Hyperpolarization-activated inward leakage currents caused by deletion or mutation of carboxy-terminal tyrosines of the Na^+/K^+-ATPase α subunit

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The Na^+/K^+-ATPase mediates electrogenic transport by exporting three Na^+ ions in exchange for two K^+ ions across the cell membrane per adenosine triphosphate molecule. The location of two Rb^+ ions in the crystal structures of the Na^+/K^+-ATPase has defined two "common" cation binding sites, I and II, which accommodate Na^+ or K^+ ions during transport. The configuration of site III is still unknown, but the crystal structure has suggested a critical role of the carboxy-terminal KETYY motif for the formation of this "unique" Na^+ binding site. Our two-electrode voltage clamp experiments on *Xenopus* oocytes show that deletion of two tyrosines at the carboxy terminus of the human Na^+/K^+-ATPase α_2 subunit decreases the affinity for extracellular and intracellular Na^+, in agreement with previous biochemical studies. Apparently, the ΔYY deletion changes Na^+ affinity at site III but leaves the common sites unaffected, whereas the more extensive ΔKETYY deletion affects the unique site and the common sites as well. In the absence of extracellular K^+, the ΔYY construct mediated ouabain-sensitive, hyperpolarization-activated inward currents, which were Na^+ dependent and increased with acidification. Furthermore, the voltage dependence of rate constants from transient currents under Na^+/Na^+ exchange conditions was reversed, and the amounts of charge transported upon voltage pulses from a certain holding potential to hyperpolarizing potentials and back were unequal. These findings are incompatible with a reversible and exclusively extracellular Na^+ release/binding mechanism. In analogy to the mechanism proposed for the H^+ leak currents of the wild-type Na^+/K^+-ATPase, we suggest that the ΔYY deletion lowers the energy barrier for the intracellular Na^+ occlusion reaction, thus destabilizing the Na^+-occluded state and enabling inward leak currents. The leakage currents are prevented by aromatic amino acids at the carboxy terminus. Thus, the carboxy terminus of the Na^+/K^+-ATPase α subunit represents a structural and functional relay between Na^+ binding site III and the intracellular cation occlusion gate.

INTRODUCTION

The Na^+/K^+-ATPase is an electrogenic ion pump, which exports three Na^+ ions and imports two K^+ ions at the expense of one ATP molecule. The reaction cycle of the Na^+/K^+-ATPase is commonly expressed as a sequence of reversible partial reactions known as the Post-Albers scheme (Fig. 1 A) (Albers, 1967; Post et al., 1972). The sequential translocation of Na^+ and K^+ ions requires strict cation specificity of the phosphorylation and dephosphorylation reactions, and the changes in the apparent affinities for the individual cation species are accompanied by alternating exposure of ion binding sites toward the intracellular and extracellular medium.

Electrophysiological experiments and relaxation studies have supported the notion that the major electrogenic event during the Na^+/K^+-ATPase's reaction cycle occurs during Na^+ transport (Fendler et al., 1985; Gadsby et al., 1985; Nakao and Gadsby, 1986; Gadsby and Nakao, 1989; Rakowski et al., 1991; Wuddel and Apell, 1995; Clarke and Kane, 2007). It has been suggested that electrogenicity arises from the passage of Na^+ ions through a narrow, high field "access channel" to the extracellular space (Läuger, 1979; Gadsby et al., 1993; Hilgemann, 1994; Sagar and Rakowski, 1994; Rakowski et al., 1997; Holmgren et al., 2000). The existence of a negative slope in the stationary current–voltage curve suggested that K^+ ions also bind within an extracellular ion well of smaller fractional depth (Rakowski et al., 1991). Structural evidence for the existence of an extracellular access channel in P-type ion pumps is missing so far, and from the viewpoint of rate theory, the movement of energy barriers along the transmembrane field would be equally sufficient to explain the experimental observations (Läuger and Apell, 1988). Nevertheless, the access channel metaphor in combination with the term "fractional depth" (or "equivalent charge") is frequently used to denote that an ion passes a certain fraction of the transmembrane electric field to reach or exit from its binding site.

According to Holmgren et al. (2000), the extracellular release of Na^+ ions occurs in three distinct steps,

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Abbreviations used in this paper: ENaC, epithelial Na^+ channel; TEVC, two-electrode voltage clamp; WT, wild-type.

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from which only deocclusion and release of the first Na^+ ion is associated with a large charge movement. This highly electrogenic release of the first Na^+ ion is most probably rate limited by the major E_1P-E_2P conformational change. After release of the first Na^+ ion, the high field access path to the other Na^+ occlusion sites is restructured such that the exit of the remaining two Na^+ ions contributes only little to the overall electrogenicity (Wuddel and Apell, 1995; Holmgren et al., 2000). In terms of the access channel model, positive voltage pulses drive Na^+ ions extracellularly out of the access channel and result in positive transient currents. Conversely, negative voltage pulses promote the movement of Na^+ ions through the extracellular access channel to enhance binding to sites in E_2P and induce negative transient currents. Due to the electrogenicity of reverse binding of extracellular Na^+, the occupancy of the Na^+ binding sites is controlled by $[Na^+]_{ext}$ and voltage. The amount of charge moved in response to certain voltage steps follows a Boltzmann-type function, which is centered at a half-maximal voltage ($V_{0.5}$), at which 50% of the pump molecules are in E_2P and 50% are in E_1P (Holmgren et al., 2000). Thus, changes in $V_{0.5}$ at a given $[Na^+]_{ext}$ indicate changes in the affinity for Na^+_{ext} (Holmgren et al., 2000; Holmgren and Rakowski, 2006).

Since 2000, the understanding of P-type ATPase function has been advanced by several crystal structures of the Ca^{2+}-ATPase from sarcoplasmic/endoplasmic reticulum (Toyoshima et al., 2000; Toyoshima and Nomura, 2002; Olesen et al., 2004, 2007; Sørensen et al., 2004; Toyoshima and Mizutani, 2004; Jensen et al., 2006), which cover the reaction cycle rather completely. The wealth of information has recently been expanded by the structures of an H^+-ATPase from plants (Pedersen et al., 2007) and the Na^+/K^+-ATPase (Morth et al., 2007; Shinoda et al., 2009).

The K^+ binding sites I and II of the Na^+/K^+-ATPase are formed by residues homologous to those coordinating the two Ca^{2+} ions in the sarcoplasmic/endoplasmic reticulum pump's E_1 conformation (Morth et al., 2007; Shinoda et al., 2009). Because this pocket most likely accommodates two Na^+ ions in the E_1 form of the Na^+ pump as well, sites I and II will be referred to as "common" sites in this study. The third binding site, which exclusively coordinates Na^+ ions and is therefore termed "unique," has been located between transmembrane domains αM5, αM6, and αM9 according to structural modeling (Ogawa and Toyoshima, 2002) and mutagenesis work (Van Huysse et al., 1993; Li et al., 2005, 2006). The unique site has been correlated to a noncanonic transport mode (Li et al., 2006), which only occurs in the absence of extracellular Na^+ and K^+ (Rakowski et al., 1991; Efthymiadis et al., 1993; Wang and Horisberger, 1995; Rettinger, 1996) and is characterized by hyperpolarization-activated, mainly H^+-driven inward currents (Vasilyev et al., 2004).

It has been suggested from the crystal structure (Morth et al., 2007) that the conserved (K/R)E(T/S)YY motif at the carboxy-terminal end of the Na^+/K^+-ATPase's α subunit is important for the formation of the unique Na^+ binding site (see Fig. 1 B). In accordance, mutations or deletions of one or both carboxy-terminal tyrosines, but also larger deletions or C-terminal extensions, decrease the affinity for extracellular as well as intracellular Na^+ (Morth et al., 2007; Tavraz et al., 2008; Blanco-Arias et al., 2009; Toustrup-Jensen et al., 2009). Changes in Na^+ affinity have also been reported in a recent electrophysiological study of a ΔKESYY-truncated *Xenopus* α_1 isoform (Yaragatupalli et al., 2009). Remarkably, these affinity changes were assigned to the effects of the ΔKESYY deletion on the two common binding sites rather than on the proximal unique site. Furthermore, the deletion led to hyperpolarization-induced inward currents, which were attributed to low affinity passive flow of Na^+ ions through the ΔKESYY-deleted pump (Yaragatupalli et al., 2009). Here, we specifically investigate the consequences of deletion or mutation of only the two carboxy-terminal tyrosines on Na^+/K^+-ATPase pump function. By analyzing stationary and presteady-state currents in two-electrode voltage clamp (TEVC) experiments on *Xenopus* oocytes, we show that the less extensive ΔYY deletion dominantly affects the apparent Na^+ affinity at the unique binding site. Furthermore, we characterize the hyperpolarization-activated inward leakage currents in more detail to provide a framework for understanding this potentially energy-dissipating operation mode of carboxy-terminally truncated Na^+ pumps.

MATERIALS AND METHODS

cDNA constructs

Human Na^+/K^+-ATPase α_2 and β_1 subunit cDNAs were subcloned into a modified pCDNA3.1 vector, which had been optimized for expression in *Xenopus* oocytes (Koenderink et al., 2005). For mutagenesis, the QuikChange site-directed mutagenesis kit (Agilent Technologies) was used. All PCR-derived fragments were verified by sequencing (Eurofins MWG Operon). Mutations Q116R and N127D were introduced to obtain an ouabain-resistant protein with an IC_{50} in the millimolar range (Price and Lingrel, 1988), which is referred to as ATP1A2 WT in this paper. Amino acid numbering refers to the human Na^+/K^+-ATPase α_2 subunit.

cRNA synthesis and oocyte treatment

cRNA synthesis was performed with the T7 mMessage mMachine kit (Applied Biosystems). Oocytes were obtained by partial ovariectomy from anesthetized *Xenopus laevis* females, followed by collagenase 1A treatment (Sigma-Aldrich). Oocytes were injected with 25 ng α_2 subunit and 2.5 ng β_1 subunit cRNA and stored in ORI buffer (contents in mM: 110 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, and 5 HEPES, pH 7.4) containing 50 mg/L gentamycin at 18°C for 3–4 d. In preceding experiments, $[Na^+]_{int}$ was elevated by incubating the oocytes for 45 min in Na^+ loading solution (contents in mM: 110 NaCl, 2.5 Na-citrate, 2.5 3-(*N*-Morpholino)-propanesulfonic acid [MOPS], and 2.5 Tris, pH 7.4), followed by an incubation

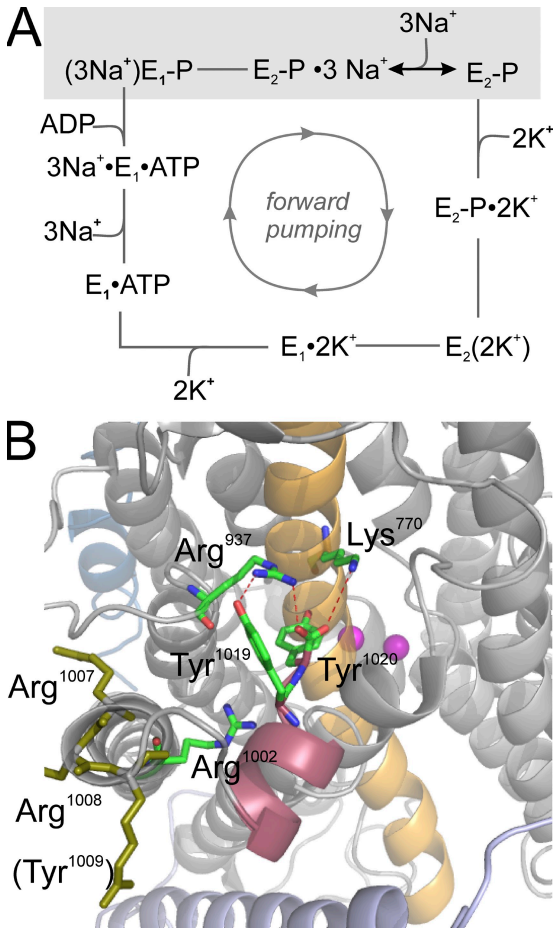

Figure 1. Reaction scheme and structural detail of the Na^+/K^+-ATPase. (A) Modified Post-Albers reaction cycle of the Na^+/K^+-ATPase. Upon intracellular binding of Na^+ ions to the E_1 conformation, a phosphointermediate with occluded Na^+ ions, $E_1P(3Na^+)$, is formed, and after a conformational change to $E_2P(3Na^+)$, the Na^+ ions dissociate to the extracellular space. Subsequently, two K^+ ions bind from the extracellular side and become occluded, a process that stimulates dephosphorylation, and after a conformational change from E_2 to E_1, the K^+ ions are intracellularly released. The gray box indicates the reaction sequence that can be studied by voltage pulses at high $[Na^+]_{ext}$ and $[K^+]_{ext} = 0$ in TEVC experiments. (B) Structure of the Na^+/K^+-ATPase according to PDB structure entry 3B8E (Morth et al., 2007). Amino acids referred to in this work are indicated in ball-and-stick representation with numbering according to the human Na^+/K^+-ATPase α_2 subunit. Helix M5 is depicted in yellow, the backbone of the carboxy terminus (V^{1014}-EKETY-Y^{1020}) is in red, and residues of a carboxy-terminal arginine cluster are in olive. Two Rb^+ ions at the binding sites are shown as magenta spheres. Note that Arg^{1005} in the 3B8E structure (pig renal α_1 subunit) corresponds to Tyr^{1009} in the human Na^+/K^+-ATPase α_2 subunit.

of at least 30 min in Na^+ buffer (contents in mM: 100 NaCl, 1 $CaCl_2$, 5 $BaCl_2$, 5 $NiCl_2$, 2.5 MOPS, and 2.5 Tris, pH 7.4), as described previously (Rakowski et al., 1991).

