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Structure of the 5th transmembrane segment of the Na,K-ATPase α subunit: a cysteine-scanning mutagenesis study

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Abstract To study the structure of the pathway of cations across the Na,K-ATPase, we applied the substituted cysteine accessibility method to the putative 5th transmembrane segment of the α subunit of the Na,K-ATPase of the toad *Bufo marinus*. Only the most extracellular amino acid position (A^{796}) was accessible from the extracellular side in the native Na,K-pump. After treatment with palytoxin, six other positions (Y^{778}, L^{780}, S^{782}, P^{785}, E^{786} and L^{791}), distributed along the whole length of the segment, became readily accessible to a small-size methanethiosulfonate compound (2-aminoethyl methanethiosulfonate). The accessible residues are not located on the same side of an α-helical model but the pattern of reactivity would rather suggest a β-sheet structure for the inner half of the putative transmembrane segment. These results demonstrate the contribution of the 5th transmembrane segment to the palytoxin-induced channel and indicate which amino acid positions are exposed to the pore of this channel.

Key words: Palytoxin; Na,K-ATPase; Cysteine scanning; P-type ion-motive ATPase; Pore structure

1. Introduction

The Na,K-ATPase is a ubiquitous P-type ion-motive ATPase (P-ATPase), responsible for the establishment and maintenance of Na and K gradients across the plasma membrane of animal cells. The large family of the P-ATPases includes a number of ion-motive ATPases which are expressed in all types of living cells from bacteria to man. The type II P-ATPases, according to the classification proposed by Palmgren and Axelsen, [1], for instance Na,K-ATPase, H,K-ATPase and the two main types of calcium ATPases, are highly related polytopic membrane proteins thought to have an intramembrane moiety formed by 10 transmembrane segments as suggested by primary structure analysis and confirmed by several experimental approaches. In the Na,K-ATPase and the H,K-ATPase, the main catalytic subunit (α subunit) is associated with a β subunit that provides an additional transmembrane segment.

The substituted cysteine accessibility method (SCAM) has made it possible to study the fine structure of various ion channels and transporters, and in particular the part of the transmembrane segments lining the channel pore or the solute pathway, by determining the amino acid positions that were accessible to water-soluble cysteine reagents [2–5]. To allow for the translocation of cations across the membrane, the Na,K-ATPase must provide a pathway and this pathway, as well as the cation occlusion cavity, is expected to be formed by the transmembrane segments [6]. Some part of this pathway may actually be similar to those found in ion channels and in this case the Na and K ions may cross part of the membrane in a way similar to ions passing through the vestibule and pore of an ion channel. The results of a large number of site-directed mutagenesis studies with the Na,K-ATPase and other type II P-ATPases point to an essential role of the 4th, 5th and 6th transmembrane segments in the intramembrane binding of the transported cations [7]. We have attempted to study the structure of the cation pathway through the Na,K-ATPase by a SCAM approach, starting with the 5th transmembrane segment (TM5).

Because the Na,K-pump is not simply a channel, we did not expect the wall of all the cation pathway to be accessible to a water-soluble reagent. This expectation was confirmed by the fact that only the most superficial position of the TM5 segment was accessible to methanethiosulfonate (MTS) cysteine modifying reagents. However, we knew that the Na,K-ATPase can be modified by the binding of palytoxin, a non-peptide toxin, and be transformed into a ouabain-sensitive cation channel [8–10]. We took advantage of this Na,K-ATPase modification to study the accessibility of the TM5 in the palytoxin-modified Na,K-pump, using the SCAM approach.

We were able to identify several amino acid positions, which, when replaced by cysteine, made the palytoxin-induced conductance highly sensitive to MTS. These positions span the whole width of the membrane and include some amino acids already well known as important for cation binding, but also other positions occupied by lipophilic amino acids in the wild type Na,K-ATPase. The pattern of reactivity does not follow that expected for an α-helix in the N-terminal part of the putative transmembrane segment, the part expected to be located in the inner half of the membrane.

