THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 51, Issue of December 19, pp. 51213–51222, 2003
Printed in U.S.A.

Electrophysiological Analysis of the Mutated Na,K-ATPase Cation Binding Pocket*

Received for publication, June 17, 2003, and in revised form, September 29, 2003
Published, JBC Papers in Press, October 7, 2003, DOI 10.1074/jbc.M306384200

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Na,K-ATPase mediates net electrogenic transport by extruding three Na^+ ions and importing two K^+ ions across the plasma membrane during each reaction cycle. We mutated putative cation coordinating amino acids in transmembrane hairpin M5-M6 of rat Na,K-ATPase: Asp^{776} (Gln, Asp, Ala), Glu^{779} (Asp, Gln, Ala), Asp^{804} (Glu, Asn, Ala), and Asp^{808} (Glu, Asn, Ala). Electrogenic cation transport properties of these 12 mutants were analyzed in two-electrode voltage-clamp experiments on *Xenopus laevis* oocytes by measuring the voltage dependence of K^+-stimulated stationary currents and pre-steady-state currents under electrogenic Na^+/Na^+ exchange conditions. Whereas mutants D804N, D804A, and D808A hardly showed any Na^+/K^+ pump currents, the other constructs could be classified according to the $[K^+]$ and voltage dependence of their stationary currents; mutants N776A and E779Q behaved similarly to the wild-type enzyme. Mutants E779D, E779A, D808E, and D808N had in common a decreased apparent affinity for extracellular K^+. Mutants N776Q, N776D, and D804E showed large deviations from the wild-type behavior; the currents generated by mutant N776D showed weaker voltage dependence, and the current-voltage curves of mutants N776Q and D804E exhibited a negative slope. The apparent rate constants determined from transient Na^+/Na^+ exchange currents are rather voltage-independent and at potentials above −60 mV faster than the wild type. Thus, the characteristic voltage-dependent increase of the rate constants at hyperpolarizing potentials is almost absent in these mutants. Accordingly, dislocating the carboxamide or carboxyl group of Asn^{776} and Asp^{804}, respectively, decreases the extracellular Na^+ affinity.

The Na,K-ATPase transports in each reaction cycle three sodium ions out of the cell and two potassium ions into the cell at the expense of one molecule of ATP. As is characteristic for P-type ATPases, the Na,K-ATPase, upon intracellular binding of Na^+ in the E_1 conformation, gets intermediately phosphorylated by ATP (forming $E_1P(Na^+)_3$; see Fig. 1). After a conformational change to E_2P, Na^+ is extracellularly released. Subsequently, K^+ can bind extracellularly and stimulates dephosphorylation. Upon a conformational change back to E_1, K^+ is intracellularly released. In order to bring about sequential translocation of Na^+ and K^+ ions in the sense of a ping-pong mechanism (1, 2), the ion pump has to obey strict cation specificity of the phosphorylation and dephosphorylation reaction (3). The binding affinities for individual cation species change during the exposure of the binding sites from intra- to extracellular, which occurs during the $E_1P \rightarrow E_2P$ conformational change (for recent reviews, see Refs. 4 and 5). Several putative cation-coordinating amino acids of the Na,K-ATPase were identified by mutagenesis studies (5, 6), including three charged (Glu^{779}, Asp^{804}, and Asp^{808}) and one polar amino acid (Asn^{776}) in transmembrane hairpin M5-M6. These residues are identical or very similar to those described earlier by MacLennan *et al.* (7) for the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA pump), and their involvement in cation binding is confirmed by homology modeling with the three-dimensional structure of the SERCA pump (8). However, their influence on electrogenic cation transport and related cation affinities is largely ambiguous.

The observation that the Na,K-ATPase generates an outward membrane current by extrusion of one net positive charge during each reaction cycle was made decades ago (9). The voltage and cation dependence of stationary pump currents have been investigated in numerous electrophysiological studies on the enzyme from native cell preparations (*e.g.* rodent heart cells (10, 11) and *Xenopus laevis* oocytes (12–14)) or after heterologous expression of wild-type enzymes in a variety of cells including *Xenopus* oocytes (15, 16) and mammalian cell lines (17–20). Furthermore, extensive work has been carried out on the study of transient currents that can be induced by voltage jumps under Na^+/Na^+ exchange conditions (10, 21, 22). There is general agreement that the major charge-translocating event occurs during the Na^+ branch of the transport cycle, which is rate-limited by the $E_1P \leftrightarrow E_2P$ conformational change (23). Several studies also indicated that electrogenicity, albeit much weaker than during Na^+ translocation, also occurs during the K^+ branch of the reaction cycle (14, 24–26).

After expression of recombinant Na,K-ATPase in different cell types became possible, many research groups focused on the biochemical characterization of mutated Na,K-ATPase. However, only very few groups studied the consequences of these mutations on properties of electrogenic transport (17–20, 27, 28). The only putative cation binding amino acids that were mutated for electrophysiological studies are Glu^{779} and Ser^{775} (17–20). The steady-state pump currents of these mutants were recorded in voltage-clamped HeLa cells (17–19) or HEK293 cells (20). The substitution of Glu^{779} by alanine resulted in a remarkably high amplitude of electrogenic Na^+/Na^+ exchange current compared with K^+-stimulated stationary currents and

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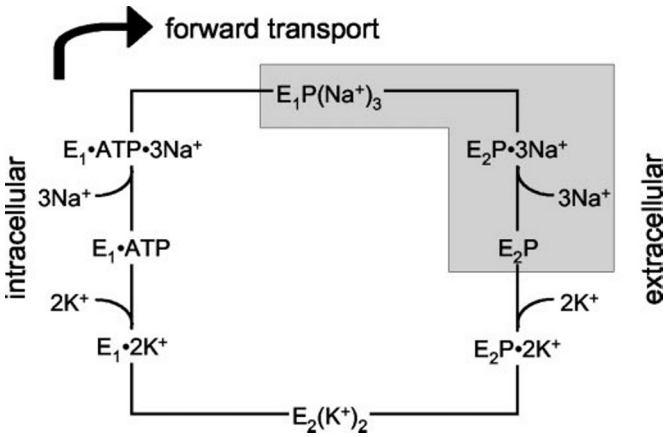

FIG. 1. **Albers-Post scheme.** To generate an outward current, Na,K-ATPase needs ATP and Na^+ in the cytoplasm of the cell and K^+ in the extracellular medium. The main electrogenic event is the Na^+ transport step that is kinetically coupled to the E_1P-E_2P transition (*gray background*).

an apparent increase of the enzyme's $K_{0.5}$ value for half-maximal activation of K^+-induced stationary currents (18, 20). Although these studies provided illuminating results, time-resolved measurements of transient currents due to the $E_1P \leftrightarrow E_2P$ transition under Na^+/Na^+ exchange conditions were not carried out, and information about the influence of other amino acids involved in cation binding is still missing.

The main electrogenic events occur during Na^+-dependent transport steps that are kinetically coupled to the $E_1P \rightarrow E_2P$ conformational change (Fig. 1) (29). Fendler *et al.* (30) demonstrated the electrogenicity of Na^+-dependent transport steps by investigating the pre-steady-state currents of Na,K-ATPase upon photolysis of caged ATP. Following pioneering experimental work by Nakao and Gadsby (10), a series of publications used voltage pulse experiments under Na^+/Na^+ exchange conditions, showing that extracellular Na^+ binding/release is the major electrogenic event (15, 21–23, 31). Analysis of transient currents yields information about the kinetics of partial reactions of the catalytic cycle. The use of *X. laevis* oocytes for investigation of transient currents (15) facilitates the study of Na,K-ATPase mutants, but so far this has been performed only for the N-terminally truncated enzyme (27).