Electrophysiology

Currents were recorded using the TEVC technique with an amplifier (Turbotec 10CX; npi electronic GmbH) and PClamp 7 software (MDS Analytical Technologies) at 21–23°C. Experimental solutions were (contents in mM): Na^+ buffer (see above), $NMDG^+$ buffer (100 NMDG × Cl, 1 $CaCl_2$, 5 $BaCl_2$, 5 $NiCl_2$, 2.5 MOPS, and 2.5 Tris, pH 7.4), and Li^+ buffer (100 LiCl, 1 $CaCl_2$, 5 $BaCl_2$, 5 $NiCl_2$, 2.5 MOPS, and 2.5 Tris, pH 7.4). All buffers contained 10 µM ouabain to inhibit the endogenous *Xenopus* Na^+/K^+-ATPase. The heterologously expressed Na^+/K^+-ATPase could be inhibited by 10 mM ouabain. To determine the voltage dependence of the currents, cells were subjected to voltage pulse protocols as follows: starting from −30-mV holding potential, cells were clamped for 200 ms to test potentials between +60 and −140 mV (in 20-mV decrements), followed by a step back to −30 mV. Unless stated differently, all currents in one experiment were normalized to the amplitude at $[Na^+]_{ext} = 100$ mM and $[K^+]_{ext} = 10$ mM and 0 mV.

K^+-induced pump currents

Solutions containing distinct K^+ concentrations were prepared by adding the appropriate amounts of KCl to Na^+ buffer, $NMDG^+$ buffer, or Li^+ buffer. The $[K^+]_{ext}$-dependent currents were calculated by subtracting currents measured in K^+-free Na^+, $NMDG^+$, or Li^+ buffer from the currents measured in the presence of a distinct K^+ concentration (added to Na^+, $NMDG^+$, or Li^+ buffer). These K^+-induced difference currents are denoted as ${}^{Na}I_{xK}$, ${}^{NMDG}I_{xK}$, and ${}^{Li}I_{xK}$, respectively, whereby the top left index symbolizes the major monovalent cation present (concentration: 100 mM) and the bottom right index "xK" denotes $[K^+]_{ext}$ (with [x] in millimolars). Alternatively, Na^+/K^+-ATPase pump currents were determined as ouabain-sensitive currents by calculating the difference between currents measured in Na^+, $NMDG^+$, or Li^+ buffer containing a certain K^+ concentration and in Na^+, $NMDG^+$, or Li^+ buffer containing 10 mM ouabain. These ouabain-sensitive, K^+-induced difference currents are denoted as ${}^{Na}I_{xK(ouab)}$, ${}^{NMDG}I_{xK(ouab)}$, and ${}^{Li}I_{xK(ouab)}$, respectively. $K_{0.5}$ values for the stimulation of stationary pump currents by extracellular K^+ were determined using fits of a Hill equation

$$I = \frac{I_{max}}{1 + \left(\frac{K_{0.5}}{[K^+]}\right)^{n_H}}$$

to the normalized currents at a given membrane potential ($K_{0.5}$ is the half-maximal concentration and n_H the Hill coefficient). Hill parameters from the fits were between 1 and 1.5.

Extracellular $[Na^+]$ dependence of ouabain-sensitive currents in the absence of K^+_{ext}

Experiments to determine the dependence on $[Na^+]_{ext}$ used Na^+[150] buffer (contents in mM: 150 NaCl, 1 $CaCl_2$, 5 $BaCl_2$, 5 $NiCl_2$, 2.5 MOPS, and 2.5 Tris, pH 7.4), Na^+[100] buffer (Na^+ buffer), Na^+[50] buffer (1:1 mixture of Na^+ buffer with $NMDG^+$ buffer), and Na^+[0] buffer ($NMDG^+$ buffer). Currents were determined by calculating the difference between currents measured in one of these buffers in the absence and presence of 10 mM ouabain.

pH dependence of ouabain-sensitive inward currents in the absence of K^+_{ext}

Na^+/K^+-ATPase currents at different pH_{ext} were measured in $Na^+_{8.1}$ buffer (contents in mM: 100 NaCl, 1 $CaCl_2$, 5 $BaCl_2$, 5 $NiCl_2$, and 5 Tris, pH 8.1), $Na^+_{5.5}$ buffer (contents in mM: 100 NaCl, 1 $CaCl_2$, 5 $BaCl_2$, 5 $NiCl_2$, and 5 MES, pH 5.5), or $NMDG^+_{5.5}$ buffer (contents in mM: 100 NMDG × Cl, 1 $CaCl_2$, 5 $BaCl_2$, 5 $NiCl_2$, and 5 MES, pH 5.5) by calculating the difference between currents measured in one of these buffers in the absence and presence of 10 mM ouabain.

Analysis of ouabain-sensitive presteady-state currents

Transient currents were measured at $[Na^+]_{ext} = 100$ mM by the application of voltage step protocols and calculating the difference between corresponding current traces, which had been measured

first in Na^+ buffer, and then in Na^+ buffer containing 10 mM ouabain. The resulting difference currents were fitted by a single-exponential function (plus a constant), whereby the first 3–5 ms were disregarded to exclude artifacts arising from the charging of the membrane capacitance. This yielded the amplitude A for the start time of the fit and a decay time constant τ. The charge Q transported during a transient current was determined as the integral of fitted currents, $A^*\cdot\tau$, in which A^* was obtained by extrapolating the amplitude of the exponential fit curve to the onset of the voltage pulse. The resulting Q-V curves were fitted to a Boltzmann function:

$$Q(V) = Q_{min} + \frac{Q_{max} - Q_{min}}{1 + \exp\left(\frac{z_q \cdot F(V - V_{0.5})}{RT}\right)},$$

where Q_{max} and Q_{min} are the saturation values of $Q(V)$ at extremely positive or negative voltages, $V_{0.5}$ is the midpoint potential, z_q is the slope factor (equivalent charge), F is the Faraday constant, R is the molar gas constant, T is the absolute temperature, and V is the transmembrane potential.

Intracellular Na^+ concentration achieved by the Na^+ loading procedure

To control the efficiency of the applied Na^+ loading procedure, oocytes were injected with RNA mixtures containing cRNAs of the α_2 subunit (25 ng/oocyte) and the β_1 subunit (2.5 ng/oocyte) of the Na^+/K^+-ATPase, and cRNAs of the α, β, and γ subunits (0.3 ng/subunit/oocyte) of the amiloride-sensitive rat renal epithelial Na^+ channel (ENaC), as described previously (Hasler et al., 1998; Crambert et al., 2000). After injection, oocytes were kept for 3 d in ORI buffer supplemented with 10 µM amiloride, and Na^+ loading was performed as described above with 10 µM amiloride in the loading/post-loading buffers. The reversal potential of the amiloride-sensitive ENaC current was measured in the presence of extracellular buffers containing 10 mM Na^+, and $[Na^+]_{int}$ was calculated using the Nernst equation:

$$\left[Na^+\right]_{int} = \left[Na^+\right]_{ext} \exp\left(\frac{V_{rev,amil} \cdot F}{RT}\right),$$

where $V_{rev,amil}$ is the reversal potential of amiloride-sensitive difference currents.

Variation of intracellular $[Na^+]$ using Na^+ uptake through the ENaC channel

These experiments used oocytes that simultaneously expressed Na^+/K^+-ATPase and ENaC, as described above. After cRNA injection, cells were kept in a Na^+-reduced ORI buffer (contents in mM: 100 NMDG × Cl, 10 mM NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 2.5 MOPS, and 2.5 Tris, pH 7.4) for 2–3 d. 18–24 h before experiments, oocytes were incubated in a Na^+-free solution (contents in mM: 110 NMDG × Cl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 2.5 MOPS, and 2.5 Tris, pH 7.4) to minimize the initial $[Na^+]_{int}$. To successively increase $[Na^+]_{int}$, oocytes were exposed to buffers containing 10 mM or (for the final increases of $[Na^+]_{int}$) 100 mM $[Na^+]_{ext}$ at −30 or −100 mV. Between these Na^+ loading steps, sets of measurements were performed in which (1) the $[Na^+]_{int}$, (2) the Na^+/K^+ pump current in response to 10 mM K^+, and (3) the ouabain-sensitive inward currents at 100 mM $[Na^+]_{ext}$ and 0 $[K^+]_{ext}$ were determined. $[Na^+]_{int}$ was determined from the reversal potentials of amiloride-sensitive difference currents measured in buffers containing 10 or 100 mM $[Na^+]_{ext}$.

Structural presentations and data analysis

Structural inspections of the Na^+/K^+-ATPase (PDB structure entry 3B8E) were performed with Swiss PDB viewer 3.7. Figures were prepared with PyMOL 1.0r1 (http://www.pymol.org). Data analysis for figure presentation was performed with Origin 7.5 (OriginLab Corporation).

Online supplemental material

Fig. S1 shows an alternative way to determine the ouabain-sensitive K^+-induced difference currents and several I-V curves of the ΔYY deletion construct at pH_{ext} 8.1. In Fig. S2, the effects of extracellular Li^+ on ouabain-sensitive currents of the ΔYY construct and ATP1A2 WT are shown. Fig. S3 depicts the voltage dependence of the ouabain-sensitive stationary and transient currents mediated by the ΔYY construct over an expanded potential range. Fig. S4 contains information about the voltage dependence of K^+-induced pump currents and transient currents of the ΔKETYY deletion construct. Figs. S1–S4 are available at http://www.jgp.org/cgi/content/full/jgp.200910301/DC1.

