A third Na⁺-binding site in the sodium pump

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The sodium pump, or Na,K-ATPase, exports three intracellular sodium ions in exchange for two extracellular potassium ions. In the high resolution structure of the related calcium pump, two cation-binding sites have been identified. The two corresponding sites in the sodium pump are expected to be alternatively occupied by sodium and potassium. The position of a third sodium-specific site is still hypothetical. Here, we report the large effects of single residue substitutions on the voltage-dependent kinetics of the release of sodium to the extracellular side of the membrane. These mutations also alter the apparent affinity for intracellular sodium while one of them does not affect the intrinsic affinity for potassium. These results enable us to locate the third sodium-specific site of the sodium pump in a space between the fifth, sixth, and ninth transmembrane helices of the α -subunit and provide an experimental validation of the model proposed by Ogawa and Toyoshima [Ogawa, H. & Toyoshima, C. (2002) *Proc. Natl. Acad. Sci. USA* 99, 15977–15982].

cation-binding site | Na,K-ATPase | sodium affinity

The sodium pump maintains the large chemical gradients of Na⁺ and K⁺ across the membrane of animal cells. These gradients provide energy for multiple cellular functions such as membrane potential, excitability, control of cell volume, and solute homeostasis through numerous secondary active transport systems. Knockout of two of the isoforms of the catalytic α -subunit of Na,K-ATPase has been reported to be lethal (1). This ATPase exports three intracellular sodium ions in exchange for two extracellular potassium ions (2). The structure of the sarcoplasmic and endoplasmic calcium ATPase (SERCA), another P-type ATPase, has been determined recently, and two cation-binding sites have been clearly identified close to the middle of the membrane (3, 4). Homology modeling indicates that the two corresponding sites in the sodium pump are expected to be alternatively occupied by sodium and potassium (5). The position of a third sodium-specific site is still hypothetical.

Na,K-ATPase is composed of two subunits, an $\approx$ 100-kDa α -subunit and an associated $\approx$ 40-kDa glycoprotein, the β -subunit (2). The general structure of the α -subunit is thought to be similar to that of SERCA, which comprises 10 transmembrane helices and for which several high resolution structures have been published (3, 6–9).

The transport cycle of the sodium pump (known as the Post-Albers cycle) is realized by the successive binding of three intracellular Na⁺ ions, their occlusion in the protein, and their release on the other side, followed by the binding, occlusion, and intracellular release of two extracellular K⁺ ions (2). The activity of the sodium pump is thus electrogenic, can be measured as a current under voltage clamp conditions, and contributes to the generation of the membrane potential. Release of sodium to the extracellular side (or binding of extracellular Na⁺ when the Na,K-pump runs in the reverse cycle) is strongly voltage-dependent and is associated with the largest charge-moving event occurring during the cycle (10–12). More precisely, this charge movement seems to be generated by the translocation of the first of the three Na⁺ ions to be released (13, 14).

In the absence of a high-resolution structure for Na,K-ATPase, the location of two of the cation-binding sites has been proposed to

be homologous with those of calcium in SERCA, roughly at mid-membrane level between the fourth, fifth, and sixth transmembrane segments (5) (see Fig. 1). This hypothetical position is largely supported by a number of mutagenesis studies (15). The position of the third Na⁺-binding site is still conjectural, and two main hypotheses have been proposed. On the basis of valence analysis of a Na,K-ATPase structural model obtained by homology with the E1 and E2 conformation of SERCA (PDB ID codes 1EUL and 1IWO), Ogawa and Toyoshima (5) proposed a location for the third Na⁺ site (designated as Na site III) between the fifth, sixth, eighth, and ninth transmembrane helices (see Fig. 1). This location was partially supported by recent mutagenesis data by Imagawa *et al.* (16) indicating a role of several residues in the eighth and ninth transmembrane helices. The apparent affinity for Na⁺ activation of Na,K-ATPase activity was reduced 1.8-fold by the V920E mutation (in M8, V927 in our numbering) and 3.1-fold by the E954A mutation (in M9, corresponding to E961 in our numbering). These authors also observed a marked effect of a mutation of threonine T774 (in M5, T781 in our numbering), suggesting a Na⁺-binding site position closer to the cytoplasmic face. Another location even closer to the intracellular side of the membrane and the fifth transmembrane helix has been proposed (15). This latter site is in close proximity to a putative access sodium site, which would provide a first step in sodium binding as suggested by Shainskaya *et al.* (17) based on the role of negatively charged residues of the intracellular loop between the sixth and seventh transmembrane helices in the initial cation recognition.

