp (TEVC) or on excised membrane patches with the patch–clamp technique. For measuring pump currents in intact cells, oocytes were pretreated for 30 min with a Na^+-loading solution (solute concentrations in mM: NaCl 110, Na citrate 2.5, MOPS 5, pH 7.6) followed with a post-loading solution (NaCl 100, $CaCl_2$ 1, $BaCl_2$ 5, TEA-Cl 20, MOPS 5, pH 7.6) for recovery. Steady-state membrane currents were recorded at the end of 200 ms voltage pulses from −90 up to +80 mV in 10-mV increments that were applied from a holding potential of −60 mV. The difference between the steady-state currents in the presence of extracellular K^+ (5 mM) and in the absence was used as a measure of the pump currents. K^+-free solution consisted of (in mM): TMA-Cl 90, TEA-Cl 20, $BaCl_2$ 5, $NiCl_2$ 2, MOPS 5, and pH 7.4. To make the extracellular K^+-containing solution, an appropriate amount of KCl was added to the K^+-free solution. The TEA^+ and Ba^{2+} were used to block all non-pump-mediated K^+-sensitive currents, and the Ni^{2+} to block Na^+/Ca^{2+} exchange. For measurements of PTX-induced currents, oocytes expressing the pump molecules were treated for 4–8 min with 30 or 100 pM PTX followed by

washing to obtain optimal, stable current responses. In both the TEVC and patch–clamp experiments, 7-s-long rectangular pulses to potentials ranging from −80 to +80 mV were applied to elicit currents, and steady-state currents were determined from the current during the last 20% of the pulse. The external solution was composed of (in mM): Na-gluconate 90, KCl 2, $CaCl_2$ 2, TEA-Cl 20, $BaCl_2$ 5, and MOPS 5, titrated to pH 7.4 with TMA-OH. Oocytes which showed signs of run-down during experiments such as an increase in leakage currents were excluded from analysis.

For patch–clamp recording of inside–out excised patches, the solution for patch electrodes contained (in mM): NaCl 90, KCl 2, $CaCl_2$ 2, and MOPS 5, titrated to pH 7.4 with Tris base. The bath solution contained (in mM): NaCl 80, Na_2ATP 5, $MgCl_2$ 5, KH_2PO_4 5, EGTA 5, and MOPS 5, titrated to pH 7.4 with Tris base. Electrophysiological experiments were conducted at room temperature (24–26 °C). All oocyte experiments were performed at the Max Planck Institute for Biophysics, Frankfurt/Main, Germany.

2.4. Measurements of ouabain binding, $^{86}Rb^+$ uptake and $^{22}Na^+$ release

The number of ouabain binding sites, which represent the number of pump molecules per oocyte, was determined by incubating Na^+-loaded oocytes for 20 min in K^+-free solution containing 2.5 μM [^{3}H]ouabain and 2.5 μM unlabeled ouabain [11]. The same batches of Na^+-loaded oocytes were used for measurements of Rb^+ uptake. The oocytes were incubated in Na^+-free test solution containing 5 mM $^{86}Rb^+$ (925 kBq/ml) for 16 min. The component of Rb^+ uptake blockable with 100 μM ouabain was taken as the pump-mediated uptake. To estimate the rate of Na^+ release mediated by the pump, oocytes were microinjected with 90 nl of ^{22}NaCl (about 25 MBq/ml). The oocyte was then placed in a perfusion chamber that was mounted on a Geiger–Müller tube, and loss of radioactivity activated by 5 mM extracellular K^+ was monitored [16]. The relative rate of efflux was determined from the exponential decline of radioactivity in the oocyte.

2.5. ATPase assay

For assay of ATPase activity, about 150 oocytes for each preparation were homogenized in 6 ml of 50 mM imidazole-HCl (pH 7.5) containing 250 mM sucrose, 1 mM EDTA and 1 mM PMSF, using Polytron for 30 s twice while chilled in ice water. The amount of inorganic phosphate released was measured colorimetrically.