2. Materials and methods

2.1. Site-directed mutagenesis

A series of cysteine mutants (from position K^{774} to A^{796}, a sequence corresponding to the putative TM5 of the α_1 subunit of the *Bufo marinus* Na,K-ATPase) were generated employing a phenotypic selection strategy (in vitro mutagenesis technique of Kunkel [11]) using the α_1 subunit subcloned into the pSD5 vector [12]. Because we had shown earlier that the first transmembrane segment contains a mercury-reactive cysteine [13], all cysteine mutants of TM5 were prepared starting from the α_1 subunit mutant in which the cysteine of the first TM was replaced by a serine ($C^{111}S$), therefore all cysteine mutants of the TM5 also bear the $C^{111}S$ mutation. All mutations were confirmed by sequencing a ~300 bp segment around the mutation.

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2.2. cRNA synthesis and expression of Bufo Na,K-ATPase in Xenopus oocytes

Wild type and mutant *Bufo* α_1 subunits and wild type *Bufo* β_1 subunit cRNA were obtained by in vitro transcription as described [14]. 7 ng of wild type or mutant α subunit cRNA and 1 ng of β subunit cRNA were injected in a total volume of 50 nl into stage V–VI *Xenopus laevis* oocytes [15]. The injected oocytes were stored for 3–5 days at 19°C in modified Barth's solution containing (in mM): 85 NaCl, 2.4 $NaHCO_3$, 1 KCl, 0.8 $MgSO_4$, 0.3 $CaNO_3$, 0.4 $CaCl_2$ and 10.0 HEPES (pH 7.4), and supplemented with 10 mg/ml penicillin, and 5 mg/ml streptomycin.

2.3. Electrophysiological measurements

Before electrophysiological measurements, all oocytes were loaded with sodium by a 2-h incubation in a K^+-free and Ca^{2+}-free solution containing 90 mM Na^+ and 0.5 mM EGTA [16]. The steady-state whole oocyte current was measured at a holding potential of −50 mV using the two-electrode voltage-clamp technique as described earlier [17]. The composition of the control solution was (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; a low-chloride solution and K channel blockers (Ba, TEA) were used to minimize the oocyte Cl^- and K^+ conductances. The experimental protocol is illustrated by the example voltage and current traces in Fig. 1. The membrane conductance (G_m) was monitored by recording the current changes produced by 1-s voltage steps from −50 to 0 mV every 20 s. The outward current induced by addition of 10 mM K^+, the Na,K-pump current, was measured first in each experiment to estimate the functional expression of the mutants. Then the oocyte was exposed to a 2 nM palytoxin solution until the membrane conductance had increased 3–10 times, within a 3-min period. If the increase of conductance was too slow or too small, the concentration of palytoxin was increased to 4 nM. After removing palytoxin, a MTS compound was added for a 2-min period. MTS solutions were prepared immediately before each measurement from a 200 mM stock solution kept on ice.

In order to eliminate the contribution of the endogenous oocyte *Xenopus* Na,K-pump, all experiments were performed in the continuous presence of 10 μM ouabain. Although this concentration of ouabain may also partially inhibit the expressed *Bufo* Na,K-ATPase with a $K_{1/2}$ of about 50 μM [14]), the uninhibited portion was largely sufficient to observe the palytoxin-induced conductance while the endogenous *Xenopus* Na,K-ATPase was completely inhibited and could not react with palytoxin [10].

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. Final dilutions were made immediately before use. Ouabain was purchased from Sigma and used from a 200 mM stock solution in dimethylsulfoxide. The MTS compounds 2-aminoethyl methanethiosulfonate (MTSEA), and [2-(trimethylammonium)ethyl]methanethiosulfonate (MTSET) were purchased from Toronto Research Chemicals (North York, Ont., Canada).

