The Fourth Transmembrane Segment of the Na,K-ATPase α Subunit

A SYSTEMATIC MUTAGENESIS STUDY*

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The Na,K-ATPase is a major ion-motive ATPase of the P-type family responsible for many aspects of cellular homeostasis. To determine the structure of the pathway for cations across the transmembrane portion of the Na,K-ATPase, we mutated 24 residues of the fourth transmembrane segment into cysteine and studied their function and accessibility by exposure to the sulphhydryl reagent 2-aminomethyl-methanethiosulfonate. Accessibility was also examined after treatment with palytoxin, which transforms the Na,K-pump into a cation channel. Of the 24 tested cysteine mutants, seven had no or a much reduced transport function. In particular cysteine mutants of the highly conserved “PEG” motif had a strongly reduced activity. However, most of the non-functional mutants could still be transformed by palytoxin as well as all of the functional mutants. Accessibility, determined as a 2-aminomethyl-methanethiosulfonate-induced reduction of the transport activity or as inhibition of the membrane conductance after palytoxin treatment, was observed for the following positions: Phe323, Ile322, Glu326, Ala330, Pro333, Glu334, and Gly335. In accordance with a structural model the cation pathway. The residues thought to form the position 323 has a critical position at the outer mouth of membrane segments 5 and 6. The phenylalanine residue in membrane segment that faces the space between transmembrane and endoplasmic reticulum calcium ATPase (Protein Data Bank the two published structures of sarcoplasmic and endo-

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Na,K-ATPase Fourth Transmembrane Segment

nature and the structure of these access channels and of the gates are still unknown.

We have recently published the results of studies in which we have examined the accessibility of the residues of the fifth and sixth TM segments by systematic mutagenesis of all the residues of these domains into cysteine (8, 9) and then probing the accessibility of the introduced thiol groups with sulphydryl reagents. These studies have allowed us to define the aspects of the corresponding TM helices that form the pathway for cations across the membrane. We used the same approach to study the role of the fourth TM segment in cation translocation. We analyzed the results using a structural model of the Na,K-ATPase based on the known SERCA structures (2, 3).

MATERIALS AND METHODS

Generation and Expression of the Mutants in the Xenopus Oocyte—Systematic mutation of each residue of the fourth TM domain to a cysteine was performed starting from the C111S mutant of the α1 subunit of the *Bufo marinus* Na,K-ATPase (cloned in the pSG5 vector) in which a reactive cysteine of the first transmembrane segment had been removed and that has physiological properties similar to the wild type protein (10). Most of the mutants were generated using a phenotypic selection strategy (11), and some mutants were generated using the Par3 procedure that was performed on the BlnI and SunI 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 described previously (12).

Stage V/VI *Xenopus laevis* oocytes were obtained from ovarian tissue of female animals that had been anesthetized by immersion in MS 222 (2 g/liter; Sandoz, Basel, Switzerland) according to the regulations of the animal care State of Vaud authorities. Seven nanograms of α subunit and 1 ng of β subunit cRNA were mixed and co-injected in a total volume of 50 nl into stage V/VI *X. laevis* oocytes (13). The injected oocytes were incubated for 3–5 days in a modified Barth’s solution and loaded with Na+ by exposure to a K+-free solution overnight before the electrophysiological measurements as described earlier (8).

Electrophysiological Measurements—The oocytes expressing the mutant Na,K-ATPase were studied by the two-electrode voltage clamp method using a Dagan TEV-200 voltage clamp apparatus (Dagan Corp., Minneapolis, MN). Current/voltage (IV) curves were recorded and analyzed using the Digidata/Pclamp package of Axon Instruments (Union City, CA).

The composition of the control solution was 92.4 mM Na+, 0.82 mM Mg2+, 0.41 mM Ca2+, 10 mM TEA−, 22.4 mM Cl−, 2.4 mM HCO3−, 10 mM HEPES, 80 mM glucose, pH 7.4; a low chloride solution and K+ channel blockers (barium and TEA) were used to minimize the occurrence (α) experiments and the non-accessible (α') currents.