2.6. Western blotting

For preparation of Triton extracts, five oocytes were disrupted by passing them through Eppendorf pipette tips in a homogenization buffer (10 μl/oocyte) containing (in mM): Tris–HCl 20 (pH 7.4), $MgCl_2$ 5, EDTA 1, NaCl 100, KCl 10, DTT 1, PMSF 1, Triton X-100 1%, and 5 μg/ml each of leupeptin, pepstatin and antipain. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. α-Subunits were immunostained with anti-*Torpedo*-α antibody and visualized with a secondary alkaline phosphatase-conjugated antibody obtained from Biozol (Eching, Germany).

2.7. Materials

PTX was purchased from Dr. László Béress, Institute of Toxicology, Christian-Albrechts University at Kiel, Germany, and from Hawaii Biotechnology Group, Honolulu, USA.

The N-terminal 31-mer peptide of the adult form of *Torpedo* Na^+,K^+-ATPase α-subunit, ASEKYQPAATSENAKNSKKSKSKTTDLDELK (with the C-terminal amidated), was synthesized by Hokkaido System Science Co., Sapporo, Japan. The purity was determined by HPLC to be 99.4%.

3. Results

3.1. Expression of pump molecules

Xenopus oocytes were injected with cRNA for either wild-type *Torpedo* or ΔK37 α-subunit (Fig. 1) together with cRNA for *Torpedo* β-subunit. Western blots of oocyte membrane proteins show that both the wild-type and truncated pumps were expressed (Fig. 2A). Measurements of ouabain binding to membrane surface of the oocytes confirm that both the wild-type and ΔK37 mutant were inserted into the membrane equally well (Fig. 2B). These results show that the truncation did not abolish expression of the α-subunit and subsequent targeting of the whole pump molecule to the plasma membrane.

3.2. Effect of N-terminal truncation on transport activity of pump molecules

Measurements of ATPase enzymatic activity, expressed as ouabain-sensitive activity in microsomes prepared from the oocytes, show that the truncated mutant is about 55% active as compared to the wild-type (Fig. 2B). The results are comparable with those of previous measurements from oocytes expressing the mutant pumps [17] and those obtained with trypsin cleavage at the T_2 site [13]. Active transport activity was assessed by measurements of ouabain-sensitive $^{86}Rb^+$ uptake and $^{22}Na^+$ efflux. After the $^{86}Rb^+$ uptake by the endogenous pumps was subtracted and the data were normalized to the ouabain binding, the uptake by oocytes expressing the truncated pump was found only at 36% level of the wild-type (Fig. 2B). Separate measurements of $^{22}Na^+$ efflux rate on different batches of oocytes