RESULTS

Dependence of pump-related currents on $[K^+]_{ext}$, $[Na^+]_{ext}$, and membrane potential

Upon expression in *Xenopus laevis* oocytes, TEVC experiments were performed in Na^+-containing buffers ($[Na^+]_{ext}$ = 100 mM) to measure the K^+_{ext}-induced ouabain-sensitive pump currents of wild-type (WT) human α_2/β_1 Na^+/K^+-ATPase and of the ΔYY construct, in which the two tyrosines at the carboxy terminus of the α subunit were deleted. Fig. 2 A shows I-V curves of the $^{Na}I_{xK(ouab)}$ difference currents for the ΔYY-truncated Na^+/K^+-ATPase α_2 subunit. In contrast to ATP1A2 WT, the ΔYY construct mediated inwardly rectifying difference currents in the absence of extracellular K^+. The amplitudes of these inward currents became smaller with increasing $[K^+]_{ext}$ but were still observed for $[K^+]_{ext}$ up to 2 mM at extremely negative potentials (−140 mV). At 10 mM K^+, the ouabain-sensitive pump currents of the ΔYY construct were positive and increased steadily with voltage (Fig. 2 A). We then determined the full K^+-induced $^{Na}I_{xK}$ difference currents of the ΔYY construct at $[Na^+]_{ext}$ = 100 mM by calculating the difference between currents recorded in the presence of a certain $[K^+]_{ext}$ and those measured at $[K^+]_{ext}$ = 0 (Fig. 2 B). The same information can be obtained by subtracting the $^{Na}I_{0K(ouab)}$ currents at 0 $[K^+]_{ext}$ from the $^{Na}I_{xK(ouab)}$ currents (Fig. S1 A). Because in the case of the ΔYY construct extracellular K^+ not only induces normal Na^+/K^+ outward pumping (as evident from the similarity to WT pump currents at positive potentials), but also diminishes the hyperpolarization-activated inward currents, the full K^+-induced $^{Na}I_{xK}$ currents (Fig. 2 B) were markedly different from those of the WT Na^+/K^+-ATPase (Fig. 2 D). For the WT enzyme, currents at $[K^+]_{ext}$ = 10 mM increased steadily with voltage, and at $[K^+]_{ext}$ below 5 mM, bell-shaped I-V curves were obtained with a maximum at +40 mV (Fig. 2 D). In contrast, the $^{Na}I_{xK}$ currents of the ΔYY construct at 10 and 5 mM $[K^+]_{ext}$ had a local minimum around −40 mV, and at lower $[K^+]_{ext}$, the I-V curves were maximal around −100 mV and

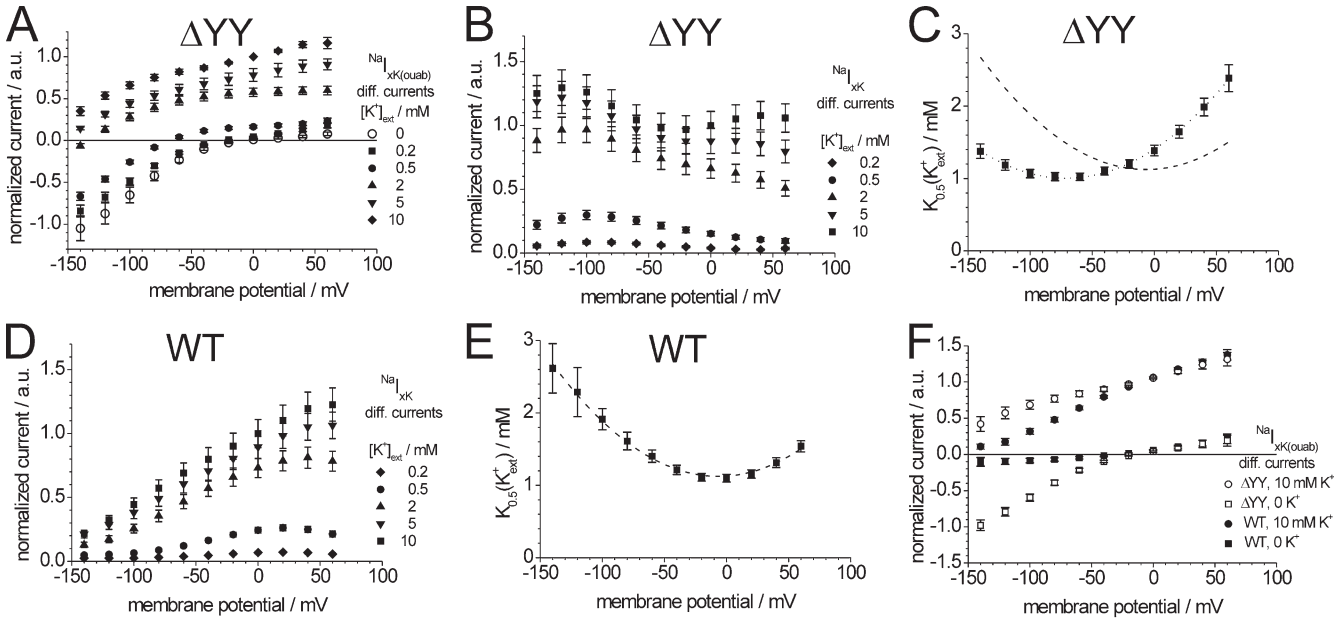

Figure 2. Voltage and $[K^+]_{ext}$ dependence of stationary currents at $[Na^+]_{ext}$ = 100 mM. (A) I-V curves of normalized ouabain-sensitive K^+-dependent currents $^{Na}I_{xK(ouab)}$ of the Na^+/K^+-ATPase ΔYY deletion construct measured at 100 mM $[Na^+]_{ext}$ and $[K^+]_{ext}$ as indicated. (B) I-V curves of normalized K^+-induced difference currents $^{Na}I_{xK}$ (at 100 mM $[Na^+]_{ext}$) of the ΔYY construct. (C) Voltage-dependent $^{Na}K_{0.5}(K^+_{ext})$ values of the ΔYY construct from fits of a Hill function to the $^{Na}I_{xK}$ currents in B at each membrane potential. The minimal $^{Na}K_{0.5}(K^+_{ext})$ was 1.02 ± 0.06 mM at −80 mV. The dashed line delineates the corresponding WT data from E for comparison. (D) I-V curves of normalized $^{Na}I_{xK}$ difference currents for ATP1A2 WT with $[K^+]_{ext}$ as indicated. (E) Voltage-dependent $^{Na}K_{0.5}(K^+_{ext})$ values for ATP1A2 WT from fits of a Hill function to the $^{Na}I_{xK}$ currents in D at each membrane potential. The minimal $^{Na}K_{0.5}(K^+_{ext})$ was 1.10 ± 0.05 mM at 0 mV. Data for both WT and ΔYY are means ± SE from 14 cells of three oocyte batches. (F) I-V curves of normalized $^{Na}I_{10K(ouab)}$ and $^{Na}I_{0K(ouab)}$ currents of ATP1A2 WT and the ΔYY construct at pH_{ext} 7.4 ($[Na^+]_{ext}$ = 100 mM). Data are means ± SE from six (WT) and seven (ΔYY) cells from two batches.

decreased steadily between −100 and +60 mV (Fig. 2 B). Fitting of the $[K^+]_{ext}$-induced $^{Na}I_{xK}$ currents at each membrane potential with a Hill equation resulted in $^{Na}K_{0.5}(K^+_{ext})$ values for the ΔYY construct (Fig. 2 C) and ATP1A2 WT (Fig. 2 E), which represent the voltage-dependent apparent affinities for extracellular K^+ in the presence of 100 mM Na^+_{ext}. Characteristically U-shaped curves were obtained for both enzymes with similar minima, but the curve of the ΔYY construct was shifted by about −80 mV.

Fig. 2 F shows normalized $^{Na}I_{10K(ouab)}$ and $^{Na}I_{0K(ouab)}$ difference currents of ATP1A2 WT and the ΔYY construct at pH_{ext} 7.4. As indicated above, the ΔYY construct generated large inwardly rectifying currents in the absence of K^+_{ext}, which were not observed with ATP1A2 WT. The direct comparison shows that the full K^+-induced ouabain-sensitive currents of the ΔYY construct at hyperpolarizing potentials are larger than the corresponding currents of the WT enzyme, resulting in a significantly smaller slope of the respective I-V curve.

To investigate which type of the cation binding site is affected by the ΔYY deletion, we measured the $[K^+]_{ext}$-induced currents in Na^+-free solutions. Replacement of extracellular Na^+ for a nontransported cation eliminates the voltage-dependent competition of extracellular Na^+ with K^+ for the binding sites and allows the determination of the "intrinsic" apparent K^+_{ext} affinity (Li et al., 2005) at the two common sites, where K^+ ions bind from the extracellular space during transport. As shown in Fig. 3 A, the K^+-induced currents ($^{NMDG}I_{xK}$) of the ΔYY construct were positive for all $[K^+]_{ext}$ tested, showing that in the absence of Na^+_{ext}, the ΔYY construct only mediates Na^+/K^+ outward pumping, and that K^+ ions apparently do not contribute to hyperpolarization-induced inward currents of the ΔYY construct. The current amplitudes for $[K^+]_{ext} \geq 2$ mM hardly changed with voltage, and at lower $[K^+]_{ext}$, the I-V curves showed a shallow decrease upon increases in membrane potential, which can be attributed to the weakly electrogenic binding of extracellular K^+ (Rakowski et al., 1991). In K^+-free $NMDG^+$ buffer, the ouabain-sensitive currents of the ΔYY construct were very small (Fig. 3 A), and even smaller than the corresponding currents of ATP1A2 WT (Fig. 3 B), showing that Na^+_{ext} is required for the hyperpolarization-activated inward currents. The large $^{NMDG}I_{0K(ouab)}$ currents of WT ATP1A2 (in the absence of extracellular Na^+ and K^+) can be attributed to the hyperpolarization-activated H^+ inward currents, as described previously (Rakowski et al., 1991; Efthymiadis et al., 1993; Wang and Horisberger, 1995; Rettinger, 1996; Vasilyev et al., 2004). Therefore, the ΔYY deletion reduces this H^+-driven inward current at neutral pH, in

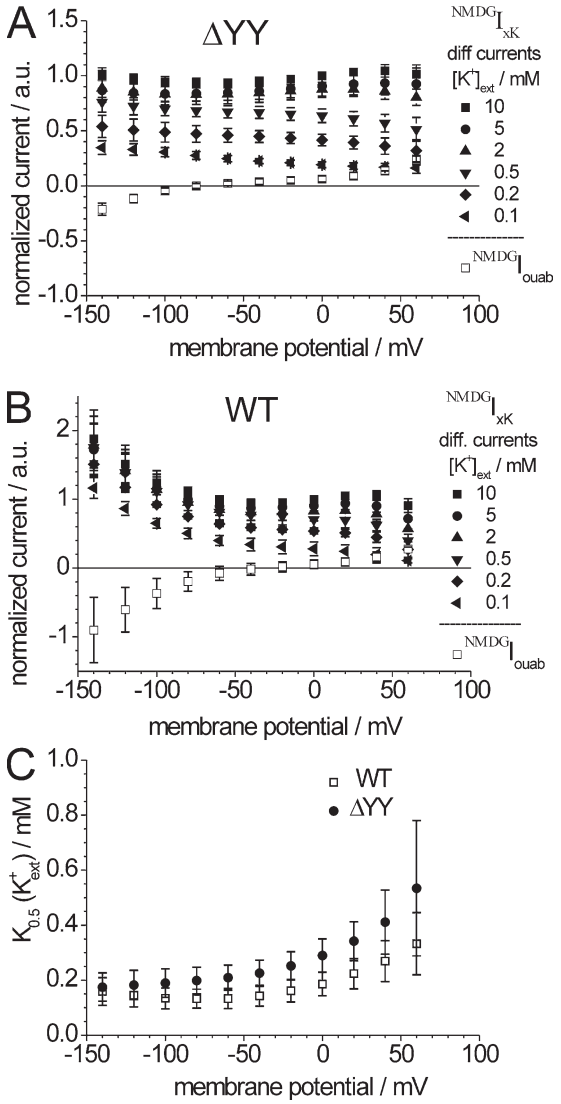

Figure 3. Voltage and $[K^+]_{ext}$ dependence of stationary currents in the absence of Na^+_{ext}. (A and B) I-V curves of normalized K^+-dependent $^{NMDG}I_{xK}$ difference currents of the ΔYY construct (A) and ATP1A2 WT (B) in the absence of extracellular Na^+ ($[NMDG^+]_{ext}$ = 100 mM, pH 7.4) with $[K^+]_{ext}$ as indicated. Also shown in A and B are the ouabain-sensitive currents ($^{NMDG}I_{ouab}$) in the absence of K^+ for the ΔYY construct and ATP1A2 WT. (C) $K_{0.5}(K^+_{ext})$ values for the ΔYY construct and for ATP1A2 WT at $[Na^+]_{ext}$ = 0. For each construct, data are means ± SD from eight oocytes out of two batches.

line with observations reported for the ΔKESYY-truncated pump (Yaragatupalli et al., 2009). In contrast to the ΔYY construct, which exhibited flat I-V curves, the K^+-induced difference currents of ATP1A2 WT increased at negative potentials for all $[K^+]_{ext}$ tested (Fig. 3 B). This distortion of the I-V curves is most likely due to the K^+-induced inhibition of the H^+ inward current, which occurs in conjunction with K^+_{ext}-induced outward pumping. However, when the ouabain-sensitive K^+-induced currents were calculated, the resulting I-V curves were only weakly voltage dependent and very similar to the corresponding currents of the ΔYY construct (not depicted). The $K_{0.5}(K^+_{ext})$ values determined from the $^{NMDG}I_{xK(ouab)}$ currents, which represent intrinsic apparent K^+ affinities, were much smaller than those measured at $[Na^+]_{ext}$ = 100 mM and did not increase at hyperpolarizing potentials (Fig. 3 C). The $K_{0.5}(K^+)$ values for the WT enzyme were slightly smaller than those of the ΔYY construct, but this difference was not significant (at $P > 0.05$ level). Thus, the intrinsic K^+_{ext} affinity at the common sites is apparently not affected by the ΔYY deletion, in line with findings obtained for the ΔKESYY construct (Yaragatupalli et al., 2009).

