Cysteine-scanning mutagenesis study of the sixth transmembrane segment of the Na,K-ATPase α subunit

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Abstract The accessibility of the residues of the sixth transmembrane segment (TM) of the *Bufo marinus* Na,K-ATPase α subunit was explored by cysteine scanning mutagenesis. Methanethiosulfonate reagents reached only the two most extracellular positions (T803, D804) in the native conformation of the Na,K-pump. Palytoxin induced a conductance in all mutants, including D811C, T814C and D815C which showed no active electrogenic transport. After palytoxin treatment, four additional positions (V805, L808, D811 and M816) became accessible to the sulfhydryl reagent. We conclude that one side of the sixth TM helix forms a wall of the palytoxin-induced channel pore and, probably, of the cation pathway from the extracellular side to one of their binding sites. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Na,K-ATPase; Palytoxin; Cysteine scanning; Cation binding site; Structure–function relationship

1. Introduction

The Na,K-ATPase is a protein, present in all animal cells, that moves Na^+ and K^+ ions across the cell membrane using the energy provided by hydrolysis of ATP. This protein is made of two subunits, the main, catalytic α subunit, which has 10 transmembrane (TM) segments [1], and the accessory β subunit. The Na,K-ATPase belongs to the large P-type ATPase family, and more precisely to the type II subfamily that includes the sarcoplasmic and endoplasmic reticulum calcium ATPases (SERCA, type IIA), the plasma membrane calcium ATPases (type IIB), and the Na,K- and the H,K-ATPase (type IIC) according to the classification proposed by Axelsen and Palgrem [2]. A large body of experimental data supports the hypothesis that P-ATPases share a common 'core' which is formed by the fourth, fifth and sixth TM segments. In the group II P-ATPases this portion of the catalytic subunit seems to be primarily involved in high affinity cation binding and occlusion [3,4]. This hypothesis has recently received strong support from the high-resolution crystal structure (2.6 Å) of SERCA [5] for which the 10 TM segments are clearly identified as well as two cation binding sites located between the fourth, fifth and sixth TM segments. In SERCA, the side chain oxygen atoms of N768 and E771 (TM5), T799 and D800 (TM6), and E908 (TM8) participate in cation binding site I. The second cation site is coordinated by the backbone oxygen atoms in an unwound part of the TM4 α helix (P308EGL) and by the side chain of N795 in TM5 and D800 in TM6.

If there is now solid evidence concerning the structure of the cation binding sites, the pathways from the intra- and extracellular solutions to these binding sites are less well defined. No obvious pathway is apparent in the SERCA crystal structure [5]. It can be expected that the parts of the TM4, TM5 and TM6 helices 'below' and 'above' the binding sites participate in the structure of this pathway. In a previous work [6], we had explored the structure of the fifth TM segment by the substituted cysteine accessibility method and we have now used the same approach to study the contribution of the sixth TM segment to the cation pathway across the Na,K-ATPase.

2. Materials and methods

2.1. Site-directed mutagenesis

Systematic mutation of each residue of the sixth TM domain to cysteine was performed starting from the C111S mutant of the α1 subunit of the *Bufo marinus* Na,K-ATPase (cloned in the pSD5 vector) in which a reactive cysteine of the first transmembrane segment had been removed and which has physiological properties similar to the wild type protein [7]. Most of the mutants were generated employing a phenotypic selection strategy [8] and some mutants were generated using the PCR procedure which was performed in a cassette defined by the *Bln*I and *Sun*I restriction enzymes. All mutations were confirmed by sequencing. Cysteine mutant *Bufo* α1 subunits as well as wild type *Bufo* β1 subunit cRNA were synthesized by in vitro transcription as previously described [9].

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

Seven ng α subunit and 1 ng β subunit cRNA were mixed and coinjected in a total volume of 50 nl into stage V–VI *Xenopus laevis* oocytes [10]. The injected oocytes were incubated for 3–5 days in modified Barth's solution and loaded with Na^+ by exposure to a K^+-free solution overnight before the electrophysiological measurements as described earlier [6].