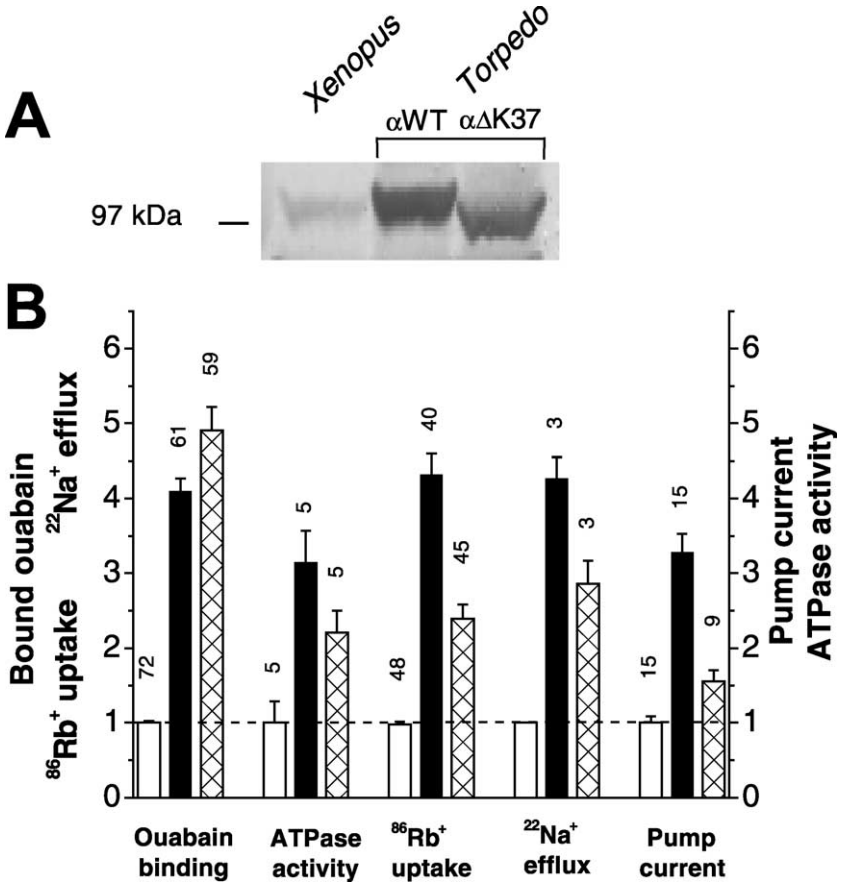

Fig. 2. Effect of deletion of 35 amino acid residues from the N-terminus of the α subunit on expression and function of the sodium pump. (A) Western blot of homogenates from non-injected control oocytes (*Xenopus*) and from oocytes injected with either cRNA of wild-type (αWT) or truncated (αΔK37) α subunits of *Torpedo* pumps together with cRNA for the β subunit. (B) Comparison of normalized data of ^{3}H-ouabain binding, ATPase activity, $^{86}Rb^{+}$ uptake, $^{22}Na^{+}$ extrusion, and pump-generated current of endogenous, wild-type, and mutant pumps. Hollow bars represent data obtained from non-injected control oocytes, filled bars from oocytes with expressed wild-type pumps, double-hatched bars from oocytes with expressed truncated pumps. Data are averages (± S.E.) with the numbers of oocytes indicated above the bars. Data obtained were normalized to the average of the control data from the same batch of oocytes. The control data for normalization were: ouabain binding, 25 fmol/oocyte; ATPase activity, 1.4 μmol Pi/mg/h; $^{86}Rb^{+}$ uptake, 3.5 pmol/min/oocyte; $^{22}Na^{+}$ efflux, 70×10^{-6}/s/oocyte; pump-generated current, 52 nA/oocyte.

showed that the mutant pumps were transporting at 48% level of the wild-type (Fig. 2B). The data show that the mutant pump also effectively extruded Na^{+} ions from the cell even though at a reduced efficacy. Thus, the measurements of ATPase enzymatic activity, ouabain binding, and fluxes demonstrate that the truncated pump is functionally inserted into the plasma membrane. On the other hand, electrogenicity was reduced as shown by steady-state pump currents (at −60 mV) activated by 5 mM K^{+} (with zero Na^{+}) externally, which were measured with the TEVC. The oocytes expressing the wild-type pump yielded an increase in pump current of 228% above the non-injected oocytes (control), whereas those expressing the truncated pump showed only an increase of 55% above the control, or 20% of the wild-type when normalized to the pump density. These results were qualitatively similar to what we observed previously [11], suggesting that K^{+} is pumped into the cell to a similar extent as Na^{+} out of the cell by the truncated pumps. The small increase of pump current over the control probably suggests that the truncated pump operates primarily in an electrically silent mode ($2Na^{+}/2K^{+}$) and only occasionally may become electrogenic.