We measured the influence of mutations in putative cation binding amino acids on stationary and pre-steady-state currents of the Na,K-ATPase. Substitution of Glu^{779} and Asp^{808} can lead to a reduced apparent affinity for K^+, and substitutions of Asn^{776} and Asp^{804} result in stationary current-voltage curves with a negative slope, which is interpreted as a large reduction of the enzyme's affinity for extracellular Na^+.

MATERIALS AND METHODS

Expression Constructs—The rat Na,K-ATPase α-subunit was amplified with the primers 5′-AAATATTCGCGAATGGGGAAGGGGGTTGGA-3′ and 5′-GGACTAGTCTAGTAGTAGGTTTCCTT-3′. The PCR product was digested with NruI and SpeI and cloned into the NruI and XbaI sites from pTLN (32). The rat Na,K-ATPase β-subunit was amplified with the primers 5′-AAATATTCGCGACCATGGCCCGCGGAAAAGCC-3′ and 5′-CGGGGTACCTCAGCTCTTAACTTCAAT-3′. The PCR product was digested with NruI and KpnI and cloned into the NruI and KpnI sites from pTLN. We used a PCR-based method for introduction of the following mutations in the Na,K-ATPase α-subunit: N776Q, N776D, N776A, E779D, E779Q, E779A, D804E, D804N, D804A, D808E, D808N, and D808A. All introduced mutations were verified by sequencing.

Expression of Na,K-ATPase in X. laevis Oocytes—*X. laevis* females were anesthetized with 2 g/liter tricane, and parts of ovaries were removed. Oocytes were separated by incubation for 2–3 h in modified Ringer's solution (110 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4, 9.5 mg/liter penicillin, and 10 mg/liter streptomycin sulfate) containing 1–2 mg/ml collagenase A. Prophase-arrested oocytes of stage V and VI were selected for injection of cRNA. cRNA synthesis was carried out with the SP6-mMessageMachine kit (Ambion, Austin, TX), using HpaI-linearized plasmid DNA. For the expression of the Na,K-ATPase α- and β-subunits, oocytes were injected with 10 and 2 ng of the corresponding cRNAs.

Functional expression in the plasma membrane was measured as the K^+-dependent (5 mM) stationary current. It increased with increasing amounts of α-subunit cRNA until a maximum was reached at 10 ng of mRNA per oocyte (data not shown). Increasing the amount of β-subunit cRNA (1–10 ng) did not increase the expression level of the functional pump at the plasma membrane. Therefore, 10 ng of cRNA α-subunit and 2 ng of cRNA of the β-subunit were injected into *X. laevis* oocytes. These oocytes express an endogenous Na,K-ATPase (~25-nA stationary current; data not shown) that can be inhibited by 10 μM ouabain (28). The current generated by the heterologously expressed rat Na,K-ATPase can be readily dissociated from the endogenous pump, since the latter is rather insensitive for ouabain (33). This phenomenon is due to two charged amino acids present in the first extracellular loop of the rat (recombinant) Na,K-ATPase α-subunit, whereas two neutral amino acids are present on this position in the *X. laevis* α-subunit (33). The specific rat Na,K-ATPase current is therefore defined as the difference in K^+-stimulated current measured in the presence of 10 μM and 10 mM ouabain. Injection of the β-subunit alone increased the ouabain-sensitive endogenous pump current, but injection of only the α-subunit could hardly increase the ouabain-insensitive current of the heterologously expressed construct (data not shown). This observation is compatible with the data of Geering and co-workers (34) and indicates that the endogenous β-subunit is the limiting factor for expression of the endogenous sodium pump. After the injections, the oocytes were incubated for 3 days at 16 °C in modified Ringer's solution. Preceding electrophysiological experiments, intracellular Na^+ was elevated by incubation of the oocytes for about 40 min at room temperature in 110 mM NaCl, 2.5 mM sodium citrate, and 10 mM MOPS[1]/Tris, pH 7.4, as described (24). After the Na^+ loading, oocytes were kept in 100 mM NaCl, 1 mM $CaCl_2$, 20 mM tetraethylammonium chloride, 5 mM $BaCl_2$, and 5 mM MOPS/Tris, pH 7.4, for at least 30 min before being used in an experiment (24).

Isolation of Total and Plasma Membranes—For the isolation of total membranes, 10 oocytes were homogenized in 100 μl of buffer (250 mM sucrose, 2 mM EDTA, and 25 mM HEPES/Tris (pH 7.0)) and centrifuged for 3 min at 1000 × *g* and 4 °C. Next, membranes were isolated by centrifugation of the supernatant for 30 min at 16,000 × *g* and 4 °C. The isolation of plasma membranes was done according to Kamsteeg and Deen (35) with some modifications. Essentially, oocytes were stripped from their follicle membrane and rotated in 1% colloidal silica, Ludox Cl (Sigma-Aldrich) in MES-buffered saline (MBSS; 20 mM MES, 80 mM NaCl, pH 6.0) for 30 min at 4 °C, washed two times in MBSS, rotated in 0.1% polyacrylic acid (Sigma) in MBSS for 30 min at 4 °C, and washed twice in modified Barth's solution (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES (pH 7.5), 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, and 0.41 mM $CaCl_2$). Subsequently, oocytes were homogenized in 1200 μl of HbA (20 mM Tris (pH 7.4), 5 mM $MgCl_2$, 5 mM NaH_2PO_4, 1 mM EDTA, and 80 mM sucrose) at 4 °C and centrifuged for 30 s at 10 × *g* and 4 °C, after which the top 1000 μl was removed and 1000 μl of HbA was added. This centrifugation and exchange of HbA was repeated three times (10, 20, and 40 × *g*). After the last centrifugation step, HbA was removed, and the plasma membranes were spun down for 30 min at 16,000 × *g* and 4 °C. The samples were solubilized in SDS-PAGE sample buffer, separated on SDS gels containing 10% acrylamide according to Laemmli (36), and blotted on Immobilon polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). The α-subunits of Na,K-ATPase were detected with the polyclonal antibody C356-M09. This antibody was generated by immunization of a rabbit with a glutathione *S*-transferase fusion protein containing the Na,K-ATPase N-terminal sequence LKKEVSMDDHKLSLDELHRKYGTDLSRGLT.

Two-electrode Voltage Clamp—Two-electrode voltage clamp experiments were essentially performed as described by Geibel *et al.* (29). The bath solution contained 90 mM NaCl, 20 mM tetraethylammonium chloride, 5 mM $BaCl_2$, 5 mM $NiCl_2$, and 5 mM MOPS/Tris, pH 7.4. The different K^+ containing solutions were obtained by replacing NaCl by KCl. All solutions additionally contained 10 μM or 10 mM ouabain. Experiments were carried out at room temperature (22–24 °C).

[1] The abbreviations used are: MOPS, 4-morpholinepropanesulfonic acid; MES, 4-morpholineethanesulfonic acid; MBSS, MES-buffered saline.

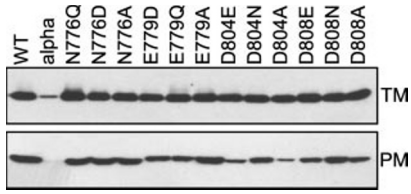

FIG. 2. **Expression of wild-type Na,K-ATPase and mutant enzymes in total membranes and plasma membranes.** Total membranes (*TM*) and plasma membranes (*PM*) were isolated from three different batches of oocytes, each containing 10 oocytes. Samples equivalent to one (*TM*) or three (*PM*) oocytes were separated by SDS-PAGE and blotted on polyvinylidene difluoride membranes, and the presence of the Na,K-ATPase α-subunit was detected with antibody C356-M09.