The effects of palytoxin and of 2-aminoethyl-methanethiosulfonate (MTSEA) after palytoxin treatment were performed following a protocol similar to that previously reported for the study of fifth and sixth TM segments. Briefly the steady-state whole oocyte current was first measured at a holding potential of −50 mV using the two-electrode voltage clamp technique. In the experiments with palytoxin, to fully inhibit the endogenous *Xenopus* Na,K-ATPase and to avoid the activation by palytoxin of any of the endogenous Na,K-pumps, the experiments were performed in the continuous presence of 10 μM ouabain. 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 nM palytoxin solution until the inward current had increased at least 3 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 palytoxin-induced conductance was calculated as the conductance after palytoxin treatment minus the conductance before palytoxin treatment. The final solutions without palytoxin and methanethiosulfonate (MTS) concentration measurements were prepared immediately before each electrophysiological measurement from 100 μM (palytoxin) or 100 μM (MTSEA or MTSET) stock solutions kept on ice.

Immunoprecipitation—The expression of the Na,K- or H,K-ATPase wild type and a few selected mutants was examined by pulse (12 h) and chase (3 h) experiments using [35S]methionine metabolic labeling. Microsomes were prepared as described previously (14) and loaded on 5–13% SDS-polyacrylamide gels. The α subunit was immunoprecipitated using *Bufo* α1 subunit antibody (15) under nondenaturing conditions as described previously (16). The dissociated immune complexes were separated by SDS-polyacrylamide gel electrophoresis, and labeled proteins were detected by fluorography.

Reagents—Palytoxin from *Palythoa caribaeorum*, 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 to avoid nonspecific adsorption on vessels and tubing. Ouabain was purchased from Sigma and used from a 200 mM stock solution in dimethyl sulfoxide. The MTSEA reagent MTSEA was purchased from Toronto Research Chemicals (North York, Ontario, Canada).

Homology Modeling—Two homology models of the Na,K-ATPase were built based on the crystal structures of SERCA in the E1 state (Protein Data Bank code 1EUL) and E2 state (Protein Data Bank code 1PWO), which are the two main conformations of the protein during the transport cycle of SERCA or Na,K-ATPase (20). First a multiple alignment was performed between all members of the Na,K-ATPase family together with all members of the SERCA family using the T-Coffee program (17). Based on this alignment, models of the *Bufo* marinus Na,K-ATPase were generated by satisfaction of spatial restraints using the MODELLER program (18). For each of the E1 and E2 states, 100 different models were computed by using different random assignment of the velocities during the simulated annealing part of the optimization. This procedure allowed the assessment of the regions of the model where the spatial restraints confined well the solution space and where there is some conformational freedom. The resulting models were clustered using aphony atom root mean square deviation of the TM4 segment. The conformation with the lowest energy was chosen from the largest cluster. No significant violations of spatial restraints were observed in the final model.

RESULTS

Functional Expression of the Cysteine Mutants—The functional expression of each mutant was evaluated in several ways: first as the outward current induced by addition of 10 mM K+ to a previously K+-free solution (we have shown earlier that when this measurement is performed in Na+-loaded oocytes at a membrane potential of −50 mV it provides a reliable estimate of the number of active Na,K-pump units expressed at the cell surface (14)), second as the current inhibited by 2 mM ouabain in the presence of 10 mM K+ in the extracellular solution, and third, in a qualitative manner, as the change in membrane conductance induced by exposure to 2 nM palytoxin. These results are presented in Fig. 1.