3.3. Effect of N-terminal truncation on channel properties of the Na^{+},K^{+}-ATPase

The results indicate that the N-terminus plays a critical role in ion translocation. Therefore, we examined the effect of N-terminal truncation on the channel properties of the Na^{+},K^{+}-ATPase by comparing PTX-induced currents between the wild-type and truncated pumps expressed in oocytes. Two different methods were used to measure PTX-induced currents, namely, the TEVC and patch–clamp methods. The currents measured before PTX application were subtracted from those measured after PTX to obtain the "difference current" due to PTX alone. The endogenous

Xenopus sodium pump is 30–100 times less sensitive to PTX than the *Torpedo* pumps. Therefore, the currents obtained in the presence of 30–100 pM PTX were almost entirely from the expressed pumps. Fig. 3A shows a family of currents obtained by TEVC from the wild-type pump treated with 30 pM PTX. At potentials negative to +40 mV the currents appeared time-independent, but at potentials above +40 mV (the top two traces) the currents exhibited a time-dependent decrease in conductance, similar to the inactivation of *Shaker* K^+ channels, but with a much slower time-course. The currents from the truncated pumps which were treated similarly with PTX are shown in Fig. 3B. At high positive potentials, the currents clearly show that the time-dependent inactivation process had been eliminated. The results are similar to the elimination of the inactivation gating of *Shaker*-B K^+ channels upon N-terminal truncation [9].

Fig. 3C shows the steady-state current-voltage (I–V) relationships of currents from wild-type (filled squares) and truncated (open squares) pumps treated with 30 pM PTX. The wild-type pump channel exhibited characteristics of an inward rectifier with reduced currents at positive potentials due to inactivation, whereas the truncated pump channel lacked such rectification.

Similar results were also obtained using the patch–clamp technique. Fig. 4A shows a series of outward currents obtained in the presence of 30 pM PTX and elicited with pulses to 20–100 mV in 10-mV increments from a membrane patch of an oocyte expressing the wild-type Na^+,K^+-ATPase. A clear pattern of time-dependent inactivation was observed, which was reflected in the inward rectification in the corresponding I–V curve (Fig. 4D). Similar results were obtained with both on-cell and inside–out patches. Fig. 4B shows a series of outward currents obtained in the presence of 30 pM PTX from an inside–out patch of an oocyte expressing the ΔK37 truncated mutant; the records and the corresponding I–V curve (Fig. 4E) clearly show that the inactivation process and inward rectification have been eliminated and thus corroborate the TEVC results (Fig. 3).

3.4. Effect of reconstitution of the N-terminal peptide to truncated pumps

If the elimination of inactivation and inward rectification is due to truncation of the N-terminus, then reintroduction of the N-terminal peptide can be expected to restore these features. An N-terminal 31-mer peptide that corresponds to the adult form of the *Torpedo* pump (Fig. 1) was prepared and purified to 99.4% homogeneity. It was then applied to the internal phase of the same membrane patch as that shown in Fig. 4B by bath perfusion at 300 μM. The outward currents obtained during the treatment now exhibited a time-dependent decay in the current traces (Fig. 4C) and a rectification in the corresponding I–V curve (Fig. 4F), suggesting a partial restoration of the inactivation process and rectification.