Voltage dependence and kinetics of ouabain-sensitive presteady-state currents in the absence of extracellular K^+

To specifically investigate electrogenic Na^+ transport, we performed voltage pulse experiments under extracellularly high Na^+ ($[Na^+]_{ext}$ = 100 mM) and K^+-free conditions to measure ouabain-sensitive transient currents. Fits of a single-exponential function to the transient currents in response to "ON" or "OFF" voltage pulses were used to determine time constants and the amount of charge (Q_{ON} or Q_{OFF} from ON or OFF transient currents) moved during a transient current. For ATP1A2 WT, the ouabain-sensitive ON and OFF transient currents follow a single-exponential function and decay almost completely to 0 (Fig. 4 A). The ON currents of ATP1A2 WT were substantially faster at negative than at positive potentials. Consequently, the reciprocal time constants increased with hyperpolarization (Fig. 4 C). Several properties of the ouabain-sensitive transient currents of the ΔYY construct (Fig. 4 B) were markedly different from the WT enzyme. First, the transient currents upon voltage pulses to negative potentials showed a large negative stationary current component (Fig. 4 B), in line with the hyperpolarization-activated inward currents described above. Second, the acceleration of transient current kinetics at negative potentials was not observed. The reciprocal time constants rather increased at positive potentials (Fig. 4 D). Third, the voltage dependence of charge moved during the transient currents was different, as apparent from the reduced amplitudes of the ON transient currents at positive potentials (or increased amplitudes at negative potentials) compared with ATP1A2 WT (Fig. 4, A and B). Accordingly, the $V_{0.5}$ values from Q-V curves of the ΔYY construct (Fig. 4 F) were shifted by about −83 mV compared with the WT enzyme (Fig. 4 E) without a significant change in z_q (Table I).

Whereas for the WT enzyme the Q_{ON} and Q_{OFF} charges were equivalent (not depicted), in the case of the ΔYY construct, the Q_{ON} values (Fig. 4 F, triangles) were significantly larger than Q_{OFF} (Fig. 4 F, squares) at hyperpolarizing potentials. The Q_{ON} and Q_{OFF} charges of the ΔYY construct saturated at positive potentials, but at negative potentials, due to the large $V_{0.5}$ shift, no onset

of saturation was apparent (Fig. 4 F). Only a subset of cells allowed for sufficiently stable current recordings to show that Q_{ON} and Q_{OFF} indeed saturate at potentials below −150 mV (Fig. S3 B). We exclude that the discrepancy between Q_{ON} and Q_{OFF} is a trivial consequence of the steady-state component of the transient currents because we applied the sum of an exponential function and a constant for fitting the transient currents, and used only the amplitude and time constant from the exponential component for calculation of the transient charge. We also do not consider the discrepancy as a voltage clamp artifact because the difference between corresponding Q_{ON} and Q_{OFF} values markedly increased at acidic pH_{ext} (see Fig. 5 E).

A direct comparison of several ON transient current signals of the WT enzyme (from Fig. 4 A) and the ΔYY construct (from Fig. 4 B) on an expanded time scale in Fig. 4 G also shows differences in the time course for the initial phase of the transient currents. Immediately after the voltage step (Fig. 4 G, dashed line), the artifacts from capacitive charging of the membrane are almost completely cancelled by the subtraction of current signals recorded in the presence of 10 mM ouabain. Fitting the sum of two exponential functions to the current traces yielded time constants for a fast rising phase (τ_1) and a slower decaying phase (τ_2). The time constant τ_2 corresponds to the kinetics of the (slow) release or deocclusion step of the first Na^+ ion released to the extracellular space, as described previously (Holmgren et al., 2000). τ_1 is of ∼1 ms and reflects the speed of the voltage clamp in TEVC experiments on oocytes. The peak of the ΔYY transient current at −140 mV occurred with a delay of ∼2 ms, and a significantly slower τ_1 of 3.3 ms was observed (Fig. 4 G). We exclude that the larger τ_1 is due to a slow voltage clamp because in the same experiment, the rising phase of the transient current at +60 mV was resolved to ∼1 ms.

Dependence of hyperpolarization-activated inward currents on extracellular and intracellular [Na^+]

To determine the Na^+ dependence of the hyperpolarization-activated inward currents of the ΔYY construct, experiments were first performed at different $[Na^+]_{ext}$ in K^+-free solutions. The resulting I-V curves in Fig. 4 H show that the amplitudes of the ouabain-sensitive inward currents increase with increasing $[Na^+]_{ext}$, and that saturation is achieved around 150 mM $[Na^+]_{ext}$. From the hyperbolic $[Na^+]_{ext}$ dependence, a half-maximal concentration of ∼70 mM was determined. For comparison, the ouabain-sensitive ATP1A2 WT currents at 100 mM $[Na^+]_{ext}$ were very small and did not show inward rectification (Fig. 4 H). In a subset of ΔYY-expressing cells that allowed for sufficiently stable recordings even at extremely negative potentials, it could be shown that the inward leak current amplitudes of the ΔYY construct at $[Na^+]_{ext}$ = 100 mM also saturate at potentials below −150 mV (Fig. S3 A).

Because the mutation or deletion of one or both carboxy-terminal tyrosines of the Na^+/K^+-ATPase α subunit strongly decreased Na^+ affinities (Morth et al., 2009; Toustrup-Jensen et al., 2009), we tested the applied Na^+ loading procedure to exclude effects resulting from insufficient saturation of Na^+ binding sites from the intracellular side. For this purpose, we coexpressed the ΔYY construct together with the amiloride-sensitive Na^+ channel ENaC and determined $[Na^+]_{int}$ from amiloride-sensitive reversal potential shifts. Before Na^+ loading, no ouabain-sensitive Na^+/K^+ outward pump currents were observed, indicating that the initial $[Na^+]_{int}$ was below the apparent K_M of 13 mM, as reported for the human α_2/β_1 Na^+/K^+-ATPase (Crambert et al., 2000). After Na^+ loading, an average $[Na^+]_{int}$ of 40 ± 4 mM was obtained. This value is ∼10-fold larger than the $K_{0.5}(Na^+)$ from Na^+-dependent phosphoenzyme formation of the ΔYY-deleted rat α_1 isoform (Toustrup-Jensen et al., 2009). When the ENaC channel was used to successively elevate $[Na^+]_{int}$, we found that the hyperpolarization-activated inward currents at 0 $[K^+]_{ext}$ saturated at a $[Na^+]_{int}$ of ∼25 mM (Fig. 4 I), with a half-maximal concentration between 10 and 15 mM, and the Na^+/K^+ outward pump currents induced by 10 mM K^+_{ext} also did not increase further at a $[Na^+]_{int}$ above 20 mM, with a half-maximal concentration between 8 and 12 mM (not depicted). Thus, for the ΔYY construct, the $[Na^+]_{int}$ achieved by the Na^+ loading procedure is not limiting for the phosphorylation reaction that leads to formation of the Na^+-occluded $E_1P(3Na^+)$ intermediate.

TABLE I

Parameters of Boltzmann fits to Q-V curves

	$V_{0.5}$	z_q	*n*
	(mV)		
WT	−0.5 ± 1.0	0.76 ± 0.02	14
ΔYY	−83.4 ± 7.9	0.81 ± 0.06	14
YY-FF	−27.1 ± 0.8	0.81 ± 0.02	13
YY-AA	−120 ± 18	0.44 ± 0.03	10
ΔKETYY	−135 ± 19	0.45 ± 0.02	8

From transient currents in response to OFF voltage pulses at $[Na^+]_{ext}$ = 100 mM and $[K^+]_{ext}$ = 0 mM. Means ± SE; *n* = number of cells.

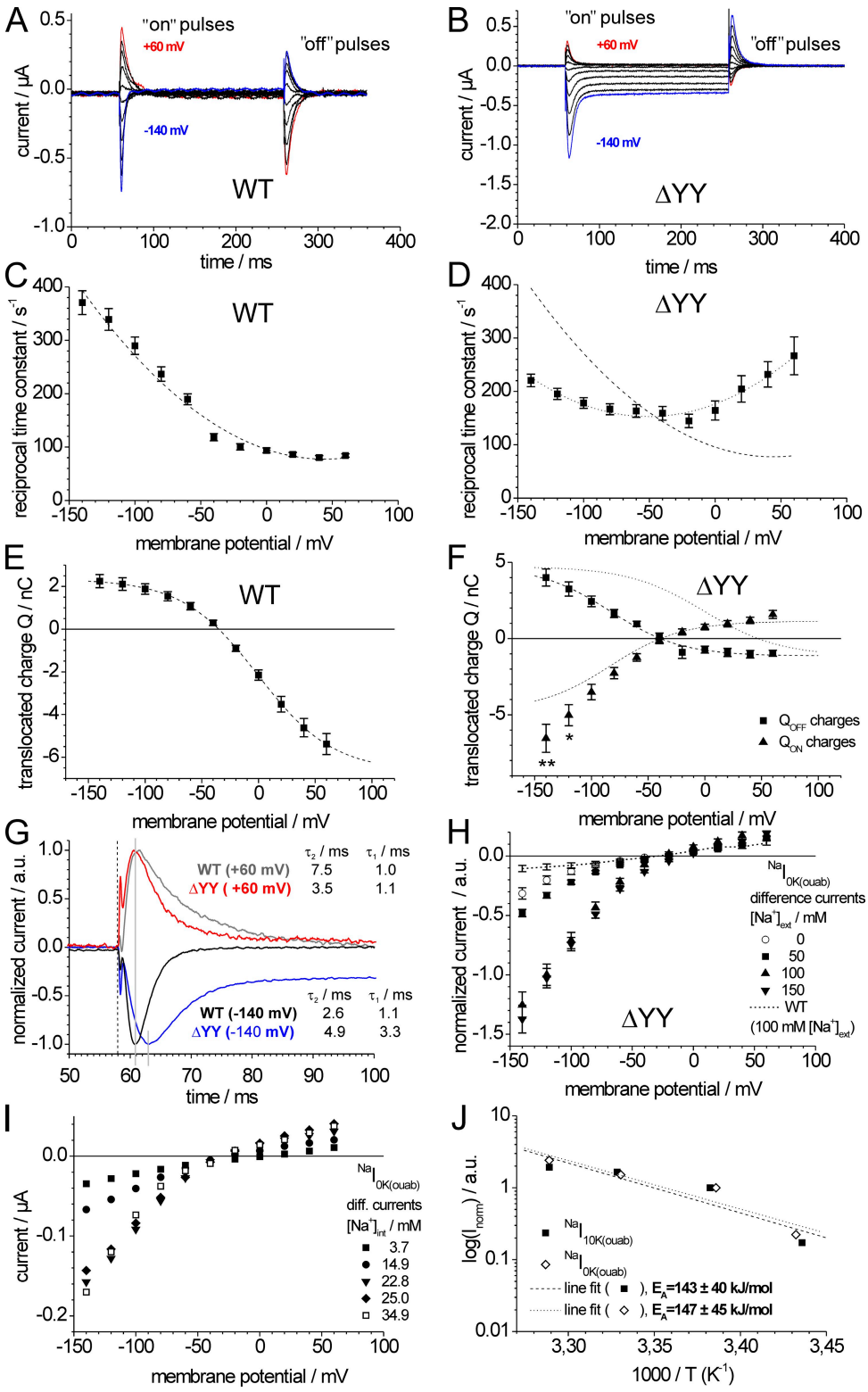

Figure 4. Properties of transient currents and hyperpolarization-induced inward currents of the ΔYY construct. (A and B) Ouabain-sensitive presteady-state currents $^{Na}I_{(ouab)}$ at $[Na^+]_{ext}$ = 100 mM and $[K^+]_{ext}$ = 0, pH_{ext} 7.4, upon voltage steps from −30 mV to potentials between +60 and −140 mV (in 20-mV decrements) for WT (A) and the ΔYY construct (B). (C and D) Voltage dependence of reciprocal time constants from ON transient currents for ATP1A2 WT (C) and ΔYY (D). Data for WT and ΔYY are means ± SE from 14 cells out of three batches. Results from a fit of a polynomial function to the WT or ΔYY data are superimposed in C and D as a dashed or dotted line, respectively. (E) Q-V curves obtained from Q_{OFF} charges of transient currents from 14 cells expressing ATP1A2 WT. The corresponding Q_{ON} charges were equivalent (not depicted). The fit of a Boltzmann function to Q-V distribution is superimposed as a dashed line (fit parameters: Q_{min} = −6.65 ± 0.12 nC; Q_{max} = 2.34 ± 0.04 nC; $V_{0.5}$ = −0.5 ± 1.0 mV; z_q = 0.76 ± 0.02). (F) Q-V curves obtained from