Determination of $K_{0.5}$ Values for the Stimulation of Stationary Pump Currents by K^+—To determine the apparent $K_{0.5}$ values for stimulation of Na^+/K^+ pump current by extracellular $[K^+]$, the amplitudes of stationary currents at a certain membrane potential from data as depicted in Fig. 4 were fitted by a Hill equation of the following form,

$$I_p = \frac{[K^+]^n}{(K_{0.5}^n + [K^+]^n)} \cdot I_{p,max} \qquad \text{(Eq. 1)}$$

where I_p represents the pump current at a given potential, $I_{p,\,max}$ is the saturation value of the current determined from the fit, $K_{0.5}$ is the half-maximal concentration for extracellular K^+, and n is the Hill coefficient.

Measurement of Voltage Jump-induced Pre-steady-state Currents under Na^+/Na^+ Exchange Conditions—Transient Na,K-ATPase currents under Na^+/Na^+ exchange conditions were obtained as the difference between current responses to a specific voltage step in the presence of 10 μM and 10 mM ouabain. The voltage step going from the constant holding potential to a variable testing potential is referred to as "on pulse"; the term "off pulse" denotes the step back from test to holding potential. These resulting transient currents were fit by a monoexponential function starting 5 ms after the voltage jump to exclude artifacts arising from capacitive charging of the oocyte membrane. The reciprocals of the time constants resulting from these fits will be referred to as (apparent) rate constants throughout this work. Accurate determination of rate constants greater than ~300 s^{-1} is difficult in voltage clamp experiments on oocytes due to the time constant for the capacitive charging of the cell membrane, which is around 1 ms.

The quantity of charge moved during the transient currents was calculated as the time integral of the fitted currents, extrapolated to onset of voltage pulses. Since the rate constants of the transient currents corresponding to "on" voltage pulses to −140 and −160 mV are higher than 300 s^{-1} (and therefore at least in part biased by residual capacitive charging currents), the charge translocated during "on" voltage pulses is not accurately determined at these potential values (Fig. 7). For data analysis and presentation, pClamp 7 (Axon Instruments, Union City, CA) and Origin 5.0 (Microcal Software, Northampton, MA) were used.

RESULTS

In the cation-binding pocket of Na,K-ATPase, the amino acid residues Asn^{776}, Glu^{779}, Asp^{804}, and Asp^{808} were each replaced by closely related amino acids or alanine residues. Asparagine 776 was replaced by glutamine, aspartic acid, and alanine; glutamic acid 779 was replaced by aspartic acid, glutamine, and alanine; aspartic acids 804 and 808 were replaced by glutamic acid, asparagine, and alanine. These 12 mutants were analyzed in *X. laevis* oocytes with the two-electrode voltage clamp method.

The expression levels in the total membranes and plasma membranes were analyzed by isolating these membrane fractions and comparing the expression levels of the mutants with that of the wild-type enzyme (Fig. 2). The expression of the Na,K-ATPase α-subunit without the β-subunit seems very low, and it is not present at the plasma membrane. This is due to the degradation of unassembled α-subunit (37). In the total membranes, the expression levels of all mutants are comparable with that of the wild-type enzyme. All enzymes (except the α-subunit alone) are present at the plasma membrane, but the quantities of D804E and D804A seem to be lower than that of the wild-type enzyme. The expression of all other mutants is comparable with that of the wild-type enzyme.

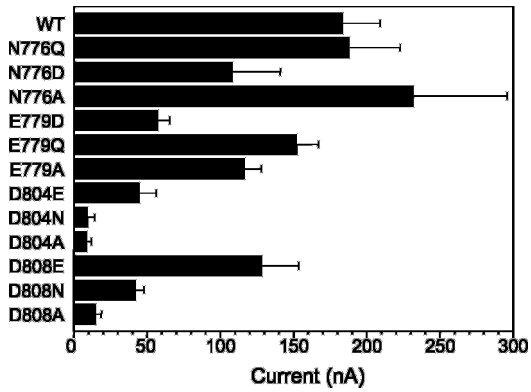

FIG. 3. **Stationary Na^+/K^+ pump currents of the wild-type and mutant Na,K-ATPases.** The currents were measured in *X. laevis* oocytes under two-electrode voltage clamp conditions in the presence of 5 mM extracellular K^+ at −20 mV holding potential. The values presented are the mean ± S.E. of 3–18 experiments.

Functional expression of the wild-type or mutated rat Na,K-ATPase constructs in the plasma membrane of oocytes was determined from the difference in K^+-dependent (5 mM) stationary currents measured in presence of 10 μM and 10 mM ouabain. Since the current generated by the heterologously expressed rat Na,K-ATPase is rather insensitive for ouabain (33), this serves to eliminate the contribution of the endogenous oocyte pump. When the current voltage relation of the wild-type rat Na,K-ATPase was studied, the extracellular addition of 5 mM K^+ generated a maximum current at a potential of −20 mV (183 ± 26 nA, n = 18; see Fig. 3).

Whereas the ATPase activity of most mutants has been well described (4, 5), little is known about electrogenic transport properties of these mutants. We therefore measured ouabain-sensitive K^+-stimulated currents in two-electrode voltage clamp experiments (Fig. 3). At 5 mM K^+ and −20 mV holding potential, the stationary currents of mutants N776Q and N776A were comparable with wild-type; mutants N776D, E779Q, E779A, and D808E produced slightly lower currents; and mutants E779D, D804E, and D808N produced significantly lower currents than the wild-type enzyme. In mutants D804N, D804A, and D808A, electrogenic cation transport was severely impaired.

The voltage dependence of the stationary currents (*I*-V curves) of the wild-type rat Na,K-ATPase and the active mutants were investigated at different K^+ concentrations. The current in the absence of K^+ was subtracted from those in the presence of 0.15, 0.5, 1.5, 5, and 15 mM K^+ (Fig. 4) at different membrane potentials (from −160 to +40 mV). Due to the occurrence of time-dependent outward currents of unknown origin at positive potentials and 15 mM K^+ stationary Na,K-ATPase, current amplitudes could not reliably be determined under these conditions and are therefore not presented. The currents of the wild-type enzyme increased both with increasing K^+ concentrations and the membrane potential. At more positive membrane potentials, the currents decreased again, forming a bell-shaped curve, which was shifted to more positive membrane potentials with increasing K^+ concentrations, as expected from other publications (12, 14, 24). Thus, the membrane potential at which the current of a certain mutant is maximal depends on the cation concentrations and has to be interpreted considering the specific affinities for these cation species (see "Discussion"). Most mutants possess a *I*-V relationship that is similar to that of the wild-type enzyme. The N776Q