The results of the evaluation of the functional expression of the Na,K-pump as K+-induced current and as ouabain-sensitive current were highly concordant except for small differences that will be discussed in a following paragraph devoted to the voltage dependence of the Na,K-pump function. As expected because the oocyte endogenous Na,K-pump was inhibited by ouabain, the oocyte injected with the cRNA of the β subunit alone did not express any potassium-activated or ouabain-sensitive current. The C111S mutant that we used as control expressed a potassium-activated current (186 ± 47 nA, n = 12) and a ouabain-sensitive current (178 ± 46 nA, n = 12) that were similar to those observed in earlier experiments (8–10). Twelve of the 23 mutants expressed an electrogenic current that was not significantly different from those of the C111S mutant, and it is worth noticing that all the mutants of the most intracellular positions of the TM4 segment (from position Ala338 to Leu344) are among this group. On the other hand, two mutants, F323C and L337C, did not express any significant potassium-activated or ouabain-sensitive current with mean values that were not significantly different from the group injected with the β subunit alone. Three other mutants, G326C, P333C, and G335C, had a very low amplitude Na,K-pump electrogenic activity (below 20 nA), but nevertheless the mean values were significantly different from the negative control, namely oocytes injected with the β subunit alone. Six other mutants (I322C, L324C, I327C, A330C, N331C, and E334C) had slightly but significantly reduced Na,K-pump activity (roughly between 20 and 90 nA).
When exposed to 2 nM palytoxin, all mutants except three reacted with an increase of the membrane conductance that was significantly larger than that observed in the \( \text{H}^{+}/\text{K}^{+} \) subunit alone group. The three non-reactive mutants, G326C, L337C and T339C, had either a very low (G326C) or no detectable (L337C) Na,K-pump activity as described above, and in the set of experiments in which palytoxin was used in the presence of \( 10^{-5} \) M ouabain, no \( \text{K}^{+} \)-activated current could be detected in either group. Surprisingly T339C presented a significant \( \text{K}^{+} \)-induced and ouabain-sensitive Na,K-pump activity but no significant response to palytoxin. In contrast, the F323C mutant, which expressed no ouabain-sensitive current, and P333C and G335C, which had a very low amplitude Na,K-pump electrogenic activity, responded to palytoxin with a large conductance increase (with amplitude similar to that observed in the control C111S group).

**Protein Expression**—To evaluate the possibility that the low level of functional expression of some mutants would be related to a low level of protein expression due to inefficient synthesis and/or rapid degradation of the protein, we examined by metabolic labeling the amount of protein synthesized in a pulse-chase experiment. Analysis by immunoprecipitation (Fig. 2) revealed that G326C and L337C were present only in very low amount compared with the control C111S mutant (with only a slightly larger amount of protein than the oocytes expressing the \( \beta \) subunit alone), although they did not appear to have a short half-life as the amount of protein did not decrease after a 48-h chase period. The E334C and T339C mutants appeared to be normally synthesized and did not appear to be degraded.

In summary, except for the L337C mutant, we observed evidence for some degree of membrane expression, although it was much reduced in some cases, for each of the mutants tested. The functional characteristics of the mutants were tested further by examining the voltage dependence of the \( \text{K}^{+} \)-induced and the ouabain-sensitive current. To avoid a too low signal to noise ratio when low expression levels were encountered, we included only recordings in which the Na,K-pump current amplitude amounted to at least 20% of the mean of the group.

**Voltage Dependence of the Na,K-pump Activity**—IV curves of the \( \text{K}^{+} \)-induced and ouabain-sensitive current of selected mutants are shown in Fig. 3. To reduce the variation due to the level of expression, all the values are normalized to the oua-
bain-sensitive current recorded at $-50 \text{ mV}$. The C111S mutant (that we used as control) presented an I/V relationship similar to what has been published earlier with very little voltage dependence in the low membrane voltage range and a slight voltage dependence (positive slope I/V curve) in the high negative membrane voltage range (19). Eleven mutants (V321C, I322C, L324C, I325C, V329C, V332C, L336C, and L337C for which the current amplitude was too small to obtain a reliable I/V relationship). To eliminate the variability due to variation in expression level, all current values are normalized to the ouabain-sensitive current recorded at $-50 \text{ mV}$ measured in the same oocyte. The normalization value is indicated in each panel. For the F323C mutant, absolute values are indicated because the ouabain-sensitive current was very close to 0. The number of measurements is between 4 and 12 in each group. ou, ouabain.

![Fig. 3. Voltage-dependent electrogenic activity of Na,K-ATPase mutants.](http://www.jbc.org/)

The ouabain-sensitive current was very small over the whole membrane potential range for the F323C, but a significant $K^+$-induced inward current was recorded mostly at negative membrane potential. The G335C mutant presented an identical and strongly voltage-dependent I/V relationship of the $K^+$-induced and ouabain-sensitive currents yielding a linear I/V relationship with a reversal potential close to $-130 \text{ mV}$.