3. Results

3.1. Expression of the cysteine mutants of the Na,K-ATPase α subunit

The expression and function of each mutant was first tested by measuring the outward current (at −50 mV) induced by addition of 10 mM K to a previously K-free solution. Because the *Xenopus* Na,K-ATPase is highly sensitive to ouabain, when *Xenopus* oocytes are exposed to ouabain concentrations as low as 0.2 μM no K-activated current can be detected at −50 mV in native or water-injected oocytes or oocytes injected with the β subunit alone of the Na,K-ATPase [18]. Therefore the K-activated outward current that we observed in the presence of 10 μM ouabain was due to the *Bufo* wild type or mutant Na,K-pumps expressed by injection of the α and β subunit cRNAs. The mean values of the K-activated Na,K-pump current measured in all the cysteine mutants is shown in Fig. 2 (left panel). A number of mutants had an activity comparable to that observed in the wild type or the $C^{111}S$ mutant and, with regard to this very simple test, appeared functionally normal. The other mutants had a decreased activity, but for all of them a measurable K-activated outward current could be detected, even if this activity amounted to 5–10% of that measured in the wild type Na,K-pump.

3.2. Effects of MTS compounds before palytoxin treatment

Before treatment with palytoxin, a 2-min exposure to 100 μM or 1 mM MTSEA had no detectable effects in oocytes expressing the wild type, the $C^{111}S$ mutant or most of the mutants with an added cysteine in TM5 (data not shown). The single exception was the $A^{796}C$ mutant for which a slow inhibition of the Na,K-pump current was observed when MTSEA was added. The inhibition of the K-induced current reached 20 ± 1% ($n = 6$) after a 2-min exposure to 1 mM MTSEA.

3.3. Effect of palytoxin

As shown in the examples of Fig. 1, exposure to 2–4 nM

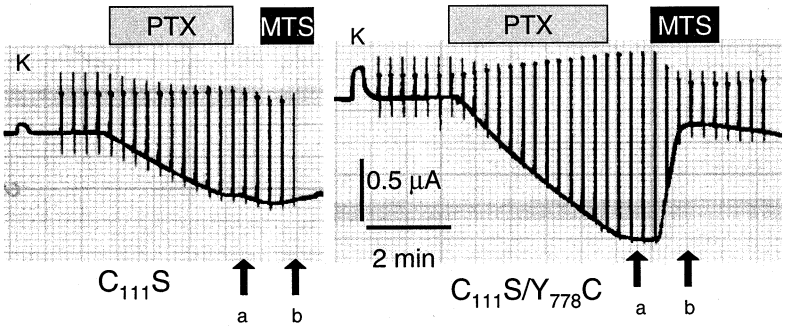

Fig. 1. Effect of palytoxin and the cysteine reagent MTSEA on the cell conductance in oocytes expressing Na,K-pump cysteine mutants. Current traces in typical experiments are shown, one (left panel) with a $C^{111}S$ mutant of the α subunit of the *Bufo* Na,K-ATPase, and the other one (right panel) with a $C^{111}S/Y^{778}C$ mutant. The outward (positive) current induced by addition of 10 mM K to a previously K-free solution was first measured (K). Then palytoxin (2 nM) was added until a large conductance was induced. Palytoxin was removed and MTSEA (100 μM) was added for a 2-min period inducing in some mutants (such as the $C^{111}S/Y^{778}C$ mutant) a large decrease in oocyte conductance. The current deflections produced by 1-s voltage clamping to 0 mV from a −50 mV holding potential were used to calculate the oocyte conductance. Ouabain was present at 10 μM in all the solutions throughout the experiment. The arrows point to the time points used to measured the palytoxin-induced conductance (arrow a, before MTS treatment) and the effect of MTS on the conductance (arrow b).