2.3. Electrophysiological measurements

The electrophysiological measurements were performed following the same protocol that was previously reported in the study of TM5 [6]. Briefly, the steady-state whole oocyte current was measured at a holding potential of −50 mV using the two-electrode voltage-clamp technique. The composition of the control solution was (in mM): Na^+ 92.4, Mg^{2+} 0.82, Ba^{2+} 5, Ca^{2+} 0.41, TEA^+ 10, Cl^- 22.4, HCO_3^- 2.4, HEPES 10, gluconate 80, pH 7.4; a low-chloride solution and K^+ channel blockers (Ba, TEA) were used to minimize the oocyte Cl^- conductance and the non-pump-related K^+ currents. In order to inhibit the endogenous *Xenopus* Na,K-ATPase and to be able to specifically measure the activity of the expressed *B. marinus* Na,K-ATPases all solutions contained 10 μM ouabain.

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The membrane conductance was monitored by recording the current changes produced by 1 s voltage steps from −50 to 0 mV every 20 s. The Na,K-pump activity was first measured as the outward current activated by changing from a K^+-free to a 10 mM K^+ solution. Then, the oocyte was exposed to a 2 or 4 nM palytoxin solution until the cell membrane conductance had increased at least three times (1–3 min). After removal of palytoxin, the methanethiosulfonate (MTSEA or MTSET) reagent was added at a concentration of 100 μM for a 1 min period. The final solutions of palytoxin and MTS compound solutions were prepared immediately before each electrophysiological measurement from 100 μM (palytoxin) or 100 mM (MTSEA or MTSET) stock solutions kept on ice.

2.4. Reagents

Palytoxin from *Palythoa cariboaerum*, purchased from Sigma, was dissolved in distilled water and kept as a 100 μM stock solution at −80°C. The final palytoxin dilution was performed in the presence of 0.1% bovine serum albumin in order to avoid non-specific adsorption on vessels and tubings. Ouabain was purchased from Sigma and used from 200 mM stock solution in dimethylsulfoxide. The methanethiosulfonate (MTS) reagents, 2-aminoethyl-methanethiosulfonate (MTSEA), and [2-(trimethylammonium)ethyl]-methanethiosulfonate (MTSET) were purchased from Toronto Research Chemicals (North York, ON, Canada).

3. Results

3.1. Functional expression of the cysteine mutants in Xenopus oocytes

The activity of the expressed cysteine mutants of the Na,K-ATPase was first tested as the steady-state outward current induced by addition of 10 mM K^+ to a previously K^+-free solution. We have shown earlier that this current provides a reliable estimate of the Na,K-pump activity under our experimental conditions [11]. In order to study specifically the expressed Na,K-ATPase, the endogenous *Xenopus* Na,K-pump was inhibited by the presence of 10 μM ouabain in all solutions. In non-injected oocytes as well as in the oocytes micro-injected with the *Bufo* β1 subunit cRNA alone, no outward K^+-activated current could be detected at −50 mV holding potential ($n = 12$). We conclude that under our experimental conditions, the K^+-activated steady-state currents which were observed in the presence of 10 μM ouabain is only due to the expressed *Bufo* Na,K-ATPase.

As shown in Fig. 1A, most of the cysteine mutants exhibited a K^+-activated outward current. For only three mutants, D811C, T814C, and D815C, no outward K^+-activated current could be recorded. In many cases the outward K^+ induced current was of similar magnitude as for our control C111S mutant, which has an activity similar to the wild type Na,K-pump [7], however some mutants showed a significantly reduced activity in comparison with the control. This was notably the cases for most of the positions of the inner half of the sixth TM segment, i.e. from proline P818 to tyrosine Y824.

3.2. Effect of palytoxin on the cysteine mutants in the sixth transmembrane segment

The presence of 10 μM ouabain in all solutions completely prevented the effect of palytoxin in oocytes that did not express the ouabain-resistant *Bufo* α1 subunit. Palytoxin, up to a concentration of 4 nM, did not increase the conductance of the non-injected oocytes ($n = 6$) or of oocytes micro-injected with β1 subunit cRNA alone ($n = 6$): the mean change in conductance observed in these pooled two groups was not significantly different from 0: 0.3 ± 0.2 μS ($n = 12$). This observation demonstrates a complete inhibition of the endogenous *Xeno-*