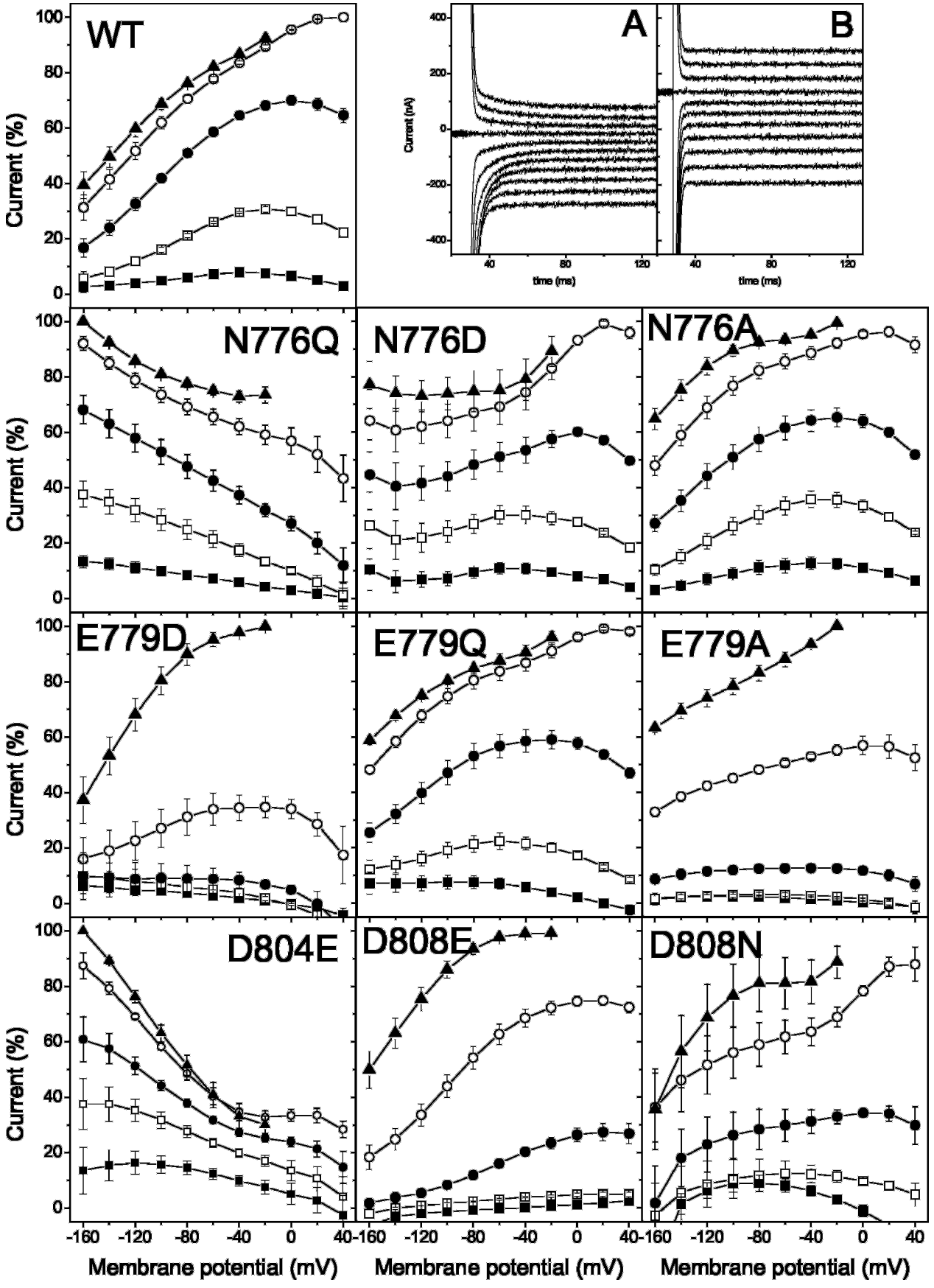

FIG. 4. **Voltage dependence of extracellular K^+-activated Na,K-ATPase current.** Steady-state voltage-current relationships of mutant and wild-type (*WT*) pumps were obtained by subtracting current measured in K^+-free solution from that measured in K^+-containing solution at each potential. For the purpose of normalization, the maximal current of each preparation was set at 100%. Values are the mean ± S.E. (n = 3–4) at the following KCl concentrations: 0.15 mM (■), 0.5 mM (□), 1.5 mM (●), 5 mM (○), 15 mM (▲). *A* and *B* (*insets* at *top right*) show original recordings of the wild-type Na,K-ATPase in the presence of 0 mM (*A*) and 5 mM KCl (*B*).

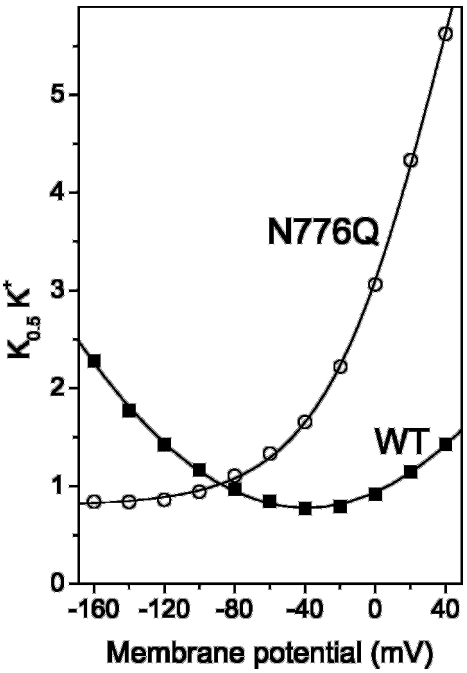

FIG. 5. **Voltage dependence of the $K_{0.5}$ value of K^+-dependent current stimulation.** The $K_{0.5}$ values were determined by fitting data in Fig. 4 with a Hill equation for the Na,K-ATPase wild type (*WT*) (■) and mutant N776Q (○).

and D804E mutants, however, show *I*-V curves with negative slope at any K^+ concentration tested (Fig. 4). Mutant N776D exhibited weaker voltage dependence than the wild-type enzyme at potentials below 0 mV.

The apparent $K_{0.5}$ value for stimulation of Na^+/K^+ pump current by extracellular K^+ was determined from the voltage dependence of stationary currents at different [K^+] (see "Materials and Methods"). The (voltage-dependent) $K_{0.5}$ of the wild-type enzyme had a minimum of 0.8 mM at −40 mV and increased to 1.4 mM (at +40 mV) and 2.3 mM (at −160 mV), respectively (Fig. 5). Some mutants hardly showed pump currents at K^+ concentrations below 1.5 mM. These mutants (E779D, E779A, D808E, and D808N) had an elevated apparent $K_{0.5}$ for K^+ (Table I). Most other mutants, except N776Q, exhibited a voltage dependence of the $K_{0.5}$ for K^+ that is similar to that of the wild-type enzyme. The N776Q mutant had its maximal apparent K^+ affinity at −160 mV ($K_{0.5}$ = 0.8 mM), which decreased when the plasma membrane was depolarized ($K_{0.5}$ = 5.6 mM at +40 mV; see Fig. 5).

The kinetics of pre-steady-state currents upon voltage jumps under Na^+/Na^+ exchange conditions were analyzed to investigate reaction kinetics of Na^+-dependent reaction steps (10). In the absence of K^+ and with high Na^+ concentrations on both sides of the membrane (and ATP within the cell), the enzyme is restricted to the electrogenic Na^+/Na^+ exchange mode (partial reaction sequence $E_1P(Na^+)_3 \leftrightarrow E_2P + 3Na^+$; see Fig. 1). Fig. 6 shows the voltage jump-induced transient currents (holding potential = −60 mV) recorded from an oocyte expressing wild-type rat Na,K-ATPase in the presence of 10 μM (*A*) and 10 mM (*B*) ouabain. Fig. 6*C* shows the transient currents, determined as the difference between traces in *A* and *B*. Between 3 and 5 ms after the voltage step, the residual capacitive charging currents (fast initial signal phases) were settled. The following, slower signal phases represent transient currents specific for Na,K-ATPase activity, which declined monoexponentially to zero, as described previously (15). The quantity of charge moved during the transient currents can be calculated from the time integral of the fitted signals (see "Materials and Methods"). In Fig. 7, the charges moved during the "on" and "off" phases of the voltage pulses are plotted against the membrane potential. The "on" and "off" charge movements are rather identical, in accordance with previous studies (15). The overall amount of translocated charge in this typical experiment is ~8 nanocoulombs, which equals $5{\cdot}10^{10}$ Na,K-ATPase molecules/

TABLE I
$K_{0.5}$, values for the stimulation of stationary pump currents by K^+ at −20 and −120 mV

Curves were fitted with a Hill function. $K_{0.5}$, and S.E. values are given.