To investigate the reason for the surprising discrepancy between the ouabain-sensitive current and the smaller $K^+$-activated current in the E334C mutant, the effect of 2 mM ouabain was measured in the presence and in the absence of extracellular $K^+$, and the effect of $K^+$ in the presence and in the absence of ouabain. While in the control group the ouabain-sensitive current measured in the presence of $K^+$ (126 ± 31 nA, current values at $-50 \text{ mV}$) was similar to the $K^+$-induced current (124 ± 29 nA, not significant), in the E334C group the ouabain-sensitive current was significantly larger (23.3 ± 5.2) than the $K^+$-induced current measured in the same oocytes (11.5 ± 3.0, $n = 9, p < 0.01$ paired t test). Ouabain added in the absence of extracellular $K^+$ had no detectable effect in the control group (0.1 ± 2.8 nA, $n = 7$), while it produced a significant inhibition of an outward current in the E334C group (9.6 ± 1.9, $n = 9, p < 0.01$). Finally, when the Na,K-ATPase had been blocked by 2 mM ouabain, increasing extracellular K$^+$ to 10 mM induced similar small inward currents in both groups.
inhibition was also observed for the F323C and the A338C motif (P333C, E334C, and G335C; genic activity in the absence of extracellular K\textsuperscript{+} results indicate that the E334C mutant has a significant electro-
Palytoxin-induced conductance remaining after a 1-min treatment with 100 \mu M MTSEA normalized to the palytoxin (PTX)-induced conductance before MTSEA treatment. The control C111S mutant was used as a negative control for the effect of MTSEA, and two cysteine mutants of the fifth transmembrane segment, L870C and Y778C described earlier (8), were used as the positive control. Stars indicate that the mean value was significantly different (p < 0.05) from 1 and from the value observed with the C111S mutant. The number of measurements is between 5 and 16 in each group.

(C111S, \(-2.9 \pm 1.9\) nA, n = 7; E334C, \(-3.5 \pm 1.2\), n = 9, not significant); these small inward currents are probably due to incomplete block of the membrane K\textsuperscript{+} conductance. These results indicate that the E334C mutant has a significant electrogenic activity in the absence of extracellular K\textsuperscript{+}. This K\textsuperscript{+}-independent activity amounts to about half the maximal K\textsuperscript{+}-stimulated activity and explains the difference between the K\textsuperscript{+}-induced and the ouabain-sensitive current.

**Residue Accessibility after Palytoxin Treatment**—Similarly to what we had studied earlier for the fifth and sixth TM segment, we have examined the accessibility of the cysteine residues introduced by mutagenesis to the sulfhydryl reagent MTSEA after exposure to palytoxin. The accessibility was estimated from the reduction of the membrane conductance of palytoxin-treated oocytes. To prevent a low signal to noise ratio, only oocytes in which a Na,K-pump current amounted to at least 20\% of the mean measured in earlier experiments (see above) and that presented a palytoxin-induced conductance at least 4 microsiemens were analyzed. For these experiments, oocytes injected with the C111S mutants were used as the negative control, and oocytes injected with Y778C and L780C, positions that have been shown to be readily accessible to MTSEA after palytoxin treatment (8), were used as the positive control.

The results of these accessibility measurements are summarized in Fig. 4. While the palytoxin-induced conductance (G\textsubscript{PTX}) was not inhibited by MTSEA in the C111S control mutant, the sulfhydryl reagent nearly completely inhibited G\textsubscript{PTX} in the two cysteine mutants of the fifth TM segment that we used as positive control. A statistically significant inhibition was also observed for the three positions of the central conserved “PEG” motif (P333C, E334C, and G335C; p < 0.001 in each case). An inhibition was also observed for the F323C and the A338C mutants (p < 0.05). In the oocytes injected with the \(\beta\) subunit alone or with G326C, L337C, and T339C, it was not possible to obtain a palytoxin-induced conductance of sufficient amplitude to measure reliably the effect of MTSEA on this conductance.