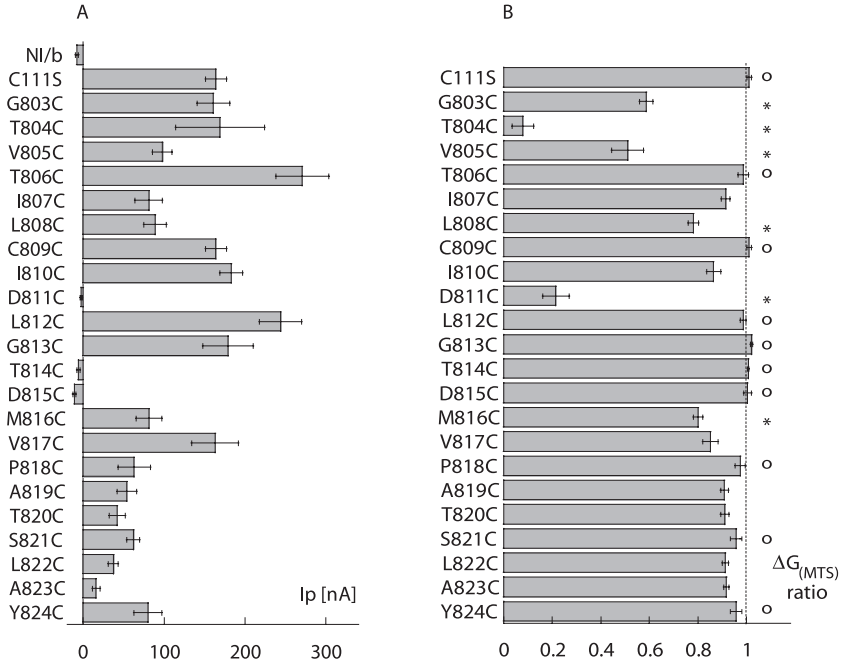

Fig. 1. K^+-activated Na,K-pump current and inhibition of the palytoxin-induced conductance by MTSEA in cysteine mutants of the α subunit of the *Bufo* Na,K-ATPase. Results obtained in non-injected oocytes (NI, $n = 6$) or oocytes injected with the β subunit alone (β, $n = 6$) were pooled. The number of measurements was 44 for the control (C111S) mutant and 7–10 for each of the other groups. A: Na,K-pump-generated current (Ip) activated by 10 mM K^+. B: The part of the palytoxin-induced conductance remaining after a 1 min exposure to 100 μM MTSEA, $\Delta G_{(MTS)}$. In B, an asterisk indicates that MTSEA induced a decrease of more than 20% of the palytoxin-induced conductance ($P < 0.001$) and a ○ indicates the absence of any detectable effect of MTSEA on the oocyte conductance. The natural amino acid in position 809 is a cysteine and the results for this position are those of the wild type sequence (C111S).

pus Na,K-ATPases and indicates that the effects observed after palytoxin treatment were entirely due to the expressed *Bufo* Na,K-ATPase mutants.

In contrast, in oocytes expressing the control α *Bufo* mutant (C111S) subunit, a large increase of the membrane conductance (16 ± 2.5 μS) was observed after palytoxin treatment, similar to what had been described earlier [6,12]. Palytoxin (2–4 nM) also induced a significant increase in cell membrane conductance in oocytes expressing all the mutant *Bufo* Na,K-ATPase α1 subunit. As our goal was to obtain a large palytoxin-induced conductance to be able to test the effect of cysteine reagents, we used various concentrations of palytoxin, starting with 2 nM and increasing to 4 nM if necessary and waiting until the increase in membrane conductance was at least three-fold over baseline. Because of the various concentrations of palytoxin that have been used and because of the various exposure time (1–3 min), the amplitude of palytoxin-induced conductance cannot be compared between individual oocytes and no conclusion can be drawn from the quantitative differences between the value of this conductance, but the important point for the following is that palytoxin-induced conductance larger than 3.0 μS (range 3.2–31.2 μS) could be obtained in each case. In particular, with the three mutants which did not demonstrate any K^+-activated Na,K-pump currents, D811C, T814C and D815C, we recorded palytoxin-induced conductances of 22 ± 4, 8.9 ± 2.4 and 13.4 ± 3 μS, respectively.