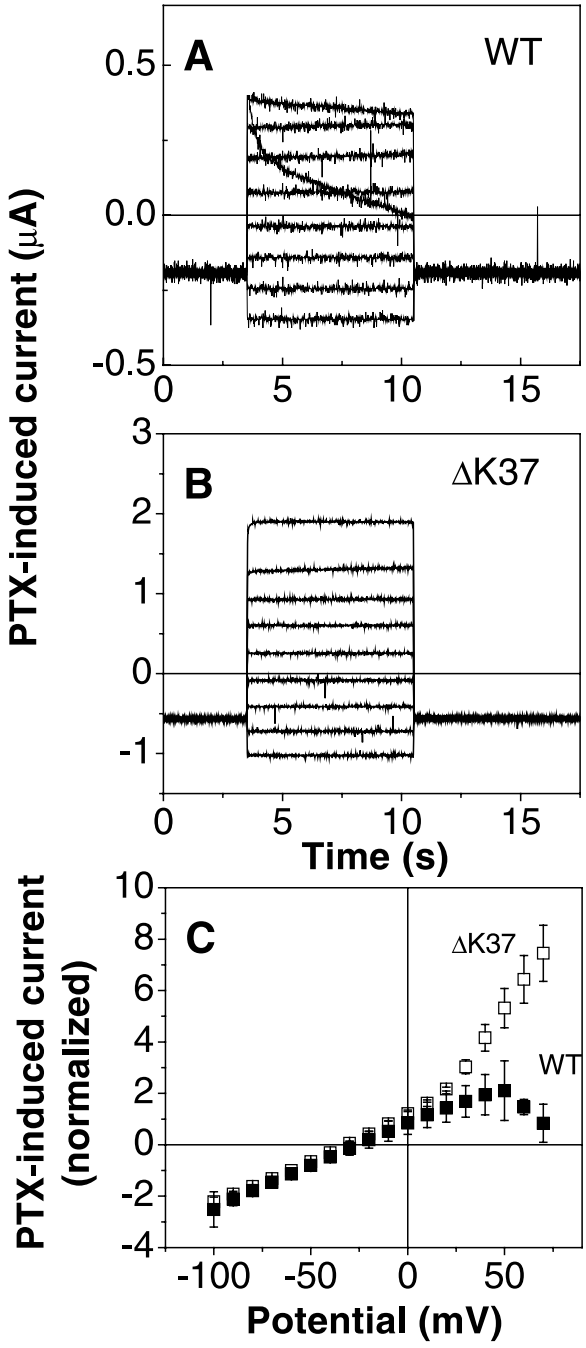

Fig. 3. (A,B) Currents obtained in the presence of 30 pM PTX in response to 7-s rectangular voltage pulses under TEVC in an oocyte with expressed wild-type (A) or truncated (B) pumps. Voltage pulses to −80 to +80 mV in 10-mV increments from a holding potential of −50 mV were given at 2-s intervals. Traces are shown at 20-mV increments for clarity. The rapidly decaying current record in (A) was from +80 mV. (C) Current–voltage curves of PTX-induced currents. Data represent the steady-state currents induced by 30 pM PTX and determined during the last 20% of 7-s rectangular pulses. Data are averages of four and three TEVC experiments (± S.E.) for oocytes with wild-type (filled symbols) and truncated (open symbols) pumps, respectively.