Temperature dependence of pump-related currents of the ΔYY construct

To investigate whether the Na^+-dependent inward leak currents were due to a transporter-like rather than an ion channel–like function of the mutant enzyme, we measured the temperature dependence of the hyperpolarization-induced inward currents and of the Na^+/K^+ pump currents. The resulting Arrhenius plots for temperatures between 18 and 30°C (Fig. 4 J) showed that the inward leak currents and the Na^+/K^+ pump currents share a very similar temperature dependence with a high activation energy of ∼145 kJ/mol, similar to values determined previously (Tavraz et al., 2008). The high activation energy strongly suggests that the inward currents of the ΔYY construct are not controlled by diffusion, as would be expected for a passively conducting ion channel.

Dependence of pump-related currents of the ΔYY construct on extracellular pH

To explore correlations between the hyperpolarization-activated inward currents of the ΔYY construct and the previously described H^+ currents of the Na^+/K^+-ATPase in the absence of extracellular Na^+ and K^+, we tested whether the inward currents of the ΔYY construct were modulated by pH_{ext}. Fig. 5 shows current recordings at −30 mV from an oocyte expressing the ΔYY construct, first at high $[Na^+]_{ext}$ (100 mM; Fig. 5 A) and then in the absence of Na^+_{ext} (Fig. 5 B). In the presence or absence of extracellular Na^+, the addition of 10 mM K^+ induced similar Na^+/K^+ pump current amplitudes (Fig. 5, A and B). Whereas in the absence of K^+ hardly any current change could be observed upon the addition of 10 mM ouabain at pH 7.4, a large ouabain-sensitive inward current was induced when the pH of the Na^+-rich buffer was lowered to 5.5 (Fig. 5 A). Upon replacement of Na^+ by $NMDG^+$, the addition of 10 mM ouabain again induced only a small difference current at pH 7.4, but a substantial ouabain-sensitive inward current occurred upon acidification to pH 5.5 (Fig. 5 B). This inward current, which can only be carried by protons in the absence of Na^+_{ext}, reaches ∼30% of the amplitude measured upon the same pH change in the presence of Na^+. The I-V curves of various pump-related currents of the ΔYY construct and ATP1A2 WT at different pH_{ext} are compared in Fig. 5 (C and D). At pH 7.4, the exclusively H^+-carried $^{NMDG}I_{0K(ouab)}$ currents were small, both for the ΔYY mutant (Fig. 5 C) and ATP1A2 WT (Fig. 5 D). Extracellular acidification to pH 5.5 increased the $^{NMDG}I_{0K(ouab)}$ currents for ATP1A2 WT, but even more so for the ΔYY mutant. Acidification to pH_{ext} 5.5 at 100 mM $[Na^+]_{ext}$ substantially increased the inward-rectifying $^{Na}I_{0K(ouab)}$ currents of the ΔYY mutant (Fig. 5 C), whereas the same pH change hardly affected the corresponding currents of ATP1A2 WT (Fig. 5 D). Comparison of the $^{Na}I_{0K(ouab)}$ current amplitudes of the ΔYY construct at pH 5.5 in the absence and presence of Na^+_{ext} shows that at least 25–30% of the inward currents is carried by H^+. The large increase of the inward current amplitudes observed upon acidification in the presence of high $[Na^+]_{ext}$ indicates that extracellular protons stimulate the Na^+-dependent inward leak current. This agrees with the observation that the leak currents of the ΔYY construct decreased at alkaline pH (Fig. S1 B).

Increased difference between Q_{ON} and Q_{OFF} charges at low pH_{ext}

To investigate the pH dependence of presteady-state charge translocation by the ΔYY construct, we measured ouabain-sensitive transient currents at pH 7.4 and 5.5 with 0 and 100 mM $[Na^+]_{ext}$. Fig. 5 E depicts ON and OFF transient currents of the ΔYY construct upon voltage pulses from −30 to −140 mV and back. At $[Na^+]_{ext}$ = 100 mM and pH 7.4, the ON current was composed of

Q_{ON} and Q_{OFF} charges of 14 cells expressing the ΔYY construct. The fit of a Boltzmann function to the Q_{OFF} values is superimposed as a dashed line (fit parameters: Q_{min} = −1.14 ± 0.17 nC; Q_{max} = 4.72 ± 0.63 nC; $V_{0.5}$ = −83.4 ± 7.9 mV; z_q = 0.81 ± 0.06). The short dashed line represents the inverted Q_{OFF} fit curve for comparison with Q_{ON} values. Q_{ON} and Q_{OFF} values of the ΔYY construct were significantly different at hyperpolarizing potentials (*, P > 0.05; **, P > 0.005; Student's *t* test). The WT Q-V curve from E is included as a dotted line in F after appropriate scaling. (G) Transient currents in response to ON voltage steps to +60 and −140 mV from WT data in A and ΔYY data in B on an expanded time scale. The voltage step occurred at 58 ms (dashed line). Fits of the sum of two exponential functions to the data (from t_0 = 59 ms) yielded time constants for the fast rise, τ_1, and the slower decay, τ_2. The peak of the transient current of ΔYY at −140 mV appeared with a delay of ∼2 ms (gray bars). (H) $[Na^+]_{ext}$ dependence of normalized ouabain-sensitive inward currents of the ΔYY construct at $[K^+]_{ext}$ = 0. The shallow dashed line shows the I-V curve of $^{Na}I_{(ouab)}$ currents of ATP1A2 WT at $[Na^+]_{ext}$ = 100 mM and $[K^+]_{ext}$ = 0. For each construct, data are means ± SE from 14 cells of four batches. (I) $[Na^+]_{int}$ dependence of ouabain-sensitive $^{Na}I_{(ouab)}$ currents (at $[Na^+]_{ext}$ = 100 mM and $[K^+]_{ext}$ = 0), measured on an oocyte coexpressing the ΔYY construct and the amiloride-sensitive Na^+ channel ENaC. Na^+ loading was achieved by exposing oocytes to $[Na^+]_{ext}$-containing solutions in the absence of amiloride, and $[Na^+]_{int}$ was determined after each loading step from the amiloride-sensitive reversal potential shift (see Materials and methods). Data from one out of three experiments with a similar $[Na^+]_{int}$ range are shown. (J) Temperature dependence (Arrhenius plots) of Na^+/K^+ pump currents ($^{Na}I_{10K}$) at 0 mV (filled squares) and hyperpolarization-induced inward currents ($^{Na}I_{(ouab)}$) at −140 mV (open squares) of the ΔYY construct. Data are from one out of four experiments across a similar temperature range (18–30°C). For both data sets, the amplitudes were normalized to the respective current at 21°C. Activation energies were derived from linear fits to the data.

a transient and a stationary inward current. The small discrepancy between Q_{ON} and Q_{OFF} charge is difficult to recognize at this pH. At pH 5.5 and $[Na^+]_{ext}$ = 100 mM, the same voltage pulse induced a transient current with a substantially increased stationary component, and the charge integral within the peak of the ON transient current was by far larger than that of the corresponding OFF current. The OFF transient currents at pH 5.5 and 7.4 are nearly identical, indicating that at both pH values a fixed amount of Q_{OFF} charge is released. Again, the ON current peak at acidic pH occurred with a delay of 2 ms with respect to the peak at pH 7.4. The subsequent relaxation to the stationary amplitude occurred with a time constant of ~20 ms, which was about fourfold slower than the relaxation at pH 7.4, showing that the pH_{ext} profoundly influences transient current kinetics. In contrast to the transient currents measured in the presence of Na^+_{ext}, the ouabain-sensitive H^+-carried transient current signal (in the absence of Na^+_{ext}) at pH 5.5 did not show a peak, but increased steadily according to a single-exponential function (time constant ~3 ms), and the subsequent OFF transient current relaxed to 0 with a similar time course.

Influence of extracellular Li^+ on pump-related currents

To investigate whether Li^+ ions contribute to the inward leak currents of the ΔYY mutant, we measured ouabain-sensitive currents at $[Li^+]_{ext}$ = 100 mM. It is known that electrogenic outward pumping of the Na^+/K^+-ATPase is stimulated by extracellular as well as intracellular Li^+ (Hermans et al., 1997). In accordance, we observed positive ouabain-sensitive currents for ATP1A2 WT in solutions containing $[Li^+]_{ext}$ = 100 mM already in the absence of K^+_{ext} (Fig. S2 A). In the absence of K^+_{ext} and at positive potentials, the ΔYY construct also mediated positive pump currents (Fig. S2 B). At hyperpolarizing potentials, however, negative $^{Li}I_{(ouab)}$ current amplitudes were observed, which were larger than the $^{NMDG}I_{(ouab)}$ currents (Fig. S2 B). Therefore, Li^+ ions apparently contribute to inward leak currents of the ΔYY construct, albeit not as efficiently as Na^+. Because currents at negative potentials are most likely a superposition of Li^+-dependent hyperpolarization-activated inward transport and Li^+-dependent outward pumping, the absolute contribution of Li^+ ions to the inward currents cannot be quantified. The addition of 10 mM K^+ to the Li^+-containing solution resulted in a further increase of pump