**Accessibility to MTSEA in the Native Na,K-pump**—We also tested the accessibility to MTSEA in the native Na,K-pump (i.e. without treatment with palytoxin) of the mutants of the outer part of the fourth TM segment. The current activated by 10 mM K\textsuperscript{+} was measured before and after a 2-min exposure to 1 mM MTSEA (in the control, K\textsuperscript{+}-free solution). Fig. 5 shows the mean values of the K\textsuperscript{+}-activated current at \(-50\) mV. As reported earlier (8), MTSEA had no effect on the C111S mutant or on most of the tested cysteine mutants. MTSEA, however, significantly inhibited the Na,K-pump current in the I322C, G326C, A330C, and E334C mutants (p < 0.05 in each case). The effect of MTSEA on the whole I/V curve is shown on these mutants in Fig. 6. In these series of experiments, the K\textsuperscript{+}-induced currents expressed by the F323C and P333C mutants were too small to allow a reliable determination of Na,K-pump inhibition by MTSEA.

When we consider not only the current value at \(-50\) mV but the potential range from \(-130\) mV to +30 mV, no effect of MTSEA on the K\textsuperscript{+}-induced current at any membrane potential could be detected in the control C111S mutant. This was also the case in most of the other mutants (data not shown), but interesting modifications of the voltage dependence of the Na,K-pump current were observed with a few mutants. The effect on the I322C mutant was a strong inhibition at negative potential, while the inhibition was much lower at positive potential. The voltage dependence of the Na,K-pump current was thus strongly increased. In these experiments with the G326C mutant and in contrast to the one described above, a small but significant electrogenic activity could be recorded. This activity proved to be nearly completely inhibited by exposure to MTSEA at least in the whole negative potential range (the large variations of the current in the positive potential range did not allow conclusions to be drawn about this part of the I/V curve). Finally the E334C mutant was weakly expressed in these experiments with a mean potassium-activated current of...
normalization values are indicated in each panel. Measurements are 11, 5, 7, and 11 for I322C, G326C, A330C, and H11002 recorded before MTSEA exposure at 50 mV in the same oocyte, and the group in the unwound part of the segment, Gly326, and Ala330, and the group in the unwound part of the segment, Pro331, Glu334, and Gly335. The other residues studied are shown in yellow. Gly326 is not visible, and Leu337, a position for which the cysteine mutant was poorly expressed, is shown in dark blue.

**DISCUSSION**

Using expression in *Xenopus* oocytes we evaluated functional aspects and residue accessibility of cysteine mutants of the fourth transmembrane segment of the Na,K-ATPase. Because of the availability of a high resolution structure for two conformations of the related calcium pump SERCA, we were able to interpret our results using homology modeling of the Na,K-ATPase structure based on SERCA structure.

The effect of cysteine mutations can result from the removal of the replaced amino acid with its specific properties but also from the addition of a sulfhydryl group at a specific position. In particular there is the possibility that the added –SH group may form a disulfide bridge with a cysteine present in the wild type sequence. But disulfide bridges do not usually form spontaneously in transmembrane segments, and in the case of the Na,K-ATPase α subunit, assuming that the structural model is at least approximately correct, of the seven cysteine residues present in the transmembrane part of the protein only one, Cys111, could be in the vicinity of any residue of TM4 (Cys111 is close to Ile325 in the E2 conformation). As Cys111 was replaced by a serine in all our mutants the formation of a disulfide bridge with any of the added cysteine seems highly unlikely.

As shown in Fig. 7, the fourth TM segment appears to be constituted of two helical segments, the outer helix from Trp317 to Asn331 and the inner helix from Leu332 to Lys354, separated by a short unwound loop consisting of Val332, Pro333, Glu334, and Gly335.

**The Outer Helix**—A scheme of the outer helix of TM4 with the position of the surrounding helices has been drawn from the structural model and is shown in Fig. 8. In the part of the outer helix that we explored in this study, most of the large hydrophobic residues can be replaced by a cysteine without major alteration of the electrogenic Na,K-pump function. This is notably the case for Val321, Leu324, Ile325, Ile328, and Val336, which, according to the structural model, seem to face the membrane lipids in the gap between the TM1 and TM3 helices (because of the tilt of TM1, this gap is very wide in the outer part of the membrane). Similarly Ile325 and Val336, which have side chains extending between TM1 and TM6, can be substituted by cysteine without any major effect on the Na,K-pump function. Ile332 is accessible to MTSEA as shown by the effect of MTSEA on the Na,K-pump function (see Fig. 6). This is not surprising considering that this residue is very close to the membrane surface. The absence of effect of MTSEA on the palytoxin-induced conductance on this mutant (Fig. 3) indicates that the binding of the sulfhydryl reagent does not block
the cation pathway opened by palytoxin. This observation can be interpreted as indicating that this residue is not very close to this cation pathway; alternatively this residue could become inaccessible in the palytoxin-bound conformation.