We have studied the effects of mutations in the ninth transmembrane helix and observed large effects of an E961A mutation on the apparent affinity for K⁺ that were present only in the presence of extracellular Na⁺, suggesting a role in extracellular Na⁺ binding for this residue. We then investigated the effects of this mutation and other mutations in the same area on the translocation of Na⁺ and made several observations that allow us to propose the location for the third Na⁺-binding site between the fifth, sixth, and ninth transmembrane helices.

Materials and Methods

Mutagenesis and Expression. All mutants were generated by the Nelson and Long PCR method (18) from the α 1-subunit of the rat Na,K-ATPase. Wild-type and mutant α -subunits were expressed by cRNA coinjection of 10–14 ng of α -subunit cRNA with 1–1.4 ng of cRNA of rat β 1-subunit in stage V–VI *Xenopus laevis* oocytes. Injected oocytes were incubated for 40–72 h at 19°C and loaded with sodium before measurements by overnight exposure to a K⁺-free solution containing 0.2 μ M ouabain to inhibit the *Xenopus* endogenous Na,K-ATPase, as described (19).

Electrophysiological Measurements. Oocytes expressing the WT and mutant Na,K-ATPases were studied by the two-electrode voltage clamp technique by using a Dagan TEV-200 voltage clamp apparatus (Dagan Instruments, Minneapolis). Data were acquired and analyzed by using a Digidata/Pclamp package from

Abbreviations: SERCA, sarcoplasmic and endoplasmic calcium ATPase; Na_{ext}⁺, extracellular concentration of Na⁺; K_{ext}⁺, extracellular concentration of K⁺; k_{1/2}K_{ext}⁺, half activation constant for extracellular K⁺; V_m, membrane potential.

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Fig. 3. Voltage-dependent inhibition of the sodium pump current by extracellular sodium. Currents activated by 1 mM K^+ were measured in the absence of extracellular Na^+ (■) and then in the presence of 30 mM Na^+ (□) and 100 mM Na^+ (○). All current values are normalized to the value at -50 mV in the absence of extracellular Na^+ (WT, 297 ± 38 nA; E960A, 677 ± 109 nA; E961A, 339 ± 65 nA; Y778F, 134 ± 19 nA; G813A, 220 ± 26 nA; T814A, 318 ± 47 nA). Data shown are means $\pm$ SE of 7–18 oocytes from two to eight different batches. Error bars are smaller than symbol size in several cases.

Extracellular Na^+ binding can also be observed as a voltage-dependent inhibition of the Na,K -pump activity by extracellular sodium (23), stimulation of the ADP-dependent Na^+/Na^+ exchange (10), or stimulation of the backward running pump (12). We studied the inhibition of the K^+ -activated current by 30- and 100-mM concentrations of Na^+_{ext} over the -130 to $+30$ mV membrane potential range and observed the known voltage-dependent inhibition in the WT Na,K -pump and the E960A mutant, but a reduced and mostly voltage-independent inhibition by Na^+_{ext} in the E961A mutant (Fig. 3).

E961 is predicted by homology modeling to extend toward the interior of the protein and was proposed to contribute with its side chain to Na^+ site III by Ogawa and Toyoshima (5). We generated mutants of other residues proposed by Ogawa and Toyoshima to participate in the structure of the sodium site III (5), namely two residues of transmembrane helix 6, G813 and T814, which contribute their backbone carbonyl, and Y778 in transmembrane helix 5 which contributes through its side chain $-OH$ to Na^+ site III (Fig. 1). Y778 was mutated to phenylalanine to produce a minimal modification due to the removal of a single oxygen atom.

Because of the very low affinity of Na^+_{ext} for its extracellular binding/release site on the E2P conformation of the Na,K -pump, and the impossibility of using very high concentrations of Na^+ in whole cell experiments, we could not obtain a direct and complete measurement of the affinity of this site. However, the effects observed with 30 and 100 mM Na^+ in the various mutants clearly indicated changes in the voltage-dependent apparent affinity for Na^+_{ext} .

First, the inhibition produced by 30 and 100 mM Na^+ was clearly voltage dependent in the WT and in E960 and G813A mutants, with much larger inhibitions at high negative membrane potentials than at depolarized potentials. In contrast, the effect of Na^+_{ext} was similar over the whole potential range for the E961, Y778F, and T814A mutants. These observations indicate that, for these three mutants, either the affinity for Na^+_{ext} is no longer voltage dependent, or rather, as suggested by the results of the pre-steady-state current measurement presented below, that the voltage dependence has been shifted so far toward negative potentials that it is no longer observable in the membrane potential range that we explored.