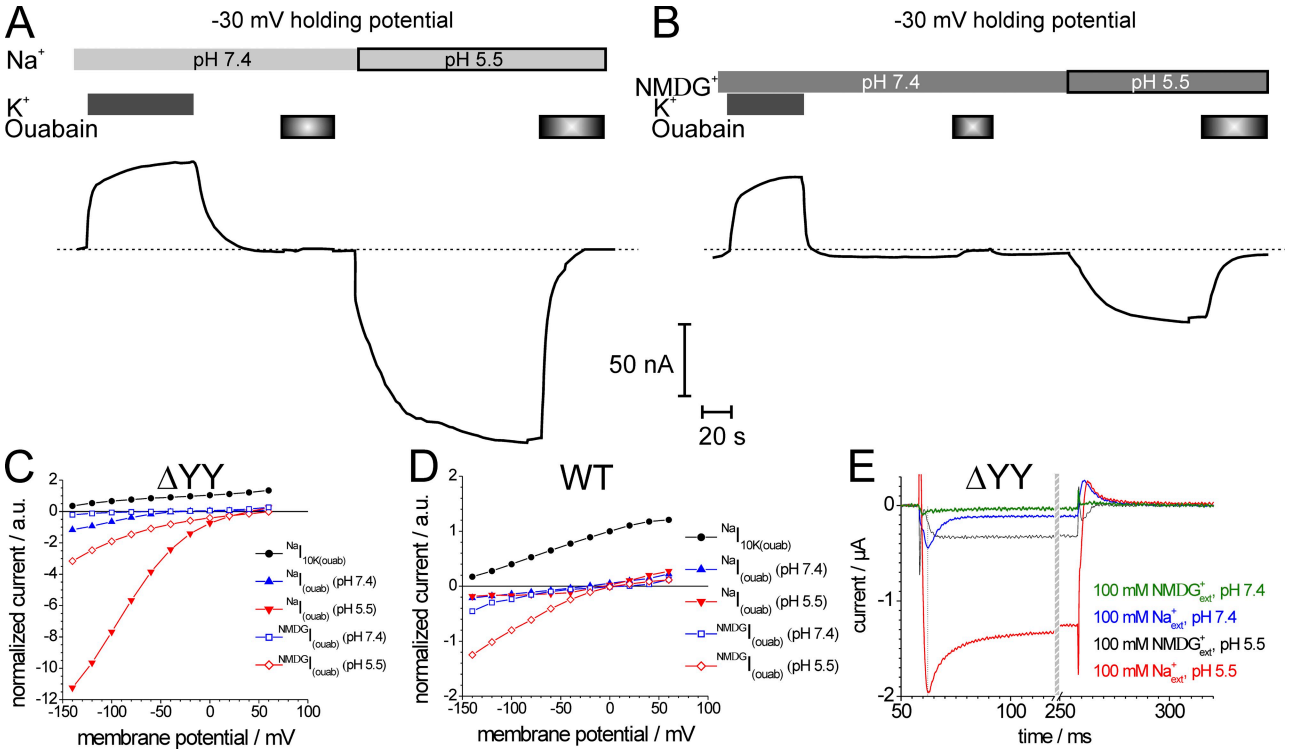

Figure 5. Voltage dependence of stationary and ouabain-sensitive transient currents of the ΔYY construct under different ionic conditions and pH_{ext}. (A and B) Current recordings at −30 mV from a ΔYY-expressing oocyte in the presence (A) or absence (B) of extracellular Na^+ at pH 7.4 and 5.5. If present, $[Na^+]_{ext}$ and $[NMDG^+]_{ext}$ were 100 mM, and $[K^+]_{ext}$ and [ouabain] were 10 mM. The composition of the extracellular solution is indicated above the current traces. Dashed lines indicate the "zero pump current" level (at 10 mM ouabain) as a reference for pump-related currents. (C and D) I-V curves of stationary currents in different extracellular solutions from oocytes expressing ΔYY (C) or ATP1A2 WT (D), each normalized to the $^{Na}I_{10K(ouab)}$ current at 0 mV. Similar data were obtained on at least three different cells. (E) Ouabain-sensitive transient currents of ΔYY at pH 7.4 ($^{NMDG}I_{(ouab)}$ and $^{Na}I_{(ouab)}$) and pH 5.5 ($^{NMDG}I_{(ouab)5.5}$ and $^{Na}I_{(ouab)5.5}$) in the absence of K^+_{ext} upon 250-ms voltage steps from −30 to −140 mV. The peak of the $^{Na}I_{(ouab)}$ current is indicated by a dotted line. Note the time axis break (hatched bar).

currents (Fig. S2, A and B), but for ATP1A2 WT as well as the ΔYY construct, the pump currents induced by 10 mM K^+ in the presence of 100 mM Li^+ were smaller than in Na^+-containing solutions, in line with previous results (Hermans et al., 1997).

Replacement of the carboxy-terminal tyrosines by alanines or phenylalanines

To explore which properties of the two carboxy-terminal tyrosines are essential for function, we mutated the tyrosines to phenylalanines (to preserve the aromatic rings but remove the OH groups) and to alanines (to eliminate the aromatic moieties), which resulted in mutants YY-FF and YY-AA. The K^+-dependent $^{Na}I_{xK}$ currents (at $[Na^+]_{ext}$ = 100 mM) of the YY-FF mutant (Fig. 6 A) were similar to those of the WT enzyme (Fig. 2 D), whereas currents of the YY-AA mutant (Fig. 6 B) resembled those of the ΔYY construct (Fig. 2 B). The voltage-dependent $^{Na}K_{0.5}(K^+_{ext})$ values for the YY-FF mutant were slightly elevated, and the minimum of the curve was shifted by about −20 mV (Fig. 6 C) compared with the corresponding WT data. In contrast, the minimal $^{Na}K_{0.5}(K^+_{ext})$ value for the YY-AA mutant was smaller (Fig. 6 D), and the $^{Na}K_{0.5}(K^+_{ext})$ curve was shifted by about −80 mV compared with WT. Furthermore, the YY-AA mutant showed ouabain-sensitive hyperpolarization-activated inward currents (albeit smaller than in the case of ΔYY), whereas the YY-FF mutant did not (Fig. 6 E). These characteristic similarities continued regarding the properties of ouabain-sensitive transient currents (Fig. 7). The reciprocal time constants for the YY-FF mutant were only slightly elevated compared with WT values (Fig. 7 A), and the $V_{0.5}$ of the Q-V distribution was only moderately shifted by about −25 mV, without a change in z_q (Fig. 7 C and Table I). The reciprocal time constants for the YY-AA mutant (Fig. 7 B) were similar to those of the ΔYY construct (Fig. 4 D), with a strong increase at positive potentials. Compared with the WT enzyme, the Q-V curve of mutant YY-AA showed a larger $V_{0.5}$ shift (by −112 mV) and a smaller slope (reduced z_q; Fig. 7 D and Table I) than the ΔYY construct.

DISCUSSION

Recent biochemical studies have supported the hypothesis that the highly ordered carboxy terminus of the Na^+/K^+-ATPase α subunit contributes to the formation of Na^+ binding site III (Toustrup-Jensen et al., 2009), as proposed previously (Morth et al., 2007). Deletions or

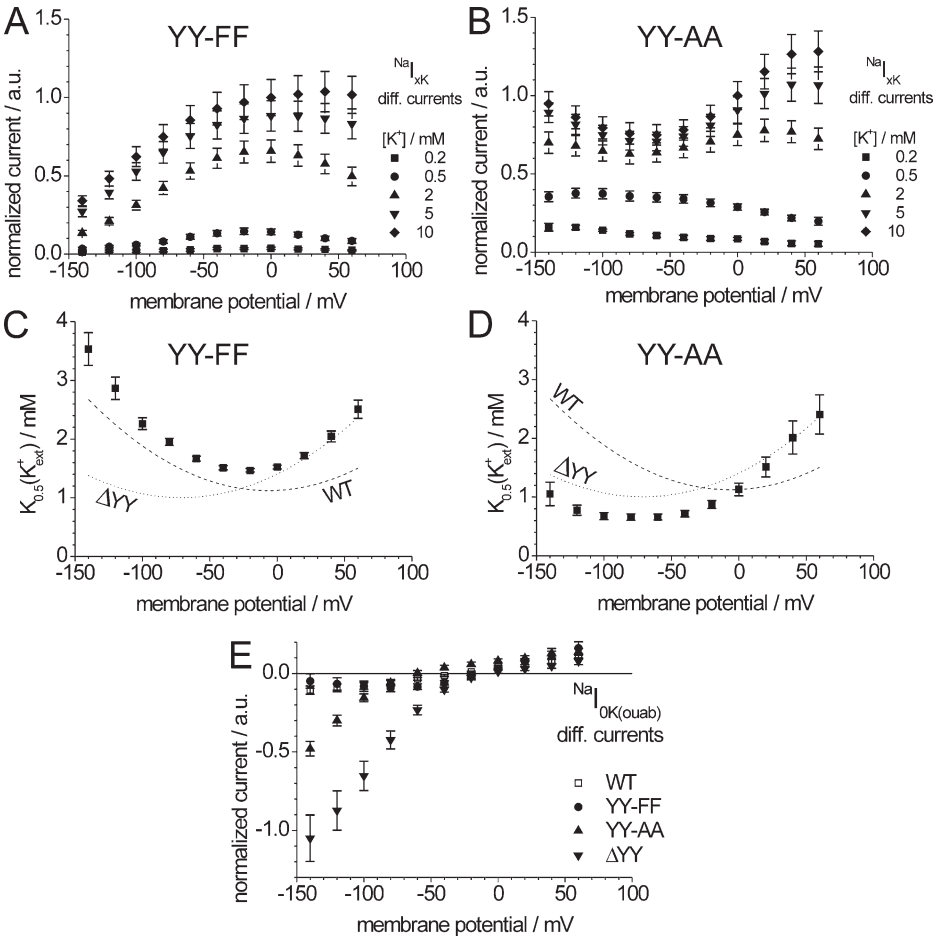

Figure 6. Properties of stationary currents of ATP1A2 mutants YY-FF and YY-AA. (A and B) Normalized I-V curves for $^{Na}I_{xK}$ currents of ATP1A2 mutants YY-FF (A) and YY-AA (B), with $[K^+]_{ext}$ as indicated ($[Na^+]_{ext}$ = 100 mM, pH 7.4). (C and D) $^{Na}K_{0.5}(K^+_{ext})$ values from $^{Na}I_{xK}$ currents for mutant YY-FF (C) and YY-AA (D) from fits of a Hill function to the data in A and B at each membrane potential. The corresponding WT data from Fig. 2 E (dashed lines in C and D) and ΔYY data from Fig. 2 C (dotted lines in C and D) are superimposed. (E) I-V curves of normalized ouabain-sensitive $^{Na}I_{(ouab)}$ currents ($[Na^+]_{ext}$ = 100 mM and $[K^+]_{ext}$ = 0, pH 7.4) for ATP1A2 WT and mutants YY-FF, YY-AA, and ΔYY. Data are means ± SE of 10–14 cells from three batches.

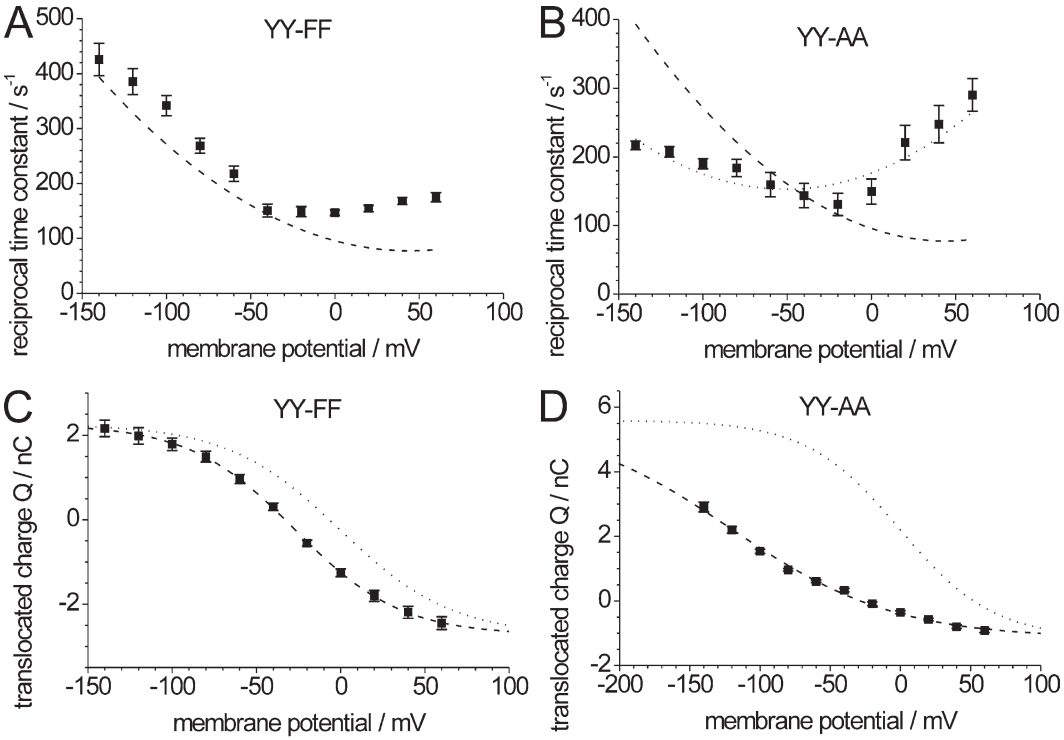

Figure 7. Properties of transient currents for mutants YY-FF and YY-AA. (A and B) Voltage dependence of reciprocal time constants from ON transient currents for mutant YY-FF (A) and YY-AA (B) ($[Na^+]_{ext}$ = 100 mM and $[K^+]_{ext}$ = 0, pH 7.4). Data are means ± SE from 13 (YY-FF) or 10 (YY-AA) oocytes from at least three batches. Curves delineating the corresponding WT data from Fig. 4 C (dashed lines in A and B) and the ΔYY data from Fig. 4 D (dotted line in B) are superimposed. (C and D) Q-V curves for Q_{OFF} charge integrals from oocytes expressing ATP1A2 mutants YY-FF (C) and YY-AA (D). Dashed lines show fits of a Boltzmann function with the following parameters: YY-FF (C): Q_{min} = −2.73 ± 0.05 nC; Q_{max} = 2.26 ± 0.03 nC; $V_{0.5}$ = −27.1 ± 0.8 mV; z_q = 0.81 ± 0.02; YY-AA (D): Q_{min} = −1.15 ± 0.13 nC; Q_{max} = 5.59 ± 0.15 nC; $V_{0.5}$ = −120 ± 18 mV; z_q = 0.44 ± 0.03. The Q-V distribution of ATP1A2 WT from Fig. 4 E is superimposed as a dotted line in C and D after appropriate scaling.

mutations within the carboxy-terminal WVEKETYY motif of the rat α_1 isoform largely decreased the Na^+ affinity from either side of the membrane (Toustrup-Jensen et al., 2009). Reduction was ninefold for the ΔYY deletion, 26-fold for the ΔKETYY deletion, and even 32-fold for the YY-AA mutation. In light of these data, our results regarding the consequences of deletions or mutations of the two carboxy-terminal tyrosines raise three major questions. Do the observed functional alterations reflect changes in cation affinity, and which type of cation binding site is affected by the ΔYY deletion? Which mechanisms could give rise to the Na^+- and pH-dependent inward leakage currents? Which side chain property of the carboxy-terminal tyrosines is crucial for correct function of the Na^+/K^+-ATPase?