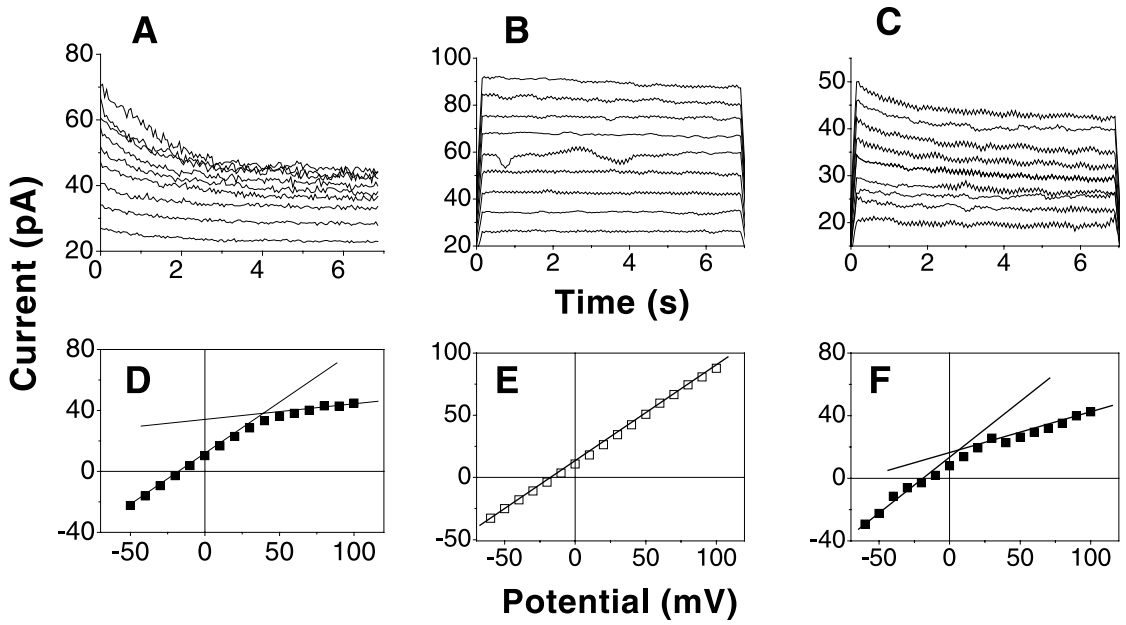

Fig. 4. Currents in response to 7-s rectangular voltage pulses to 20–100 mV from membrane patches with 30 pM PTX present in the patch pipette. (A–C) Currents from an on-cell patch with expressed wild-type pumps (A), and from an inside–out patch with truncated pumps (B,C) induced by 30 pM PTX. Currents in panel B were in the absence and those in panel C in the presence of 300 μM of the synthetic 31-mer peptide in the bath solution, both recorded from the *same* membrane patch. The peptide-induced inactivation of the current responses (C) and inward rectification in the steady-state I–V curves (F) were qualitatively similar to those of the wild-type pumps (A,D), which were absent from the truncated mutant without the peptide (B,E). Data are fitted with a single line in E and with two asymptotic lines in D and F. Similar results were observed in 13 patches from the wild-type pumps, 16 patches from the truncated pumps, and 3 patches from the truncated pumps reconstituted with the peptide.

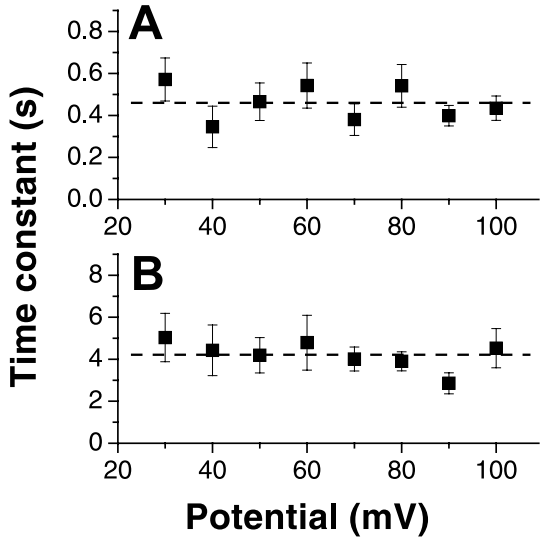

Fig. 5. Time constants for the inactivation of current obtained in the presence of 30 pM PTX as shown in Fig. 4A at potentials more positive than +20 mV. The time constants are plotted as a function of membrane potential for τ_1 in A and τ_2 in B (averages from 10 patches). The broken lines indicate the average values of $\tau_1 = 0.46$ and $\tau_2 = 4.22$ s. Statistical analyses of τ_1 ($n = 69$) and τ_2 ($n = 71$) indicate no significant voltage dependence for either time constant.

3.5. Lack of voltage-dependence of the time-constants of inactivation

The time course of current decay of the wild-type pump channel could be fitted by a two-component exponential curve to yield a fast and slow time constant. The two time constants (averages of 10 patches) are plotted as a function of membrane potential in Fig. 5A for the fast component τ_1 and in Fig. 5B for the slow component τ_2. Statistical analyses of τ_1 and τ_2 values collected from the 10 patches at 30–100 mV indicate no significant voltage dependence for either time constant, with averaged values of $\tau_1 = 0.46 \pm 0.03$ and $\tau_2 = 4.22 \pm 0.24$ s. This is similar to the lack of voltage dependence for the kinetics of the inactivation mechanism of *Shaker* K^+ channels.