Second, with the G813A mutant, the inhibition by Na^+_{ext} occurred with a much higher apparent affinity. At -50 mV, for instance, 30 mM Na^+ inhibited $49 \pm 2\%$ ($n = 9$) of the K^+ -induced current compared with $14 \pm 1\%$ in WT ($n = 18$), $P < 0.001$, and 100 mM Na^+ inhibited $89 \pm 1\%$ ($n = 9$) of the K^+ -induced current compared with $40 \pm 2\%$, in WT ($n = 18$), $P < 0.001$. Because of the much higher affinity of this mutant for Na^+_{ext} , the $K_{1/2}$ value could be estimated to ≈ 30 mM at -50 mV (30 mM Na^+ inhibits $\approx 50\%$ of the K^+ -induced current).

In the presence of an “ion well,” the effective concentration of Na^+ at its binding site, and thus the apparent affinity for extracellular Na^+ , is dependent on the membrane potential (11). In the G813A mutant, the shift toward the positive membrane potentials that we observed for inhibition of the K^+ -activated Na,K -pump current by Na^+_{ext} is thus equivalent to a increase in the affinity of a Na^+ -binding site located in an ion well.

Third, there seem to be different effects on the voltage-independent apparent affinity for extracellular Na^+ in some mutants. For instance, in the E961A mutant, there was no significant effect of 30 mM Na^+ and only a $23 \pm 3\%$ ($n = 8$) inhibition at 100 mM Na^+ at 10 mV, whereas, in the WT Na,K -ATPase, 30 mM Na^+ produced a significant inhibition ($18 \pm 3\%$, $n = 18$, $P < 0.02$) and 100 mM a significantly larger inhibition ($40 \pm 3\%$, $n = 18$, $P < 0.02$) than in the E961A mutant. In contrast, the T814A mutant showed a significantly larger inhibition by 30 and 100 mM Na^+ ($27 \pm 5\%$, $n = 10$, $P < 0.05$ and $59 \pm 2\%$, $n = 9$, $P < 0.001$) than the WT Na,K -pump.

Thus, even though we cannot obtain precise absolute values for the apparent affinity for Na^+_{ext} , we can state that the mutations that we studied affect the apparent affinity for Na^+_{ext} both at high negative membrane potentials, a potential range where the apparent affinity for Na^+_{ext} seems highly voltage dependent, and in the depolarized potential range, where there is little voltage dependence of the apparent affinity for extracellular Na^+ .

The kinetics of the release/binding of extracellular Na^+ can also be studied by measurements of ouabain-sensitive pre-steady-state currents after fast voltage perturbations under Na^+/Na^+ exchange conditions (24–26). We studied the slow component of ouabain-sensitive pre-steady-state current during series of 50-ms voltage steps (from -170 to $+30$ mV) in WT and mutant Na,K -ATPases. The mean ouabain-sensitive charge dis-

Fig. 4. Effect of mutations on the ouabain-sensitive pre-steady-state currents under Na^+/Na^+ exchange conditions. (A) Voltage dependence of the slow component of the ouabain-sensitive pre-steady-state charge translocation (Q) in WT and mutant Na,K-ATPases. The holding potential was -50 mV, and currents were recorded during a series of 50-ms voltage pulses ranging from -170 mV to $+30$ mV, before and after addition of 2 mM ouabain. (Inset) Original recordings of the ouabain-sensitive current obtained in oocytes expressing the WT and the E961A mutant Na,K-ATPase during 50-ms steps (only two traces corresponding to the $+30$ mV and -170 mV step are shown for clarity). The smooth curves represent the best fitting Boltzmann equation:

$$Q(V) = Q_{\min} + Q_{\max} \left(1 + \exp \left(z(V_m - V') \frac{E}{RT} \right) \right), \quad [2]$$

where $Q(V)$ is the charge displacement produced by the voltage step, $Q_{\min}$ the charge displaced at maximal large negative membrane potential, $Q_{\max}$ the maximal displaceable charge during a negative to positive potential jump, V_m the membrane potential, V' the mid-point potential, z the apparent valence, which was set to one, F the Faraday constant, R the universal gas constant, and T the absolute temperature. (B) Voltage dependence of the relaxation of the slow component of the ouabain-sensitive pre-steady-state currents. The smooth curves represent best fitting curves corresponding to the following equation

$$k(V) = k(0) \exp\left(-\frac{VF}{RT}\right) + k'(0) \exp\left(\frac{VF}{RT}\right), \quad [3]$$

which describes the voltage (V) dependence of the relaxation rate constant k as the sum of forward and backward voltage-sensitive rate constants with an effective charge z and values of $k(0)$ and $k'(0)$ at 0 mV, respectively. The fit for the G813A mutant is simply linear.