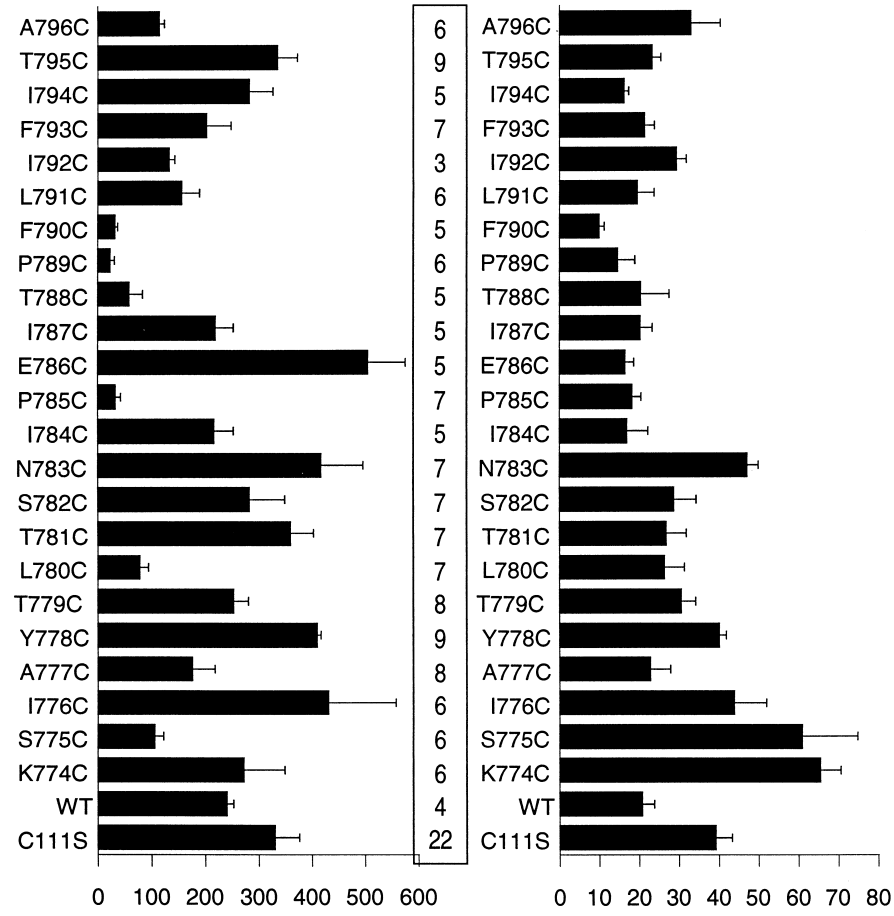

Fig. 2. Na,K-pump current and palytoxin-induced conductance in oocytes expressing the wild type and cysteine mutants of the *Bufo* Na,K-ATPase. I_K (left panel) is the K-induced outward current measured at −50 mV as described in Fig. 1. ΔG_{PTX} (right panel) is the increase in whole oocyte conductance (measured between −50 and 0 mV) produced by exposure to palytoxin. These values are to be compared with the oocyte conductance before treatment by palytoxin, which averaged 4.9 ± 0.3 μS (mean of all groups pooled together). There was no obvious relationship between the amplitude of the K-induced current and the palytoxin-induced conductance. Some mutants expressed a low K-induced current; however, in all mutants it was possible to induce a large increase in oocyte conductance with 2 or 4 nM palytoxin. The numbers of measurements are indicated in the middle column.

palytoxin induced a progressive increase in cell membrane conductance in oocytes expressing wild type or mutant *Bufo* Na,K-ATPase, similar to what we have described earlier for the *Xenopus* or *Bufo* Na,K-ATPase [10]. We checked that the effects of palytoxin were not due to interaction with the endogenous *Xenopus* Na,K-ATPase, which was inhibited by the continuous presence of 10 μM ouabain: no effect of palytoxin (4 nM) could be detected in non-injected oocytes ($n = 8$) or oocytes injected with the cRNA of the β subunit alone ($n = 8$) indicating a complete inhibition of the endogenous *Xenopus* Na,K-ATPase. In all cysteine mutants a large (> 3-fold) increase in conductance could be obtained by treatment with 2 or 4 nM palytoxin. The absolute value of the palytoxin-induced increase in conductance is shown in Fig. 2 (right panel). The amplitude of palytoxin-induced conductance was not evaluated as such because various concentrations (2–4 nM) and exposure times (1–3 min) were used to obtain in each case a sizeable palytoxin-induced increase in oocyte conductance, as the purpose was to evaluate the MTS sensitivity of the palytoxin-induced conductance.