4. Discussion

The Na^+,K^+-ATPase undergoes conformational changes between E_1 and E_2 states during its transport cycle. In the ATP-bound E_1 state the enzyme has a high affinity for intracellular Na^+, and in the phosphorylated E_2 state a high affinity for extracellular K^+. In the E_1 state, the T_2 site is accessible to trypsin digestion, whereas in the E_2 state, the site is hidden [13]. Thus, the conformational changes involve a movement of the flexible N-terminus. Truncation of the N-terminus at the T_2 site, by either molecular bio-

logical methods or limited trypsin digestion, results in a loss of pump current [11] and reduction of ATPase enzymatic activity [13,17]. Jørgensen [18] concludes that charged residues in the N-terminus are engaged in ionic interactions of importance for the E_1-E_2 transition. The predicted movement of the N-terminus is confirmed by a recent study of the crystal structure of Ca-ATPase, showing that Domain A, which contains the N-terminus, undergoes a large movement in the E_1-E_2 transition [19].

Two previous studies with purified Na^+,K^+-ATPase or α-subunit proteins reconstituted in lipid bilayers have made an initial observation that the pump channel can be inactivated when the membrane is depolarized to high positive potentials [20,21]. The present study with the PTX-induced channel of the Na^+,K^+-ATPase shows conclusively that: (a) the channel exhibits an inactivation gating behavior with similar lack of voltage dependence as that of *Shaker* K^+ channels; (b) the inactivation is eliminated upon truncation of the charged N-terminus; and (c) the inactivation is partially restored upon application of the synthetic N-terminal peptide. It has been well established that truncation of the N-terminus of the *Shaker*-B K^+ channels removes the inactivation gating, whereas application of the synthetic N-terminal peptide to the truncated channels restores the inactivation gating, and the inactivation process can be explained by the ball-and-chain model [9,10]. Thus, all three features of the pump channel are similar to the characteristics of *Shaker* K^+ channels, and the role of the N-terminus in inactivation of the pump channel seems consistent with the ball-and-chain model for the inactivation process.

In *Shaker* K^+ channels, the inactivation gate enters the inner pore and lodges its N-terminus into the central cavity [22,23]. The lack of voltage dependence in the *Shaker* channel inactivation gating is attributed to a rate-limiting step to form a preinactivated state, an additional step which occurs outside the membrane electric field [22]. The lack of potential dependence of the time constants for inactivation of the pump channel (Fig. 5) may be explained by a similar model. Furthermore, similar to *Shaker* K^+ channels, there may be a fast voltage-dependent step preceding the inactivation which positions a docking site or receptacle inside the membrane, making it available for the inactivation ball. The degree of inactivation, but not the kinetics, will then depend on the membrane potential. Although the inactivation is much slower than that of *Shaker* K^+ channels from *Drosophila*, the time constants of the pump channel inactivation are actually very close to those of the mammalian Kv1 channels which have been reported to be in seconds [24–26].

It is known that as a gate closes, voltage-gated channels may trap blocking ions inside the channel pore [27]. Therefore, the ball-and-chain mechanism for inactivation gate offers a good model for the occlusion of at least one of the ions within the sodium pump. This would be consistent with the observed loss of translocation of one Na^+ ion, leading to the $2Na^+/2K^+$ stoichiometry in the truncated pump. In addition, following the model for *Shaker* K^+ channels, it suggests that the pore region forming the docking site for the gate would most likely contain negatively charged residues in the transmembrane regions.

Because truncation of the N-terminus eliminates the inactivation gating of the PTX-induced pump channel, alters the Na^+/K^+ transport stoichiometry, and abolishes the electrogenicity of the pump, the N-terminus acting like an inactivation gate is likely to be involved in the gating of ion pathway inside the pump. In conjunction with the previous findings from crystal structures [19], the present results suggest that the apparent channel inactivation gating mediated by the N-terminus may be involved in the E_1-E_2 conformational change.

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