3.3. Accessible cysteine residues in the palytoxin-modified Na,K-pump

The effects of MTSEA on the palytoxin-induced conductance are reported in Fig. 1B as the proportion of the palytoxin-induced conductance remaining after a 1 min exposure to 100 μM MTSEA. As reported earlier, MTSEA had no effect on the control mutant (C111S) which possesses one cysteine residue (C809) in the middle portion of the TM6, suggesting that this residue is not accessible. For eight other cysteine positions, the 1 min MTSEA exposure did not induce any detectable decrease of the conductance (indicated by empty circles in Fig. 1B). Seven of these positions are regularly spaced, every three residues, along the TM6 segment: T806, C809, L812, D815, P818, S821, Y824, and thus may be expected to form a slowly winding helix making a full turn over the whole length of an idealized α-helix. Two other positions, G813 and T814, together with L812 and D815,

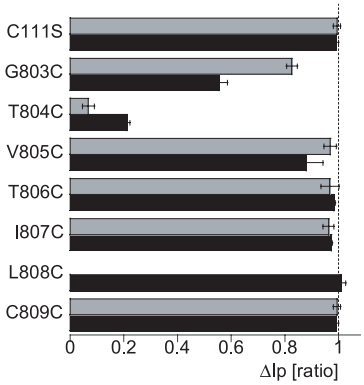

Fig. 2. Effect of MTSEA and MTSET on the activity of the native conformation of the Na,K-pump. The bar graph shows the mean values of the ratio of the K^+-induced current (ΔIp) measured immediately before and after a 2 min exposure to 100 μM MTSEA (black columns) or 100 μM MTSET (gray columns).

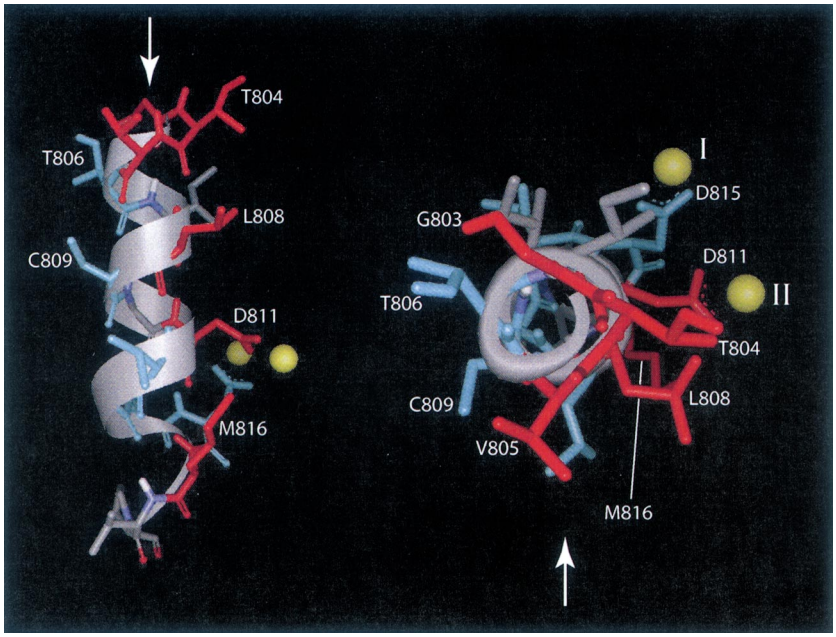

Fig. 3. Three-dimensional model of the sixth transmembrane segment of the Na,K-ATPase α subunit. For clarity, only the outer two-thirds of the TM segment are shown. The model was obtained by homology with the structure of SERCA (1EUL) using the GeneMine/Look software and the graphic presentation was done with the WebLab ViewerLite software of Molecular Simulation Inc. The left panel shows a side view of the α-helix (from the direction indicated by the arrow in the right panel) and the right panel shows a top view of the α-helix from the external side of the membrane as indicated by the white arrow in the left panel. The yellow balls indicate the position of two cations, corresponding to the calcium sites I and II defined by Toyoshima et al. in SERCA [5]. The amino acid positions in which a substituted cysteine is readily accessible to MTSEA (inhibition larger than 20%) are colored red. The positions for which no effect of MTSEA at all could be detected are colored blue.

form a block of four residues located at the center of the transmembrane segment which do not seem accessible at all.