3.4. Effects of MTS compounds after palytoxin treatment

The effect of MTS compounds was quantified as the portion of the palytoxin-induced conductance remaining after a 2-min exposure to the compound (see Fig. 1). The results of the exposure to MTSEA are summarized in Fig. 3. In wild type Na,K-ATPase, in the $C^{111}S$ mutant and in 14 out of the 23 TM5 cysteine mutants, 100 μM MTSEA had no detectable effect on the palytoxin-induced conductance. In seven other cysteine mutants a large part (50% or more) of the palytoxin-induced conductance was inhibited. For two other mutations, at positions close to the intracellular end of the putative transmembrane segment ($I^{776}C$ and $K^{774}C$), MTSEA induced a small but significant decrease of the palytoxin-induced conductance.

The larger MTS compound (MTSET) was tested on the four most external amino acid positions ($F^{793}C$, $I^{794}C$, $T^{795}C$, $A^{796}C$) and on the eight other positions that were reactive to MTSEA ($K^{774}C$, $I^{776}C$, $Y^{778}C$, $L^{780}C$, $S^{782}C$, $P^{785}C$, $E^{786}C$, $L^{791}C$). A 2-min exposure to 100 μM MTSET reduced the palytoxin-induced oocyte conductance in the $A^{796}C$ mutant by 75 ± 27% ($n = 6$) but had no significant effects on $C^{111}S$ or any of the other tested mutants (data not shown).

4. Discussion

K-activated outward current and response to palytoxin treatment indicated that all cysteine mutants were expressed at the cell surface and able to form palytoxin-induced channels. The amplitude of the electrogenic Na,K-exchange activity was very much reduced in some of the mutants. This may be due either to a low rate of expression of the mutant protein at the cell surface, to a reduced activity of the expressed protein, or to both; our results do not allow us to distinguish between these possibilities. However, in all cases a large increase in conductance was induced by the interaction of palytoxin with the expressed mutant Na,K-ATPase (see Fig. 2), indicating that a sufficient density of Na,K-pump was present at the oocyte surface. It was therefore possible to test the accessibility to cysteine reagent of each position in the TM5 segment.

Of the 23 positions tested, corresponding to the putative TM5 segment, seven were readily accessible to sulfhydryl reagents as shown by the inhibition of the palytoxin-induced conductance by MTSEA. Considering the rather low concentration (100 μM) of MTSEA that we used and the fast rate of inhibition (see Fig. 1), it is highly probable that the cysteine residues placed in these positions were accessible through a water-filled pathway from the extracellular solution. The fact that the same mutants were not sensitive to MTSEA, even at a 10 times higher concentration, without exposure to palytoxin argues against the possibility that MTSEA would reach these cysteine residues by diffusing through the membrane lipids.