Fig. 5. Apparent affinity for intracellular sodium ($K_{1/2} Na^+$). The activation by intracellular Na^+ was studied as described earlier (19, 27) by measuring the K^+ activated sodium pump current and the intracellular Na^+ concentration by using the reversal potential of the amiloride-sensitive current in oocytes coexpressing Na,K-ATPase and the epithelial sodium channel. Data are means $\pm$ SE of 7–14 oocytes from 4–12 different batches. *, $P < 0.05$; ***, $P < 0.0001$.

placement (Q), calculated as the time integral of the current transient, is shown for each mutant in Fig. 4A. The Q/V_m relation could be well fitted by a Boltzmann relation with a $Q_{\max}$ of 1,530 nC and 1,010 nC and a mid-point potential at -40 mV and -38 mV for the WT protein and the E960A mutant, respectively. These values for the WT are similar to what has been described earlier (25) and indicate only a slightly lower level of expression for the E960A mutant. The three mutants with a decreased voltage-dependent inhibition by Na_{ext}^+ (Y778F, T814A, and E961A) showed small charge displacement after positive voltage jumps but much larger Q amplitudes with negative voltage jumps. Although the parameters of the Boltzmann distribution could not be determined precisely (due to the fact that the explored voltage range covers only part of the Boltzmann curve), it is clear that there was a large shift to the left of the voltage dependence curve. The G813A mutant presented the opposite change, a large displacement of the Boltzmann curve toward positive potentials.

The voltage dependence of the relaxation rate of the pre-steady-state current (Fig. 4B) was very similar in the WT and the E960A mutant to what has been described earlier (25, 26), with increasing rates at negative potentials and little voltage dependence in the depolarized potential range. Three mutants (Y778F, T814A, and E961A) showed less voltage dependence or even a reverse voltage dependence for the E961A mutant. In contrast, the G813A mutant showed quasi-linear voltage dependence along the whole voltage range, with faster rate at high negative membrane potentials.

Finally, we also studied the activation by intracellular Na^+ during progressive Na^+ loading of oocytes coexpressing the epithelial Na^+ channel (19, 27). The E961A, Y778F, and T814A mutants resulted in a significant decrease of the apparent affinity for intracellular Na^+ whereas the G813A mutation had the opposite effect (Fig. 5).

Discussion

Our results indicate that three of the mutants (Y778F, T814A, and E961A) have a lower voltage-dependent affinity for Na^+_{ext} in the negative membrane potential range whereas the reverse alterations were observed for the G813A mutant. The effects of the Y778F mutation are expected to be due to the removal of the side chain oxygen whereas the effects of the T814A and G813A are expected to be related to modifications of the geometry of the helix backbone and the positions of the carbonyl oxygen. Our experimental data thus demonstrate the role of four residues

located around the Na⁺ site III proposed by Ogawa and Toyoshima (5) for the third Na⁺-binding site. This site seems strictly sodium-specific in the E2 conformation because, at least for the mutant E961A, the intrinsic affinity for extracellular K⁺ is not modified (see Fig. 2B). The sodium site defined by our mutations is thus clearly distinct from the two high-affinity K⁺-binding sites. In addition, the predominant effect of mutations altering this site is a major modification of the voltage-dependent extracellular release of Na⁺.

Concerning the binding of intracellular Na⁺ to the E1 conformation, our measurements also indicate some effects of the same mutations on the apparent Na⁺ affinity. Imagawa *et al.* (16) observed a slightly larger effect, a 3-fold decrease of the same parameter, but, considering the very different techniques used, these results can be considered in reasonably good agreement. Small changes in the ATPase activation by Na⁺ were also observed by Van Huysse *et al.* (28) for a mutation of the homologous residue in the rat α 1-subunit (E956Q in their numbering), but their measurements of ATPase activity would not have detected the effect of extracellular sodium and these authors did not consider that the effect of this mutation indicated the proximity of a cation-binding site. Indeed, the larger effects that we observed on the voltage-dependent release/binding of extracellular Na⁺ than on activation by intracellular Na⁺ indicate that the mutations that we have studied affect more the mechanism of Na⁺ release from the E2 conformation than the Na⁺-binding site in the E1 conformation. Indeed, the larger effect (7.5-fold) on the apparent affinity for intracellular Na⁺ of the T774A (T781 in our numbering) mutation (16) suggests that this residue is more important for the Na⁺ site III in the E1 conformation or controls the access to this site from the cytoplasmic side.