Six positions showed a clear inhibition of the palytoxin-induced conductance by MTSEA (inhibition larger than 20%, $P < 0.001$ in each case, indicated by an asterisk in Fig. 1B). Three of them, corresponding to the most external residues G803, T804 and V805, are strongly inhibited. L808C, the substitution of a lipophilic residue located in the outer third of the membrane, was only slowly, but significantly inhibited while the more deeply located D811 was highly sensitive to the cysteine reagent. A 20% inhibition was also observed for M816.

Only very limited inhibition was observed for most of the mutants of the inner half of the transmembrane segment. Even though this inhibition was statistically significant, it hardly reached 10% for A819C, T820C, L822 and A823. With these mutants of the inner half of TM6, 100 μM MTSEA produced a very slow inhibition that did not reach equilibrium within the 1 min exposure period, suggesting a strongly reduced accessibility.

3.4. Effect of MTS compounds on the native Na,K-ATPase

To study the accessibility of the residues of the TM6 segment in the native conformation of the Na,K-pump, we also measured the K^+-activated current before and after a 2 min exposure to 100 μM MTSEA or MTSET in cysteine mutants of the outer half of TM6. As reported earlier [6] this treatment had no effect on the control mutant (C111S). Thus, the effects of MTS reagents observed with other mutants were related to the presence of the introduced cysteine. As shown in Fig. 2, qualitatively similar results were obtained with MTSEA and MTSET. Cysteine substitution at two positions located close to the membrane/extracellular fluid interface, positions 803 and 804, resulted in a high sensitivity to the MTS reagents. No significant inhibition of the Na,K-pump activity could be detected at any deeper position. These results are consistent with those of similar measurements performed with substituted cysteine in the TM5 segment [6] and indicate that in the native conformation of the Na,K-pump only the most superficial residues of the 'core' transmembrane segments are accessible from the external solution.

4. Discussion

The purpose of the present work is to understand the structural basis for the cation transport pathway across the Na,K-ATPase. Asano and collaborators [13] have recently explored by alanine scanning the corresponding region of the rabbit gastric H,K-ATPase, another member of the type IIC P-ATPases. Our results obtained by cysteine scanning in the *B. marinus* Na,K-ATPase α1 subunit are similar to theirs in several points, but the comparison also reveals interesting differences.

A first important point is the confirmation of the essential role of D815 in cation transport. This residue or its homologue (D808 in pig α1 Na,K-ATPase, D826 in rat gastric H,K-ATPase) cannot be mutated to another residue without loss of function [13–15]. Our result demonstrate, however, that this mutant is well expressed at the membrane as demonstrated by the effect of palytoxin. Surprisingly, when D815 was replaced by a cysteine, the thiol reagent MTSEA had no effect on the conductance induced by palytoxin. This observation indicates that the side chain of the substituted cysteine is not accessible to water-soluble reagents added at the extracellular side of the membrane, even though the corresponding residue of SERCA (D800) is known to participate in both calcium sites [5] and other residues located deeper in the membrane (M816 for instance) can be reached by MTSEA. Several hypotheses can be proposed to explain this observation. First, the side chain of cysteine is slightly shorter than that of aspartate and the thiol group may not reach the free space of the pore as readily as the carboxyl of aspartate. Second, palytoxin may stabilize a conformation in which the side chain of this residue is removed from its close contact of the cation binding sites, resulting in a lower binding energy of the cation and thereby allowing a low-affinity interaction with channel type of cation transport rather than the high-affinity interaction that is expected in an ion pump.

The other negatively charged residue in this segment, D811 (corresponding to D804 in pig α1 Na,K-ATPase and E820 in the in rat gastric H,K-ATPase) also appears very important for the transport function of the Na,K-pump as already shown by several reports [14–18]. In the rabbit H,K-ATPase, the alanine mutation at the corresponding position (E822A) resulted mostly in a large decrease in affinity for stimulation of the dephosphorylation by K^+ and a reduced (to about 20% of wild type) ATPase activity at 15 mM K^+ [13]. In our case, no activity could be observed with 10 mM K^+. This mutant was, however, well expressed and, after palytoxin treatment, it could be rapidly inhibited by MTSEA indicating that this residue position was readily accessible from the extracellular solution. The side chain of the corresponding residue in SERCA, N796, contributes to the calcium site II [5] and, assuming the same role for D811 in the Na,K-ATPase, this observation suggests that the pathway opened by palytoxin shares a common structure with the equivalent of the calcium site II in the Na,K-ATPase.