The I327C and N331C mutants exhibited only reduced current amplitude and a small difference between the K⁻-induced and the ouabain-sensitive current, which may result from some degree of ouabain resistance. In the model, these residues are one above the other with their side chains facing the TM5 helix (Ile298 or Asn331). Indeed the side chain of Ile298 comes into close contact (in the E1 conformation) with a phenylalanine residue in TM5 (Phe790 or Phe803 in the rat α1 Na,K-ATPase sequence) that has been recently shown to play a key role in ouabain binding (21). Asn331 is a rather less conserved residue: an asparagine is found in all Na,K-ATPases and non-gastric H,K-ATPase sequences, but it is a tyrosine in all gastric H,K-ATPases and an alanine (Ala106) in most SERCAs. According to the published SERCA structure, Ala106 does not participate directly in the coordination of calcium ions, and Asn331 does not participate directly with the Na⁺ and K⁺ binding sites in the model of the Na,K-ATPase published by Ogawa and Toyoshima (20).

In contrast, mutations of the three residues directly facing the space between TM5 and TM6, Phe323, Gly326, and Ala330 (see Fig. 7), result in a large decrease in the transport activity and/or major alterations in the voltage dependence of the cation transport as seen from the K⁻-activated and ouabain-sensitive I/V curves (data in Fig. 2). To our knowledge, few mutagenesis studies have been published on single residues in the segment corresponding to this outer helix in the Na,K- or H,K-ATPase. The N331L mutant (N326L of the rat α1 Na,K-ATPase) has been found to have a low apparent affinity for Na⁺ and a significant K⁺-independent ATPase activity (22). A triple mutant in which residues of the rat α1 Na,K-ATPase were mutated into the amino acids present in the gastric H,K-ATPase, L319F, N326Y, and T340S (corresponding to position Leu⁷93, Asn⁷94, and Thr⁸25 in our sequence), displayed a change in cation selectivity evidenced by a pH-dependent ATPase activity in the absence of sodium (23). Results obtained with SERCA have shown that most positions of this segment are relatively resistant to mutations with little alterations (except for some decrease in Vmax with the exception of Ala330 (corresponding to Ile328 in our isoform of the Na,K-ATPase) for which a significant decrease of calcium affinity was observed (24). It should be noted that the sequences of this outer helix are rather divergent between the SERCAs on one hand and the Na,K- and H,K-ATPases on the other hand, although they are conserved within the SERCA (P-2A subfamily) or the Na,K- and H,K-ATPase (P-2C) subfamily.

This is the case for instance for Phe323, which is absolutely conserved in all known Na,K- and H,K-ATPases of animals, while an isoleucine is found at this position in most SERCA sequences. While little or no effect of mutation of Ile328 was observed in SERCA (24, 25), several observations indicate a crucial role of Phe323 in the Na,K-ATPase family. First, mutation of Phe323 into a cysteine led to the near complete absence of ouabain-sensitive function, while a small K⁺-induced inward current observed at negative membrane potential suggested a K⁺ “leak” through this mutant that is well expressed at the cell surface as shown by the robust response to palytoxin. Phe323 is probably located on the cation pathway as MTSEA added after palytoxin resulted in a significant reduction of the membrane conductance. The position of this residue in the structural models makes it appear to be a gate in the pathway leading from the extracellular medium to the binding sites (most directly to binding site II) and its movement between the “E1” and the “E2” conformation linked to the large outward translocation of the whole TM4 in relation to the TM5-TM6 hairpin and a slight counterclockwise rotation (when viewed from the intracellular side) of TM4 also suggests an important role of this residue in the control of the access to the cation binding site from the extracellular solution.

Gly326 is also highly conserved in the P-2C subfamily, while an alanine or a serine is present in most SERCAs at the corresponding position. While mutation of Ala301 in SERCA had no effect on Ca²⁺ transport, little transport activity or response to palytoxin could be detected with the G326C mutant of the Na,K-pump. This reduced function is probably related to a low level of expression in oocytes as shown in Fig. 2. In the experiments in which some activity could be detected, this activity was strongly inhibited by exposure to MTSEA indicating that the side chain of the cysteine replacing this glycine was accessible from the opened cation pathway.