The direction of the changes, namely the decrease of the apparent affinity for both intracellular and extracellular Na⁺ in the case of the Y778F, T814A, and E961A mutants and the increase of the apparent affinity for both intracellular and extracellular Na⁺ in the case of the G813A mutant strongly suggests that the mutations produce their effects by alteration of the Na⁺-binding site itself rather than through modifications of the kinetics of a conformational change. As illustrated with the kinetic model presented in the supporting information, which is published on the PNAS web site, a shift in the E1–E2 conformation equilibrium would rather produce opposite effects on the apparent affinities for intracellular and extracellular cations, whereas a modification of the structure of the binding site itself affecting both the binding of intracellular and extracellular Na⁺ is expected to modify the apparent affinity for extracellular and intracellular Na⁺ in the same direction, even though the magnitude of the change might not be similar considering the rather large reorganization of the cation site resulting from the E1 to E2 transition.

According to the model of Hilgeman (13), the release of Na⁺ to the extracellular side of the membrane occurs in two steps: the release of a first Na⁺ ion associated with a large charge movement is followed by a reorganization of the binding sites, which modifies the high field access pathway to the Na⁺ occlusion sites so that the two remaining Na⁺ ions are released with little charge translocation. Because mutations around Na⁺ site III result in a major alteration of the voltage-dependent, charge-translocating release of

Na⁺, we can make the hypothesis that the first Na⁺ ion to be released to the external medium producing the large charge-carrying event is released from this Na⁺ site III. Although these changes seem to be due in part to alterations of the structure of the binding sites themselves, our results do not allow us to determine how much of these changes might also be related to the reorganization of the binding sites linked to extracellular Na⁺ release or alterations in the cation access pathways.

Some mutations also seem to moderately affect the voltage-independent component of Na_{ext}⁺ binding (E961A with a lower affinity, and T814A with a higher affinity than WT; see Fig. 2), but these effects were of smaller amplitude and were not observed for all of the mutants. In addition, and in contrast to the mutations in transmembrane helix 9, the mutations in transmembrane helices 5 and 6 had also some effects on the intrinsic affinity for extracellular K⁺ (data not shown), which is not surprising considering the contribution of transmembrane helices 5 and 6 to cation-binding site I.

The pathway of Na⁺ ions from the cytoplasm to their binding sites in the membrane is still not clearly defined. An entry port for Ca²⁺ between the M1 and M2 segments of SERCA has been proposed (7), but this hypothesis needs more experimental support in the case of Na,K-ATPase. In addition, it is not known whether all three Na⁺ ions enter the protein through the same pathway. The presence of a Na⁺ occlusion site at the location defined by our mutations is not incompatible with the presence of another site that could be occupied by a Na⁺ ion on its way from the cytoplasm to the occlusion site; this could be the case, for instance, for the space between the M4, M5, and M6 helices, close to their cytoplasmic ends, which has been proposed to be occupied by a partially hydrated Na⁺ ion (15, 29).

The α - and β -subunits of Na,K-ATPase can be associated with a third subunit belonging to the FXYD family, and this association can modulate the affinity for Na⁺ and K⁺ ions (2, 30). We have recently provided evidence for an interaction between the transmembrane segment of FXYD proteins and the ninth transmembrane segment of the α -subunit (22). A similar position of FXYD protein is supported by other approaches (31) while other locations have also been proposed recently (32). The role of the ninth transmembrane helix in Na⁺ transport and the close interaction between the transmembrane domain of FXYD proteins and the ninth transmembrane helix suggest that interaction between these transmembrane segments could be responsible for the functional effects of the association of these proteins with the α/β complex, even if interactions between cytoplasmic domains may also affect Na,K-ATPase function.

The requirement for the binding and occlusion of three Na⁺ ions in Na,K-ATPase, instead of two Ca²⁺ ions in SERCA, results in profound differences in the cation transport cycle between these two P-type ATPases. The experimental identification of a site located close to the ninth transmembrane segment raises the possibility that the third Na⁺ ion follows a different pathway than the two other cations through the membrane-associated part of Na,K-ATPase.

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