Influence of the carboxy-terminal tyrosines on common and unique cation binding sites

Shifts of the Q-V curves obtained from the slow component of presteady-state currents directly indicate changes in the apparent affinity for extracellular Na^+. Based on models for extracellular release or reverse binding of Na^+ ions through an access channel with a fractional depth (or z_q) of 0.7, a shift in the Q-V curve by −25 mV is equivalent to a twofold reduction in Na^+_{ext} affinity (Holmgren et al., 2000; Holmgren and Rakowski, 2006). The Q-V curve of the ΔYY construct was shifted by −83 mV compared with the WT enzyme, which together with a z_q of 0.75–0.8 indicates a 12- to 14-fold reduced affinity for extracellular Na^+. This agrees well with the ninefold reduction in apparent Na^+ affinity of phosphoenzyme formation for the ΔYY deletion of the rat α_1 isoform (Toustrup-Jensen et al., 2009). The negative $V_{0.5}$ shift also indicates an increased preference of the ΔYY enzyme for the E_2P state at 0 mV, in agreement with the substantial E_2P stabilization found in biochemical studies (Toustrup-Jensen et al., 2009). Due to the decreased affinity for extracellular Na^+, more strongly hyperpolarizing potentials are required to saturate the Na^+ binding sites of the ΔYY construct from the extracellular side and to drive the enzyme subsequently from E_2P to E_1P. The half-maximal $[Na^+]_{int}$ from the stimulation of Na^+/K^+ pump currents was between 8 and 12 mM, which is similar to the value of 10.8 ± 0.6 mM reported for the Na^+ dependence of phosphoenzyme formation for the ΔKETYY construct (Toustrup-Jensen et al., 2009). However, this half-maximal Na^+_{int} concentration is comparable to the value of 12.8 ± 2.2 mM reported for the WT

human α_2/β_1 pump from pump current measurements (Crambert et al., 2000), which indicates that pump current measurements might not be adequate to reveal changes in the affinity for intracellular Na^+.

The shifted Q-V curve of the ΔYY construct can be attributed to a change in affinity at the unique Na^+ binding site III (see below). Because the slow presteady-state charge movement reflects the strongly electrogenic extracellular deocclusion/release of the first Na^+ ion (Holmgren et al., 2000), the major electrogenic event is most likely Na^+ deocclusion/release from site III. Alternatively, if the deletion affected the Na^+ affinity at the two common sites, the shifted Q-V curve could result from the fact that more hyperpolarizing potentials are required to saturate these sites, a process that has to be complete before the major electrogenic reuptake of the third Na^+ to site III can take place. However, we disfavor the interpretation of an affinity change at the two common sites for the following reasons. The α subunit's carboxy terminus is proximal to the unique Na^+ binding site. Therefore, it is unlikely that the ΔYY deletion would exclusively affect the more distant sites I and II. Furthermore, we observed that the ΔYY deletion changed neither the intrinsic apparent affinity for extracellular K^+ in the absence of extracellular Na^+ ($K_{0.5}(K^+_{ext})$ values) nor the maximal apparent K^+ affinity in the presence of high $[Na^+]_{ext}$ (minimum of the $^{Na}K_{0.5}(K^+_{ext})$ values). Thus, even under conditions that allow for competition of extracellular Na^+ with K^+ ions at the common binding sites, the ΔYY deletion does not change the maximal K^+ affinity and probably does not change the cation affinity at the common sites in general. Of note, the voltages at which the $^{Na}K_{0.5}(K^+_{ext})$ curves are minimal, and the shift between these minima, absolutely coincide with the $V_{0.5}$ values from the corresponding Q-V curves obtained from transient currents of the WT enzyme and the ΔYY construct. It is conceivable that the decreased affinity at the unique Na^+ binding site III, which leads to a shifted voltage dependence of charge release/reuptake from/to site III, is simultaneously responsible for the shift of the $^{Na}K_{0.5}(K^+_{ext})$ curves. This modulatory role of site III on the voltage dependence of the $^{Na}K_{0.5}(K^+_{ext})$ values can be explained as follows. Potentials more positive than $V_{0.5}$ promote the release of Na^+ from site III, which in turn allows for Na^+ release from the two common sites. The increase of the $^{Na}K_{0.5}(K^+_{ext})$ values at positive potentials can then be attributed to the subsequent weakly electrogenic binding of K^+ ions to the common sites. At potentials more negative than $V_{0.5}$, which promote Na^+ reuptake to site III, increasing concentrations of K^+ would be required to overcome the inhibitory effect of extracellular Na^+ on Na^+/K^+ pumping, thus giving rise to the increase of $^{Na}K_{0.5}(K^+_{ext})$ upon hyperpolarization. However, the $^{Na}K_{0.5}(K^+_{ext})$ values at negative potentials should be considered with some caution because the full K^+_{ext}-induced currents of the ΔYY construct are not exclusively Na^+/K^+ outward pump currents (in contrast to the WT pump), and a substantial contribution arises from the inhibition of the hyperpolarization-activated inward currents. It is likely that both processes rely on the same event, namely the binding of extracellular K^+ to the pump in E_2P (which at the same time stimulates outward pumping and prevents the pump from entering the inward leak current mode), and therefore occur with the same apparent K^+ affinity.

Whereas we propose that the ΔYY deletion changes Na^+ affinity at the unique binding site without a major effect on the common sites, Yaragatupalli et al. (2009) arrived at the opposite conclusion based on their findings on a ΔKESYY deletion of the *Xenopus* Na^+/K^+-ATPase α_1 isoform. These authors reported a $V_{0.5}$ shift of the Q-V curve by about −100 mV and observed similar ouabain-sensitive inward leak currents. In contrast to the ΔYY-deleted Na^+ pump, the voltage-dependent $^{Na}K_{0.5}(K^+_{ext})$ values of the ΔKESYY construct were not only shifted to hyperpolarizing potentials, but also the minimum of the curve was significantly lower. We tested whether the decreased minimum of the $^{Na}K_{0.5}(K^+_{ext})$ curve could be the result of the more extensive five–amino acid deletion and found this indeed to be the case (Fig. S4, A and B). Functional analysis of a homologous ΔKETYY deletion of the human α_2 isoform showed that the $^{Na}K_{0.5}(K^+_{ext})$ values were significantly lower than those of the ΔYY construct (Fig. S4 B) and similar to the values reported for the ΔKESYY-truncated *Xenopus* α_1 isoform. Therefore, the ΔKETYY deletion apparently causes a larger structural disruption with additional long-range consequences also on the distant common sites, whereas the ΔYY deletion mainly affects the proximal unique Na^+ binding site. The properties of the ΔKETYY construct were similar to the more severe phenotype of the YY-AA mutation, which further indicates that the functional consequences of the ΔKETYY deletion are more substantial. The $^{Na}K_{0.5}(K^+_{ext})$ values of the ΔKETYY construct and mutant YY-AA were similar (compare Fig. 6 D and Fig. S4 B), and the $V_{0.5}$ shift of the Q-V curve from transient currents (Fig. S4 F) was larger than that of the ΔYY construct with a similarly reduced slope factor z_q (Table I). Regarding the properties of ouabain-sensitive transient currents, the ΔKETYY and ΔYY constructs were equivalent. Stationary inward leak currents reached similar amplitudes (Fig. S4 D), and the reciprocal time constants showed the same reversed voltage dependence (Fig. S4 E). Furthermore, the amount of Q_{ON} charges at negative potentials also exceeded the Q_{OFF} values (Fig. S4 F).

The assignment of the consequences of the ΔKESYY deletion to the two common cation binding sites resided on two experimental aspects: the −100-mV shift of the Q-V curve and the overlapping I-V curves of ouabain-sensitive, 10-mM K^+-induced currents for the WT enzyme and the ΔKESYY construct (Yaragatupalli et al., 2009).

Based on a kinetic scheme for Na^+/K^+-ATPase function, these authors argued that a shift of identical magnitude would occur in both the I-V and the Q-V curves if the deletion only affected Na^+ binding at the unique binding site. In contrast, an effect only at the common sites would shift the Q-V curve, but not the I-V curve. However, the comparison between the $^{Na}I_{10K(ouab)}$ curves of the WT enzyme and those of the ΔKESYY construct critically assumes that, even at the most hyperpolarizing potentials, the $^{Na}I_{10K(ouab)}$ currents of ΔKESYY are exclusively Na^+/K^+ outward pump currents. Any residual inward current component would result in a downward deflection of the $^{Na}I_{10K(ouab)}$ curve and conceal a possible horizontal shift. Our data show that the I-V curve of the $^{Na}I_{10K(ouab)}$ currents of the ΔYY construct is indeed shifted compared with WT data (Fig. 2 F), which supports our conclusion that the ΔYY deletion changes the Na^+ affinity at the unique site also by kinetic arguments (Yaragatupalli et al., 2009).

Mechanisms involved in the generation of inward leak currents

The hyperpolarization-activated inward current of the ΔYY construct occurs in the absence of extracellular K^+, a condition that prevents Na^+/K^+ outward pumping. In the absence of K^+_{ext}, the WT Na^+/K^+-ATPase hardly generates any ouabain-sensitive current, except for the pH-sensitive ATP-dependent and mainly H^+-driven current, which only occurs if extracellular Na^+ is also absent (Rakowski et al., 1991; Efthymiadis et al., 1993; Wang and Horisberger, 1995; Rettinger, 1996; Li et al., 2006). The inward leak currents of the ΔYY construct depended on intracellular $[Na^+]$ in a very similar way as the normal outward pump currents, indicating that Na^+_{int}-dependent phosphoenzyme formation is equally involved. Furthermore, the inward leak currents required extracellular Na^+ and showed saturating concentration dependence.