Mutant	$K_{0.5}$ at −20 mV	$K_{0.5}$ at −120 mV
Wild type	0.79 ± 0.02	1.42 ± 0.08
N776Q	2.22 ± 0.09	0.86 ± 0.02
N776D	0.98 ± 0.05	1.36 ± 0.09
N776A	0.91 ± 0.05	1.66 ± 0.09
E779D	13.78 ± 2.09	—[a]
E779Q	1.15 ± 0.03	1.46 ± 0.21
E779A	5.48 ± 0.12	5.56 ± 0.58
D804E	0.44 ± 0.10	0.78 ± 0.10
D808E	3.20 ± 0.02	6.75 ± 0.96
D808N	2.68 ± 0.04	4.18 ± 1.37

[a] $K_{0.5}$ value was too large to be reliably determined.

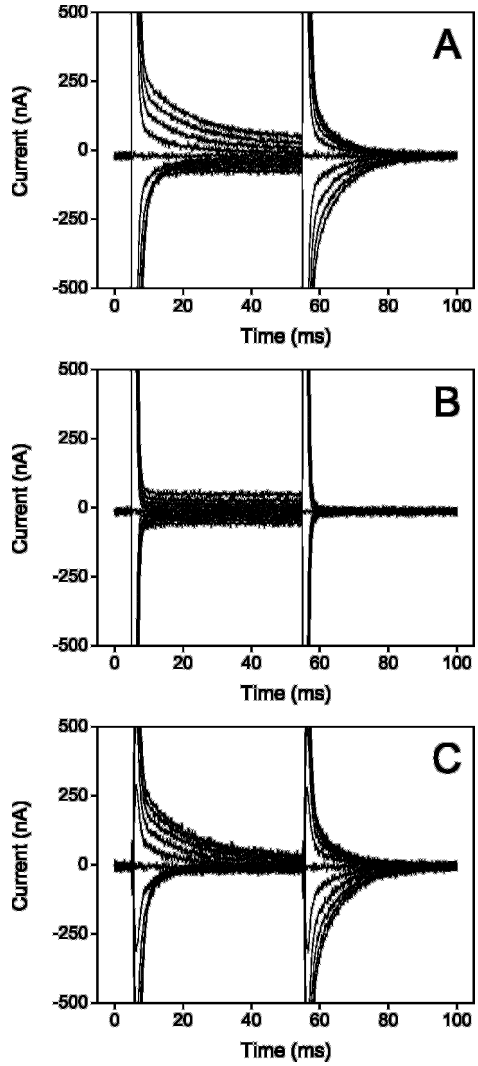

FIG. 6. **Original current recordings of the wild-type Na,K-ATPase subjected to voltage pulses in the absence of extracellular K^+.** Transient currents were recorded in the presence of 10 μM (*A*) and 10 mM (*B*) ouabain. The difference between *A* and *B* is shown in *C*. The exponential decrease of the current reflects the apparent rate constant of the $E_1P(Na^+)_3 \leftrightarrow E_2P + 3Na^+$ reaction.

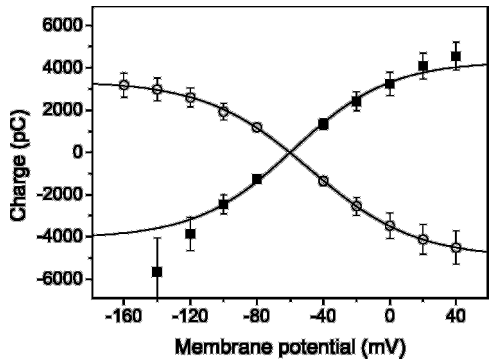

FIG. 7. **Voltage dependence of charge transferred during transient currents of wild-type Na,K-ATPase.** The quantity of charge moved during the transient currents was calculated as the time integral of the monoexponentially fitted currents, extrapolated to onset of voltage pulses (*filled symbols*, "on" voltage pulses; *open symbols*, "off" voltage pulses).

oocyte (assuming a net transport of one unitary charge per pump cycle). Together with the average pump current (183 nA), this results in a turnover number of 23 s^{-1}, which is in good agreement with published data for the Na,K-ATPase under these conditions (21 °C) (38, 39).

In Fig. 8, the apparent relaxation rate constants are plotted against the membrane potential. For the wild-type enzyme, the rate constants are not or are only weakly voltage-dependent at depolarizing potentials (~100 s^{-1} between −40 and +40 mV) and increase strongly at negative voltages. This is usually interpreted in terms of a voltage-dependent rebinding of Na^+ (partial reaction sequence $E_2P + 3Na^+ \rightarrow E_2P(Na^+)_3$), which is favored by negative potentials (Fig. 1). Within the forward reaction sequence ($E_1P(Na^+)_3 \rightarrow E_2P + 3Na^+$), however, the electrogenic event (Na^+ release) is rate-limited by the preceding conformational change ($E_1P \rightarrow E_2P$), resulting in essentially voltage-independent rate constants at depolarizing potentials (10, 40).

Asn^{776} Mutations—The transient currents of N776A decayed with apparent rate constants greater than ~250 s^{-1}, which could accurately be resolved only at membrane potentials above −20 mV, due to limited time resolution of two-electrode voltage clamp experiments on oocytes. The N776D mutant exhibited a voltage-independent rate constant of ~100 s^{-1} up to voltages of −100 mV, resulting in a negative shift of the distribution of rate constants compared with wild-type. The apparent rate constant of the N776Q mutant was nearly completely voltage-independent and at membrane potentials above −100 mV higher than that of the wild-type enzyme.

Glu^{779} Mutations—At membrane potentials >−60 mV, the distribution of rate constants for mutant E779Q was comparable with wild type (voltage-independent value of ~100 s^{-1}), with a somewhat weaker increase at more negative potentials. The E779A mutant showed at potentials >−100 mV significantly faster rate constants (~170 s^{-1}) than the wild-type enzyme, which were weakly voltage-dependent and showed only a moderate increase at hyperpolarizing potentials. The rate constants of the E779D construct, which could be determined accurately only at potentials greater than −60 mV, were around 300 s^{-1} and therefore also faster than those of the wild-type enzyme.

Asp^{804} and Asp^{808} Mutations—The D804E mutant exhibited fast and apparently voltage-independent rate constants of 250–300 s^{-1} under Na^+/Na^+ exchange conditions. This is also true for the D808N mutant, for which the rate constants could accurately only be determined between −80 and +40 mV. The D808E construct remarkably exhibited fast rate constants (≥~300 s^{-1}) at depolarizing membrane potentials that gradually decreased to ~180 s^{-1} at −160 mV.