The other cysteine positions that render the Na,K-ATPase highly sensitive to thiol reagents are the three most external residues of the putative sixth TM segment. The fact that these residues are accessible is not surprising considering their location close to the membrane surface. Indeed, two of them, G803 and T804, are readily accessible to MTSEA or MTSET even in the native conformation of the Na,K-pump, while the third, V805, becomes accessible only after treatment with palytoxin. Cysteine residues in the positions of I806, L808 and I810 also became accessible to MTSEA after palytoxin treatment but were not sensitive to the reagent in the native conformation of the Na,K-pump. This pattern of accessibility suggests the presence of a size-restrictive barrier, an external 'gate', approximately at the level of T804. This conclusion is consistent with our previous observation that only the most extracellular position of TM5, A796, could be reached without palytoxin treatment [6]. The effect of palytoxin could be produced by induction of a significant conformation change in this external gate controlling the access of large molecules such as MTSEA (66 $Å^3$) from the extracellular side to the cation binding sites.

In contrast to the results obtained by Asano et al. [13], substitution of L808 and M816 (L819 and I827 respectively in the rabbit gastric H,K-ATPase) did not abolish the Na,K-pump activity, but only produced a moderate reduction ($\sim$50%) in electrogenic transport. Leucine 808 is highly conserved in all vertebrate Na,K- or H,K-ATPases and even in SERCA (L793), but its mutation into a cysteine only moder-

ately affects the overall transport function of the Na,K-ATPase. The available SERCA structure (1EUL) shows that the side chain of L793 points away from the cation binding site, towards the TM1 and TM2 helices. Conversely, the T814C mutation abolished all Na,K-pump activity while the corresponding T825A mutation in the gastric H,K-ATPase had only a moderate effect on the activity of this enzyme. This threonine is also a very highly conserved residue in all known vertebrate Na,K- or H,K-ATPases. These differences may be related to the different cation selectivity (H^+ or Na^+) of the two ATPases in question and suggest a specific role of these residues in the selectivity for the cation transported in exchange for potassium as also proposed by Koenderink et al. [19]. Another possible explanation is the fact that methionine and cysteine are both sulfur-containing residues and their main difference is the larger size of methionine. It is possible that the methionine to cysteine mutation may be better tolerated than the isoleucine to alanine mutation in the case of the H,K-ATPase.

Our results are less informative concerning the intracellular part of the sixth TM segment. A reduced Na,K-pump activity is observed for most of the cysteine substitutions in this region (A819C, T820C, S821C, L822C, A823C and Y824C). These results are similar to those of Asano et al. [13], supporting their hypothesis that the inner half of this segment is important for the mechanism of energy transduction between the K^+ binding site and the phosphorylation site.

Fig. 3 summarizes our findings concerning the outer two-thirds of the sixth transmembrane segment. This structural model was obtained by homology with the high-resolution (2.6 Å) published SERCA structure (1EUL) [5]. In their recent review, Sweadner and Donnet [20] proposed a model of the Na,K-ATPase built by homology with SERCA structure. Because of the high degree of homology in the sixth TM segment (identity 9/23, similarity 15/23) and the presence of several residues that are conserved in most members of the Na,K- and H,K-ATPases as well as in the SERCA family, the sequence alignment can be made without any ambiguity and the structural homology is rather likely. Fig. 3 shows that, except for the two superficial residue G803 and V805, the most reactive positions (T804, L808, D811 and M815) are located on the same side of the α-helix, the side that directly faces the cation binding site II, according to the model of Toyoshima et al. [5]. In contrast, the side chains in direct contact with site I are not, or only poorly, accessible (D815, I807).

Palytoxin transforms the Na,K-pump into a non-selective monovalent cation channel. Our results allow us to describe the part of the sixth transmembrane segment of the Na,K-ATPase that contributes to form part of the 'wall' of this channel-like structure. Because several of the residues involved in this channel are also known to be essential constitutive parts of the cation binding site of the normal Na,K-ATPase, these data support the hypothesis that the channel opened by palytoxin is largely made of the same elements as the mechanism of cation translocation in the normal Na,K-pump. Our data thus allow us to propose that one side of the sixth TM α-helix lines the entry or exit pathway for cations into or out of their occlusion site in the core of the Na,K-pump transport mechanism.

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