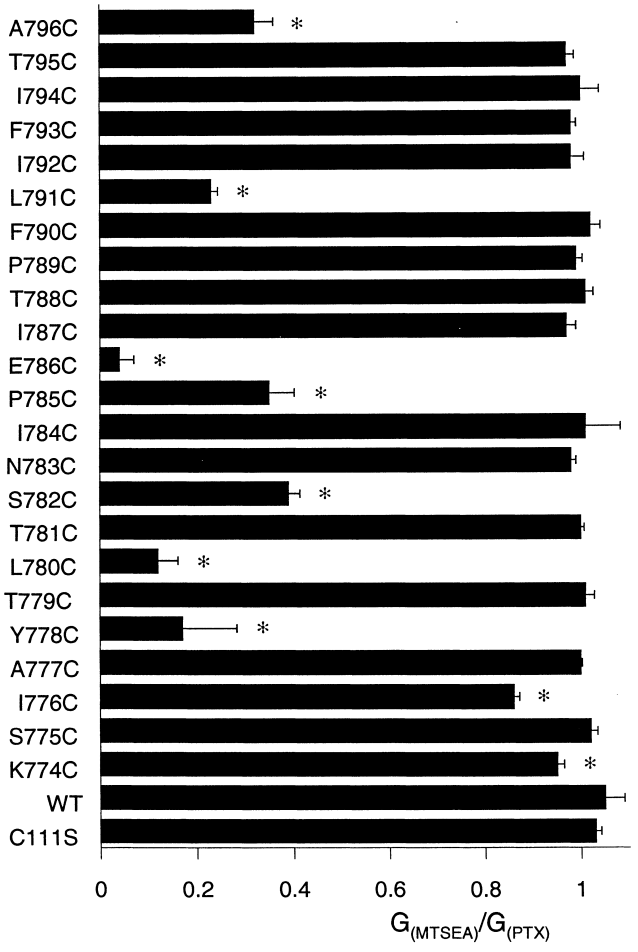

Fig. 3. Effect of the cysteine reagent MTSEA on the palytoxin-induced conductance. The fraction of the palytoxin-induced conductance remaining after a 2-min exposure to 100 μM MTSEA is shown. A value of 1.0 indicates the absence of effect of MTSEA. Values significantly different from 1 ($P < 0.01$) are indicated by an asterisk. The numbers of measurements are the same as those indicated in Fig. 2.

A remarkable observation was that MTSEA was able to reach positions all the way across the membrane, indicating that the pathway opened by palytoxin is wide enough to allow the passage of this molecule. In contrast, the larger MTSET (molecular volume of MTSET 109 Å^3 versus MTSEA 66 Å^3) could reach only the most superficial position (A^{796}). By analogy with the interpretation proposed for a similar observation with the voltage-gated Na channel [19], we suggest that a size-restricting filter is present close to the extracellular surface of the cation pathway opened by palytoxin in the Na,K-pump.

Among the seven readily accessible positions, one (A^{796}) is the most superficial; its accessibility is not surprising but the conductance blocking effect of MTSEA (or MTSET) binding at this position indicates that, even if it is located close to the surface of the membrane, this residue occupies a critical position along the cation pathway. The six other positions are located deeper in the membrane. Three of these, Y^{778}, S^{782} and E^{786}, are oxygen-containing amino acids and have been shown to hold a critical position for cation binding [20–23] and with regard to this point, our data are in good agreement with these earlier results. Three other positions shown to be accessible in the present work (L^{780}, P^{785} and L^{791}) were not known for a specific role in cation transport. Three other positions, T^{779}, T^{781} and N^{783} (T^{772}, T^{774} and N^{776} in the rat isoform) have been shown earlier to influence cation affinity when mutated to other amino acids [21], but are not accessible in the palytoxin-induced pore according to the present results.

4.1. The nature of the palytoxin-induced ion channel

The binding of palytoxin to the Na,K-ATPase and the antagonist effect of ouabain suggested that the ion conductance was produced by the interaction of palytoxin with the Na,K-ATPase rather than the mechanisms of action known for other ionophores [8]. This hypothesis was subsequently confirmed by studies in which the palytoxin conductance could be produced in cells artificially expressing functional Na,K-pumps [10,24]. We now provide evidence that one of the transmembrane segment, TM5, of the Na,K-ATPase α subunit directly contributes to the structure of the pore of the cation channel opened by palytoxin. As this 5th transmembrane segment is known to provide essential components of the Na^+ and K^+ binding site of the native Na,K-ATPase (see above), our results also strongly suggest that the palytoxin-induced channel is made, at least in part, by a modification of the pathway used by Na^+ and K^+ when they are carried across the membrane during the physiological transport cycle of the Na,K-pump.