DISCUSSION

For the SERCA pump and the Na,K-ATPase, the cation-coordinating residues were identified by mutagenesis studies (5, 6, 41), and for the Ca^{2+}-ATPase they were confirmed by the crystal structure (7, 8, 42). The two calcium ions are bound in two binding sites. Sequence alignment with Na,K-ATPase suggests that in the Na,K-ATPase E_1 conformation, site I contains Asn^{776}, Glu^{779}, and Asp^{808}, and site II harbors Asp^{804}, Asp^{808}, and some backbone carbonyl groups of transmembrane segment M4 (43). These amino acids are involved in cation binding also in the E_2 conformation (43).

The effects of mutations at amino acids Asn^{776}, Glu^{779}, Asp^{804}, and Asp^{808} on cation binding properties of the Na,K-ATPase have been studied in a number of publications, mostly by the application of biochemical techniques (44–49), whereas extensive electrophysiological measurements focused only on the E779A mutant (17–20). To investigate the influence of the above residues on properties of electrogenic cation transport, either the length of the side chain was changed by one CH_2 group, or the carboxyl group was replaced by a carboxamide or reverse, and finally the side chain was reduced to a methyl group (mutation to alanine). The voltage dependence of K^+-stimulated Na^+/K^+ pump current was measured for all mutants that showed significant pump currents. From these data, the voltage-dependent $K_{0.5}$ values for the K^+-stimulated stationary currents were calculated. It is important to mention that observed changes in K^+ affinity can be due to indirect kinetic effects. Furthermore, pre-steady-state currents evoked by voltage pulses under ATP-dependent Na^+/Na^+ exchange conditions were analyzed. For the interpretation and classification of the observed effects, several considerations regarding the electrogenicity of partial reactions of the Na,K-ATPase pump cycle must be taken into account as described below.

A major electrogenic event of the Na,K-ATPase reaction cycle occurs during Na^+ transport. Fendler *et al.* (30) demonstrated electrogenicity of Na^+-dependent transport steps by measuring pre-steady-state currents upon photolytic release of ATP from caged ATP. From these and subsequent measurements (39, 50), it was concluded that either the conformational change $E_1P(Na^+)_3 \rightarrow E_2P(Na^+)_3$ is electrogenic or that this partial reaction may be itself nonelectrogenic but is rate-limiting for the subsequent, electrogenic Na^+ release step(s). In a series of publications in which voltage pulse experiments were carried out under Na^+/Na^+ exchange conditions (10, 15, 21–23, 31), it was shown that extracellular Na^+ binding/release is the major electrogenic event, as originally proposed by Läuger (51). In voltage clamp experiments on oocytes, only the slowest phase (with decay time constants between ~2 and ~20 ms), which presumably corresponds to the major electrogenic release/rebinding of one Na^+ ion, can be accurately determined (15, 22). Additional experimental effort is required to resolve faster electrogenic signals associated with release/rebinding of the other two Na^+ ions (21, 23). The apparent rate constants (reciprocals of time constants) of the above mentioned signal component are nearly voltage-independent at positive membrane potentials (~100 s^{-1}, 21–24 °C) and increase exponentially at negative potentials (10, 15). This is interpreted as a result of electrogenic Na^+ binding at the extracellular side. Electrogenic Na^+ rebinding, which is favored at negative potentials, gives rise to the strong increase of rate constants upon hyperpolarization. Na^+ release, however, which is favored by positive potentials, is rate-limited by the preceding slower conformational change $E_1P(Na^+)_3 \rightarrow E_2P(Na^+)_3$; thus, the apparent rate constants at positive potentials appear voltage-independent.

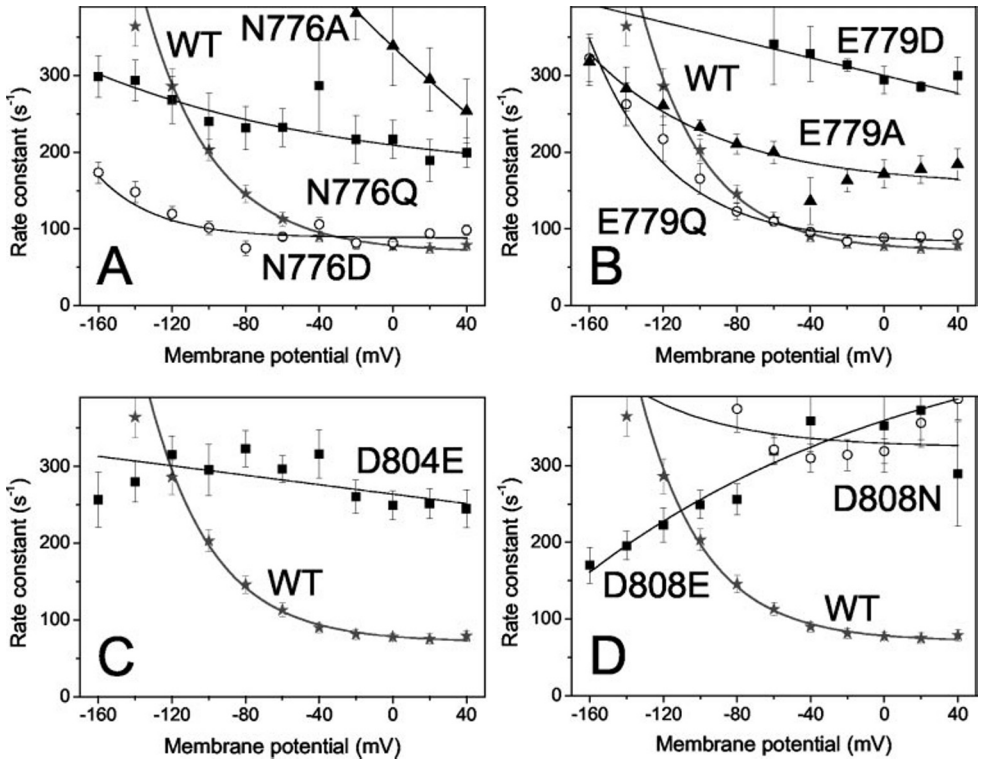

FIG. 8. **Voltage dependence of the relaxation rate constant of the pre-steady-state currents of wild-type (*WT*) Na,K-ATPase and mutant enzymes.** The relaxation rate constant was determined as the reciprocal of time constants from monoexponential fits of transient current traces (as in Fig. 6). *A*, Asn^{776} mutations; *B*, Glu^{779} mutations; *C*, Asp^{804} mutations; *D*, Asp^{808} mutations. In each *panel*, the corresponding wild-type data are shown in *gray* for comparison.

This behavior is explained on the basis of a kinetic pseudo-two-state model that accounts for a reaction sequence $E_1P(Na^+)_3 \leftrightarrow E_2P(Na^+)_3 \leftrightarrow E_2P + 3Na^+$ (Fig. 1) (10, 15). Within that model, the apparent rate constant k, which determines the time course of the transient currents, is given as the sum of the rate constants for the forward reaction (first-order voltage-independent rate constant k_1) and the pseudo-first-order reverse rate constant k_{-1}. The latter is equal to $k_{-1}(0)\cdot[Na^+]_b$, where $k_{-1}(0)$ is the second-order rate constant for the Na^+ binding reaction and $[Na^+]_b$ is the "effective" Na^+ concentration at the extracellular binding site. Electrogenic Na^+ binding results in a Boltzmann expression for the equilibrium concentration of Na^+ between the extracellular medium ($[Na^+]_o$) and its binding locus ($[Na^+]_b$) of the form, $[Na^+]_b^n = [Na^+]_o^n \exp(-z_k FV/RT)$, where the slope factor z_k represents an apparent valence and is a measure of the electrogenicity of the reaction sequence. F and R are thermodynamic constants, T is the temperature, V is the membrane voltage, and n is the Hill coefficient. Therefore, the backward reaction rate constant is given by $k_{-1} = k_{-1}(0)\cdot[Na^+]_o^n \exp(-z_k FV/RT)$ (see Ref. 15 for details). The distribution of the observed k values therefore is negatively shifted upon a decrease in $[Na^+]_o$. For the discussion of the results in this study, it is important to note that a negative shift of the voltage dependence of the apparent rate constants is interpreted as a decrease in Na^+ affinity. This change of external Na^+ affinity can also be due to a change in the rate of the preceding conformational change.