The third position of the outer helix leading to a major functional alteration of the Na,K-pump was Ala330, a residue that is conserved throughout the type II family. Mutation of the corresponding residue (Ala305) in SERCA leads to a complete loss of Ca²⁺ transport related to a strong stabilization of the E2-P conformation of SERCA mutants (25). The A330C mutant of the Na,K-pump still had a significant electrogenic transport activity, reduced to about 20% of the control protein, and the voltage-dependence was altered with a negative slope of the I/V curve that could be explained by a change in the voltage-dependent binding of extracellular K⁺. This in turn could be due either to a direct alteration of potassium binding or to a slower K⁺-induced dephosphorylation step leading to a stabilization of the E2-P state, which would be similar to what has been described for SERCA.

The Central Loop—Many more data are already available concerning the four residues of the short central loop (Val332, Pro333, Glu334, and Gly335). This motif, VPEG (or IPEG in the calcium pumps), is absolutely conserved in the type II P-ATPases.

Despite this high degree of conservation, the V332C mutation did not induce any detectable change in the Na,K-pump function. This position was not accessible to MTSEA after palytoxin treatment. Mutations of the residue at the homolo-
gous position in SERCA, Ile307, were characterized by a moderately reduced transport activity or no change (24, 25). As was the case for the other "silent" mutations in the outer helix, the structural model shows that the side chain of this residue points toward the lipid between the TM1 and TM3 helices.

For the P333C mutation we observed a very much reduced electrogenic activity, although the large amplitude of the response to palytoxin indicated that the mutant was expressed at the plasma membrane. The low level of functional expression did not allow characterization of the function more precisely, but our results are compatible with earlier studies showing that mutation of this residue in the rat α1 subunit (P328A) results in reduced affinity for Na⁺ and K⁺ (26), and a similar reduction in calcium affinity has been observed for mutations (P308A and P308G) of the corresponding position in SERCA (24). Pro333 is obviously critical for the geometry of the backbone around the second cation site (2).

There are numerous reports of mutation of Glu334 (Glu327 or Glu329 in the Na,K-ATPase sequences of other species) or Glu309 in SERCA. Most studies have reported a strong reduction or an abolishment of the transport activity most probably by interference with cation binding (27–32). However, the E329Q mutant of the rat α1 Na,K-ATPase provides a sufficient transport activity to sustain life of cells expressing only this mutant with a normal Vₘₚ, but decreased affinity for Na⁺ and K⁺ (33). Our results with the E334C mutant show a moderately reduced activity. In addition, the effects of MTSEA after palytoxin treatment also indicate that this residue is in direct contact with the cation pathway. All these observations are in complete agreement with the role of the side chain of Glu309 of the mutant to provide adequate Na⁺ binding, although the affinity could be reduced due to diminution of the number of negative charges.

The observation of a significant K⁺-independent activity (observed in our case as the presence of an outward ouabainsensitive current in the absence of extracellular K⁺) is similar to that reported by de Pont and his collaborators (34–36) for a mutation of residues in the fifth and sixth transmembrane segments of the gastric H,K-ATPases. The same group has recently published data supporting the hypothesis that removal of a negative charge (Glu290) releases the positively charged group of a lysine (Lys791) that is able then to mimic a K⁺ ion and activate the dephosphorylation step and shift to the E1 conformation (37). Because of the absence of any positively charged residue in the membrane part of the Na,K-ATPase, a homologous mechanism is not possible for the Na,K-ATPase. Indeed several positions belonging to the core TM segments surrounding a central "pocket" around the cation binding sites are accessible to MTSEA (Pro333, Glu334, and Gly335 in TM4; Ser782, Pro785, and Glu786 in TM5 (8); and Asp811 in TM6 (9)) after palytoxin treatment as if once the MTSEA molecule has reached this central pocket it could bind to several positions and block the palytoxin-opened pathway.

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The Fourth Transmembrane Segment of the Na,K-ATPase α Subunit: A SYSTEMATIC MUTAGENESIS STUDY
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