4.2. Structure of M5 in the palytoxin-transformed Na,K-ATPase

As shown in the helical wheel diagram of Fig. 4, the distribution of the accessible positions does not show a periodicity compatible with the hypothesis that one side of the putative helix forms the wall of a water-filled cation pathway. Cysteine residues located at opposite sides of the wheel are

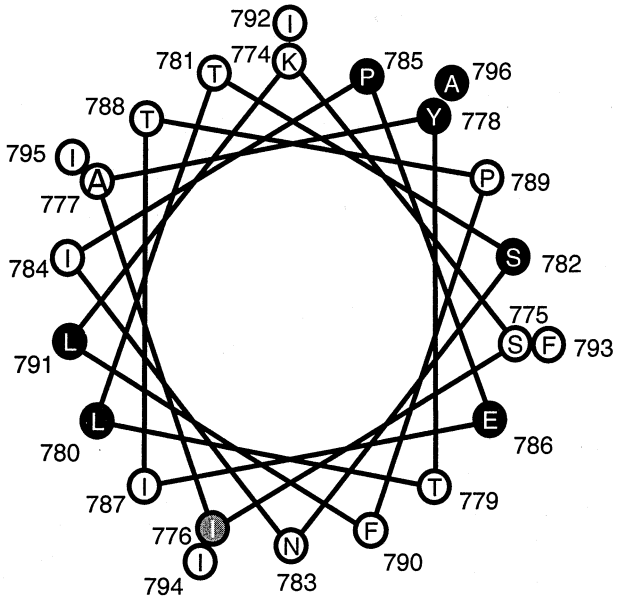

Fig. 4. Helical wheel representation of the putative 5th transmembrane segment of the Na,K-ATPase α subunit. The highly reactive positions are indicated by a white letter against a black background, the weakly reactive positions by a white letter against a gray background and the non-reactive positions by a white background. The numbering corresponds to the *Bufo* α_1 isoform. The distribution of the reactive residues around the helix model makes it highly unlikely that this structure is present in the palytoxin-transformed Na,K-pump.

highly sensitive to the effect of sulfhydryl reagents. Between the position S^{775} and N^{783}, there is a pattern of alternating MTS reactivity rather suggesting the presence of a β-sheet structure for most of the inner half of the putative 5th transmembrane segment.

The recently published high resolution structure of the sarcoplasmic and endoplasmic reticulum calcium ATPase (SERCA) [25] clearly indicates that the homologous domain (M5) of SERCA forms a long α-helix, located in the center of the group of 10 transmembrane segments, spanning the whole membrane width and extending into the 'neck' region of the intracytoplasmic domain. From the high degree of sequence similarity, it is expected that the Na,K-ATPase has a similar overall structure. How can our results be reconciled with the observation on SERCA?

A first explanation could be that, by binding to the Na,K-ATPase, palytoxin induces, or stabilizes, a specific conformation of the Na,K-ATPase that is largely different from the state that has been crystallized in SERCA. With the currently available data, it cannot be determined if the conformation stabilized by palytoxin is close to or very different from a 'natural' conformation adopted by the Na,K-pump during its transport cycle. As a second possibility, it can be hypothesized that, even though the primary structure of the Na,K-ATPase is highly similar to that of SERCA, the different function, in particular the ability to occlude three cations (Na^+ ions) simultaneously, may require a different structure for the ion binding pocket and a different M5 structure. A third explanation is that in the palytoxin-bound state, the α-helical M5 is highly mobile and may expose different aspects to the pore of the channel. This last possibility seems less probable because it would make it difficult to understand why binding of MTSEA to some specific positions results in a rapid channel block, while others are completely insensitive. Finally it is also possible that some non-conservative mutations such as $L^{780}C$ or $L^{791}C$ would produce structural changes. Our present results do not allow us to choose between these possibilities.

In summary, our results first provide additional evidence supporting the hypothesis that the palytoxin-induced channel is largely made of the transmembrane segments of Na,K-ATPase, and highlights the role of the 5th transmembrane segment. These results also suggest that in the palytoxin-bound state, the inner half of the putative 5th transmembrane segment of the Na,K-ATPase α subunit does not have an α-helical secondary structure. They are rather compatible with an extended, β-sheet structure and they indicate which of the amino acid residues of the M5 segment may line the pore of the cation channel through the palytoxin-modified Na,K-ATPase.

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