Whereas K^+-dependent transport steps of the Na,K-ATPase were initially reported to be voltage-independent (52), the detection of a negative slope in steady-state current-voltage relationships of the Na,K-ATPase at positive potentials and subsaturating extracellular K^+ concentrations (14, 24) and of strongly voltage-dependent $K_{0.5}$ values for the stimulation of stationary Na^+/K^+ currents in the absence of extracellular Na^+ (24) provided evidence for electrogenic partial reactions within the K^+ branch of the pump cycle. This was corroborated by measurements of transient currents under K^+/K^+ exchange conditions upon substitution of extracellular K^+ by Tl^+ (26). A description of the complex current-voltage dependence of Na,K-ATPase stationary currents is given in Ref. 12 on the basis of the so-called "access channel" model, in which both Na^+ and K^+ ions pass a major fraction of the transmembrane electric field during extracellular release or (re-)binding. The stationary current in the absence of extracellular Na^+ and at saturating extracellular $[K^+]$ is nearly voltage-independent at negative membrane potentials. In the presence of Na^+, however, the pump current decreases at negative potentials due to the stimulation of Na^+-dependent backward reaction steps. At subsaturating extracellular K^+ concentrations, positive potentials reduce the effective K^+ concentration at the binding sites, and K^+ binding becomes rate-limiting for the turnover, which leads to a decreasing current-voltage curve upon depolarization (12).

Mutants

According to the voltage and $[K^+]$ dependence of the stationary currents, the mutants could be subdivided into three groups: 1) mutants that behaved rather similarly to the wild-type enzyme (N776A and E779Q); 2) mutants that showed a decreased apparent K^+ affinity (E779D, E779A, D808E, and D808N); and 3) mutants with severely altered voltage dependence (N776Q, N776D, and D804E) and a decreased Na^+ affinity at the extracellular sodium-binding site.

Asn^{776} Mutants—The N776A mutant exhibited K^+-stimulated stationary currents with amplitudes and voltage dependence comparable with wild type, leaving the $K_{0.5}$ for K^+ also unchanged. The enzyme activity found by others was 0% (49) or 26% (53) of that of the wild-type activity. The expression level in the total and plasma membrane fraction of all three Asn^{776} mutants was comparable with that of the wild-type enzyme. The discrepancy found must therefore be due to differences in

determining the enzyme activity and/or the expression system. The apparent rate constants of pre-steady-state currents under Na^+/Na^+ exchange conditions, however, were significantly higher than for the wild type. This corresponds to the results of Argüello *et al.* (53), who reported a high level of Na^+-ATPase activity for mutant N776A. Such a behavior has previously also been shown for the E779A mutant, for which both high Na^+-ATPase activity (54, 55) and a high level of stationary Na^+/Na^+ exchange currents in the absence of K^+ have been demonstrated (18, 20). We observed for N776A and E779A stationary, ouabain-sensitive Na^+/Na^+ exchange currents of ~40 and 50 nA, respectively (at −20-mV holding potential). For all other mutants, we did not observe Na^+/Na^+ exchange currents that were significantly different from that of the wild type. The high rate constants of N776A from transient Na^+/Na^+ exchange currents reflect the acceleration of reaction kinetics under these conditions probably due to an increased Na^+-stimulated dephosphorylation ($E_1P(Na^+)_3 \leftrightarrow E_2P + 3Na^+ \leftrightarrow E_2(Na^+)_2 + Na^+ + P_i$) (17). However, this kinetic change does not interfere with the competition of Na^+ and K^+ in the voltage-dependent regulation of the Na^+/K^+ pump current (compare Fig. 5, wild type and N776A).

The substitution for a charged side chain in the N776D mutant led to a decreased voltage dependence of the K^+-induced stationary currents, paralleled by a decrease in K^+ affinity. The voltage dependence of the rate constants from Na^+/Na^+ exchange currents indicates that the forward reaction within the Na^+ transport branch is unchanged, whereas the characteristic increase in rate constants occurs only at strongly hyperpolarizing potentials, in accordance with the weaker voltage dependence of stationary currents. This suggests a decreased Na^+ affinity of this mutant.

The stationary current of N776Q (at −20 mV) was comparable with that of the wild-type enzyme. This is in contrast to the enzyme activity found by others, which was 7% (49) and 22% (53) of that of the wild-type activity. This discrepancy is probably due to differences in determining the enzyme activity and/or the expression system. Dislocation of the carboxamide group in the N776Q mutant leads to stationary currents with a negative slope at all $[K^+]$ tested. This behavior is reminiscent of the wild-type enzyme in the absence of Na^+ at subsaturating $[K^+]$ and indicates that the voltage dependence of the stationary currents mediated by mutant N776Q is determined only by K^+-dependent reaction steps at any potential tested. Since perfusion buffers contained 100 mM Na^+, we interpret this behavior in terms of a greatly reduced affinity for extracellular Na^+. The absence of voltage dependence of the $K_{0.5}$ for K^+ at hyperpolarizing membrane potentials also supports the reduced affinity for extracellular Na^+. This is also supported by the voltage dependence of the apparent rate constants; although they are higher at potentials above −100 mV than for the wild-type enzyme, the voltage-dependent rise at negative potentials is nearly completely absent in this construct.

Glu^{779} Mutants—The expression level in the total and plasma membrane fraction of all three Glu^{779} mutants was comparable with that of the wild-type enzyme. The stationary currents of the E779A mutant have been investigated in HeLa cells (17–19) and HEK293 cells (20). In accordance with these published data, we observed a prominent stationary current due to electrogenic Na^+/Na^+ exchange in the absence of K^+ (data not shown). This behavior is paralleled by a high level of Na^+-ATPase activity in the absence of K^+ (17). As in the case of mutant N776A, the apparent rate constants obtained from pre-steady-state currents under Na^+/Na^+ exchange conditions are accelerated for the E779A construct, which could be due to the significant contribution of Na^+-stimulated dephosphorylation to the observed relaxation kinetics. The voltage dependence of the apparent rate constants is weaker than for wild type. This suggests a decreased affinity for extracellular Na^+ and agrees with the somewhat weaker voltage dependence of the stationary currents. From the $[K^+]$ dependence of Na^+/K^+ pump currents, we found a decreased affinity for $[K^+]$, in agreement with previous data (17, 19, 20). The observation of voltage-dependent *I*-V curves agrees with the findings of Zillikens *et al.* (20), who also studied the E779A mutant of the rat Na,K-ATPase. However, Argüello *et al.* (17), observed voltage-independent stationary currents in the presence of extracellular Na^+ for the sheep orthologue. The reasons for these discrepancies are unclear, but they may reflect species differences.

Upon dislocation of the carboxyl group in the E779D mutant, we observed a drastic decrease in affinity for the K^+ stimulation of stationary currents, whereas the voltage dependence of these currents was comparable with wild type. This agrees with the results of Nielsen *et al.* (47), who observed that high affinity occlusion of Rb^+ or Tl^+ was abolished. Stationary currents mediated by that construct were only ~30% of wild type, which could explain why an ouabain-insensitive E779D construct could not confer ouabain resistance to HeLa cells (54, 55). Rate constants of pre-steady-state currents under Na^+/Na^+ exchange conditions were ~300 s^{-1}, even higher than for the E779A mutant. This construct, however, did not possess an increased level of Na^+/Na^+ exchange currents.

Upon removal of the negative charge of Glu^{779} (E779Q mutant) the overall pump current properties were very similar to those of the wild-type enzyme. The $K_{0.5}$ of $[K^+]$-dependent stimulation of pump currents was comparable with that of the wild type, and a somewhat weaker increase of apparent rate constants of pre-steady-state currents at negative potentials was found. Peluffo *et al.* (19) also observed an unchanged $K_{0.5}$ for $[K^+]$. In summary, changes in side chain length at position 779 lead to pronounced changes in electrogenic cation transport properties, whereas removal of the negative charge has only mild effects.

Asp^{804} Mutants—The expression level at the plasma membrane of D804A and D804E was reduced compared with that of D804N and the wild-type enzyme. The importance of the Asp^{804} residue for proper function of the Na,K-ATPase is underlined by the fact that mutants D804A and D804N yielded only very small currents. Previously, it was demonstrated that the amino acids Asp^{804} (and also Asp^{808}) are critical for enzyme catalytic function and cation binding (45, 46, 48). Van Huysse *et al.* (46) reported that none of the mutations D804E, D804N, D804L, and D804A (when inserted into an ouabain-resistant enzyme) could confer ouabain resistance to HeLa cells upon transfection. Since the expression level at the plasma membrane of D804E was reduced and the current produced by the D804E mutant still was 25% of the wild-type level, a carboxyl group at that position seems to be required for Na^+/K^+ pumping. However, the *I*-V curves of K^+-stimulated stationary currents for D804E were severely different from wild type, since they exhibit a negative slope for all K^+ concentrations tested. This is interpreted as a reduced affinity for extracellular Na^+, which is supported by the nearly absent voltage dependence of the apparent rate constants from pre-steady-state currents under Na^+/Na^+ exchange conditions. Our findings support earlier findings of Kuntzweiler *et al.* (45), who found an elevated $K_{0.5}$ value for the antagonistic effect of Na^+ on ouabain binding. In the D804E mutant, high affinity Rb^+ and Tl^+ occlusion was abolished (47), which is in agreement with earlier studies indicating an essential role of Asp^{804} in K^+ binding (44, 45). However, we could only detect mild effects of the D804E mutation on the apparent $K_{0.5}$ of K^+-stimulated stationary cur-

rents, although the negative slope of the *I*-V curves indicates that K^+ transport into the cell is rate-limiting under all conditions. It was observed that mutants D804A and D804N, which did not produce pump currents in our experiments, both possessed ATPase activity. Whereas mutant D804N showed only ~30% of wild-type ATPase activity (44), the activity of the D804A mutant was comparable with the wild-type enzyme, although the apparent affinity for ATP was increased. Both mutants possessed a Na^+-ATPase activity that was only slightly lower than the activity measured in the presence of Na^+ and K^+ (44). Together with these findings concerning ATPase activity, the absence of K^+-stimulated stationary currents in mutants could also be interpreted in terms of an essentially functional enzyme that has lost its net electrogenicity.

Asp^{808} Mutants—The expression level in the total and plasma membrane fraction of all three Asp^{808} mutants was comparable with that of the wild-type enzyme. According to stationary current measurements, mutation D808E reduces and mutations D808N and D808A nearly completely abolish Na^+/K^+ pumping, underlining the role of the carboxyl group at that position. Mutants D808E and D808N showed at all membrane potentials an increased $K_{0.5}$ for the K^+ stimulation of pump currents. This is paralleled by strong alterations in kinetics and voltage dependence of electrogenic Na^+ transport steps. Whereas the apparent rate constants from pre-steady-state currents under Na^+/Na^+ exchange conditions of the D808N construct were greatly enhanced compared with wild type, those of the D808E mutant showed an inverted voltage dependence. In terms of the pseudo-two-state model for electrogenic cation transport described above, this behavior could indicate that extracellular release of Na^+ is no longer rate-limited by a preceding electroneutral reaction step (usually the $E_1P(Na^+)_3 \rightarrow E_2P(Na^+)_3$ transition). Asp^{808} has been implicated in coordinating K^+ during transport (45, 46, 48). In the report by Van Huysse *et al.* (46), only the D808E mutant could confer ouabain resistance to transfected HeLa cells. This mutant was found to have a reduced turnover and Na^+ affinity of Na,K-ATPase activity; however, K^+ affinity of Na,K-ATPase activity was unchanged. In agreement with the increased $K_{0.5}$ for K^+-stimulated stationary currents determined in the present study, Kuntzweiler and colleagues reported a decreased affinity for the antagonistic effect of K^+ on ouabain binding to the D808E mutant enzyme (45).

Structural Implications

In this study, we investigated the consequences of mutations at amino acids Asn^{776}, Glu^{779}, Asp^{804}, and Asp^{808} on the electrophysiological behavior of the Na,K-ATPase. The important role of these amino acids in electrogenic cation transport is confirmed. The presence of a negative charge on amino acids Asp^{804} and Asp^{808} is essential for electrogenic cation transport as electrogenic pump activity is abolished or greatly reduced upon removal of the carboxyl group. Analysis of mutations at Glu^{779} revealed that the side chain length is more important than the charge for the preservation of pump properties, since currents of the E779Q mutant were very similar to wild type, but E779D and E779A reduced K^+ affinity. Mutations at Asn^{776} produce complex effects. Asn^{776} points toward the cations, as does Asp^{804}. Remarkably, mutants D804E and N776Q behaved very similarly, exhibiting current-voltage curves with negative slope. Dislocating the carboxamide group of Asn^{776} in the N776Q mutant decreases the affinity for extracellular Na^+ in a similar fashion as found upon dislocation of the carboxyl group of Asp^{804} in D804E. Interference with cation binding possibly originates from the longer side chains of these mutants, since they would protrude deeper into the cation-binding pocket. Complete removal of the carboxamide group in the N776A mutant had no measurable effect on the voltage sensitivity of the Na,K-ATPase, although it accelerated the $E_1P(Na^+)_3 \leftrightarrow E_2P + 3Na^+$ relaxation drastically. Replacement of the carboxamide group by a carboxyl group without changing side chain length in N776D reduces the voltage sensitivity of the current. It is concluded that substitution of Glu^{779} and Asp^{808} results in a reduced apparent affinity for K^+ and that substitution of Asn^{776} and Asp^{804} results in stationary *I*-V curves with negative slope and a concomitant decrease in extracellular Na^+ affinity. The drastic change in voltage dependence of the Na,K-pump current after mutagenesis of Asn^{776} and Asp^{804} demonstrates that these polar residues in the membrane-spanning region M5-M6 of the protein essentially control the electrogenic properties of the Na,K-ATPase, whereas Glu^{779} and Asp^{808} have an effect on the apparent K^+ affinity. This study shows, that the combined analysis of stationary and transient currents mediated by Na,K-ATPase mutants yields valuable information about the impact of cation coordinating residues on cation affinities, voltage dependence and electrogenicity of transport.

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