Yaragatupalli et al. (2009) also observed such hyperpolarization-induced inward currents for the ΔKESYY-truncated pump and attributed these currents to a "low affinity Na^+ passive flow" through the Na^+ pump because saturation of inward current amplitudes could not be observed at $[Na^+]_{ext}$ up to 125 mM. However, our data vote against a passive, ion channel–like transport mechanism for the following reasons. First, the inward current amplitudes of our ΔYY construct saturated at hyperpolarizing potentials and at rather moderate $[Na^+]_{ext}$, which would not be expected for a channel-like conductance. Because the ΔKESYY-truncated pump conceivably has an even lower affinity for extracellular Na^+ than the ΔYY construct (reduction 26-fold vs. ninefold; Toustrup-Jensen et al., 2009), much higher $[Na^+]_{ext}$ than 125 mM would be required to achieve saturation of the ΔKESYY inward currents. Second, a comparison of the relative current amplitudes of the ΔYY construct shows that the inward leak currents at −140 mV are only 1.2-fold (pH 7.4) or 12-fold (pH 5.5) larger than the maximal Na^+/K^+ pump currents at 0 mV. This, together with a Na^+/K^+ pump turnover rate of 13 s^{-1} for the human α_2/β_1 Na^+/K^+-ATPase (Tavraz et al., 2008) would yield ion permeation rates of 15 to 160 s^{-1} per pump molecule, which is by several orders of magnitude smaller than in ion channels. Third, the high activation energy of the inward leak currents argues against a channel-like, diffusion-controlled mechanism. Because the activation energy is equivalent to that of Na^+/K^+ outward pumping, a major conformational transition might be similarly involved for the inward leak currents. It seems possible that after extracellular Na^+ binding to the pump in E_2P, the $E_2P \leftrightarrow E_1P$ conformational change is involved. This can be inferred from the equivalent voltage dependences of the inward leak currents and the Q-V distributions of the ΔYY construct (Fig. S3) under the assumption that for the ΔYY-truncated pump, the Q-V distribution also reflects the poise of the dynamic equilibrium between E_1P and E_2P, as commonly accepted for the WT enzyme (Holmgren et al., 2000). In this interpretation, the saturating inward leak currents at hyperpolarizing potentials coincide with maximal abundance of the Na^+-occluded E_1P state. Under these conditions for the dynamic equilibrium, the enzyme would toggle between the Na^+-loaded E_2P state and the Na^+-occluded E_1P state, and the inward currents could then arise from cation release ("leak") from the Na^+-occluded E_1P state. As will be outlined below, we suggest that the ΔYY deletion destabilizes the Na^+-occluded E_1P state of the enzyme.

Yet, one cannot exclude that cation leak already occurs from E_2P, although such a mechanism would lack the direct coupling to a major conformational transition or to a change in accessibility of cation binding sites. Furthermore, the inward leak currents could be due to a Na^+_{ext}-dependent E_2P-E_2-E_1 cycle, which might be correlated with the increased Na^+-dependent ATP hydrolysis rate of carboxy-terminally truncated pumps (Toustrup-Jensen et al., 2009). However, these rates were found to still be 10-fold smaller than the maximal hydrolysis rates under Na^+/K^+ turnover conditions (Toustrup-Jensen et al., 2009). Because the ΔYY-truncated pump apparently works with a $3Na^+/2K^+$ stoichiometry under Na^+/K^+ turnover conditions, such an E_2P-E_2-E_1 cycle would imply that each extrusion of $3Na^+$ ions must be followed by inward translocation of much more than three cations to account for the inward current amplitudes. The ATP/cation inward transport ratio might even be variable because pH_{ext} has a profound influence on the inward current amplitudes. It remains to be determined whether the inward cation leak mechanism requires ATP hydrolysis at all, and which fractions of the current are carried by Na^+ and H^+.

Interference with the intracellular ion occlusion gate of the Na^+ pump

Active transporters avoid energy-dissipating leakage currents by strictly coordinated operation of two access gates for the transport sites from either side of the membrane (Gadsby, 2009). Holmgren and Rakowski (2006) analyzed the underlying mechanism of the Na^+ pump by measuring the effects of intracellular and extracellular Na^+ on ouabain-sensitive presteady-state currents (Scheme 1 in Fig. 8 and Appendix). Because intracellular Na^+ binding was shown to be weakly electrogenic (Wuddel and Apell, 1995; Pintschovius et al., 1999), it was asked whether nonsaturating $[Na^+]_{int}$ could change kinetics, voltage dependence, or amplitudes of the presteady-state currents. However, $[Na^+]_{int}$ had no effect on the $V_{0.5}$ or z_q of the Q-V distributions, but rather determined the total amount of charge Q_{tot} available for presteady-state charge translocation. At depolarizing potentials, neither charge transients that reflected electrogenic intracellular binding of Na^+ nor increasing rate coefficients of transient currents (k_{tot}) were observed. It was concluded that the transient charge movement arises only from extracellular Na^+-sensitive current, and that the voltage dependence of the pseudo first-order rate coefficient for reverse binding of Na^+ through a deep extracellular ion well largely determined the observed k_{tot} (Eq. 1 in Appendix). To explain why intracellular Na^+ binding does not affect the extracellular Na^+-sensitive current, it was proposed that a slow occlusion step follows intracellular Na^+ binding. This slow occlusion reaction kinetically isolates intracellular Na^+ binding from the rapid extracellular release steps, which is the kinetic equivalent to closure of an intracellular occlusion gate (Holmgren and Rakowski, 2006).

At this point, the reversed voltage dependence of the reciprocal time constants from presteady-state currents of the ΔYY mutant needs consideration (Fig. 4 D). According to Holmgren and Rakowski (2006), exactly such a behavior would result if the intracellular Na^+ occlusion step were not rate limiting. Vasilyev et al. (2004) reported such an effect on the reciprocal time constants of presteady-state currents of the Na^+/K^+-ATPase as a consequence of extracellular acidification. Whereas lowering the extracellular pH did not modify $V_{0.5}$ and z_q of the Q-V distributions from transient currents, the reciprocal time constants at $pH_{ext} \leq 6.6$ exhibited a suspicious increase at positive potentials. In addition, steady-state components were observed in the transient current signals at acidic pH, which correspond to the acid-induced inward currents described previously (Rakowski et al., 1991; Efthymiadis et al., 1993; Rettinger, 1996). Vasilyev et al. (2004) suggested that protonation at some regulatory site reduces the activation energy of the $3Na^+ + E_1 + ATP \leftrightarrow E_1P(3Na^+)$ occlusion reaction, which would in effect lead to a proportional increase of the rate coefficients for the forward and backward reaction.

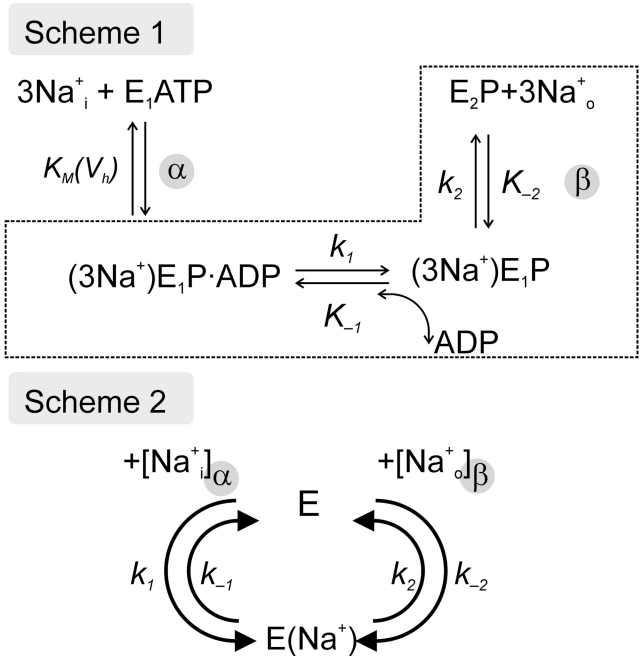

Figure 8. Partial reaction schemes for the Na^+ transport limb of the Na^+/K^+-ATPase cycle. Scheme 1 was introduced by Holmgren and Rakowski (2006) to describe the dependence of Na^+/K^+-ATPase presteady-state currents on intracellular $[Na^+]$. At saturating intracellular concentrations of Na^+ and ATP, the intracellular Na^+ binding (and phosphorylation) is at rapid equilibrium, and the subsequent slow occlusion step kinetically isolates the intracellular Na^+ binding step from the more rapid extracellular Na^+ release steps. Scheme 2 refers to Vasilyev et al. (2004), who provided a concept for the mainly H^+-driven inward currents of the Na^+/K^+-ATPase observed in Na^+- and K^+-free extracellular solutions. At low pH_{ext}, the energy barrier for equilibration of occluded Na^+ ions with the intracellular space is reduced so that the occluded state can be reached from the extracellular as well as the intracellular solution. See Appendix for details.

Consequently, the kinetic model (Scheme 2 in Fig. 8 and Appendix) included that at acidic pH_{ext}, the Na^+-occluded state of the pump is kinetically accessible from both the extracellular side (passage along a deep ion well of fractional depth α) as well as from the intracellular side (through a shallow access channel of fractional depth β). As a result, the rate coefficients k_{tot} of the transient currents (Eq. 2 in Appendix) increase at negative potentials as well as at positive potentials. The destabilization of the intracellular Na^+ occlusion gate was confirmed by tracer flux measurements, which showed that in addition to protons, Na^+ ions also contributed to the inward currents with a H^+/Na^+ permeation ratio of $\sim 3 \times 10^4$ (Vasilyev et al., 2004).

Correlation of the Na^+-dependent leak currents to acid-induced inward currents of the Na^+/K^+-ATPase

From the pH_{ext} dependence and the similar behavior of the reciprocal time constants from transient currents, we propose that the inward leak currents of the ΔYY-truncated pump and the acid-activated inward currents

of the Na^+/K^+-ATPase share a common mechanism. We suggest that the ΔYY mutation, in addition to the change in Na^+ affinity at binding site III, also reduces the energy barrier for access of the Na^+-occluded state from the intracellular solution, which results in an increased open probability of the intracellular occlusion gate. Because the inward leak currents of the ΔYY construct already occurred at neutral pH, it is conceivable that an increased Na^+/H^+ permeation ratio accompanies the impairment of the ion occlusion gate. Thus, the large inward leak current at neutral pH_{ext} might be due to a large Na^+ component, whereas the exclusively H^+-carried component requires a more acidic pH than the WT pump because the ΔYY deletion most likely reduces the H^+ affinity at the proposed regulatory H^+ interaction site. In the WT enzyme, the regulatory H^+ site destabilizes the intracellular cation occlusion gate and gives rise to a large H^+ component of the inward leak currents (Vasilyev et al., 2004). Because the exclusively H^+-carried leak currents of the ΔYY construct at neutral pH are smaller, it is likely that the decreased potency of protons to destabilize the cation occlusion gate is overcompensated by the direct destabilizing effect of the ΔYY deletion on Na^+ occlusion. From a structural viewpoint, it is unclear how the ΔYY deletion might interfere with Na^+ binding at site III with the molecular mechanism of cation occlusion, and with the proposed H^+ binding site that also modifies cation occlusion, although these effects are tightly coupled on a functional level. The regulatory H^+ site most likely does not share amino acid residues with Na^+ binding site III, otherwise extracellular acidification should affect Na^+ affinity and therefore shift the Q-V distribution.

The interference of extracellular Na^+ and protons was investigated by mutagenesis of residues, which were suggested to contribute directly to Na^+ binding at site III (Li et al., 2005, 2006). Most mutations reduced the voltage-dependent inhibition of pump currents by extracellular Na^+, in agreement with a lower affinity for Na^+_{ext}, but also decreased the exclusively H^+-carried inward leak currents at acidic pH. From this, it has been suggested that Na^+ and H^+ ions use the same extracellular high field access channel to reach the binding sites (Li et al., 2006). Of note, none of these site III mutants exhibited stationary inward leak currents at high $[Na^+]_{ext}$, suggesting that mutations, which directly interfere with Na^+ coordination, apparently do not destabilize the intracellular Na^+ occlusion gate. Thus, the carboxy-terminal residues of the Na^+/K^+-ATPase α subunit might be unique as the link between correct Na^+ coordination at the unique site and function of the ion occlusion mechanism. Again, on a structural level things might be more complex. Abriel et al. (1999) reported that a 34–amino acid deletion of the cytoplasmic amino terminus of the Na^+/K^+-ATPase β subunit caused Na^+ inward leak currents similar to those of the ΔYY mutant, a strong hyperpolarizing shift of the Q-V curves obtained from transient currents and reversed voltage dependence of reciprocal time constants. Therefore, the N terminus of the β subunit on the cytoplasmic side might also interfere with Na^+ occlusion, although it remains to be determined whether the effects of the βN-terminal deletion are secondarily caused by disruption of critical α/β subunit interactions, as outlined by Shinoda et al. (2009).

pH_{ext}-dependent asymmetry between Q_{ON} and Q_{OFF} charges and changes in transient current kinetics

The discrepancy between Q_{ON} and Q_{OFF} charges appears to be the specific consequence of the ΔYY deletion that destabilizes the intracellular cation occlusion gate. Such an effect has not been reported for the WT Na^+/K^+-ATPase, not even at low pH (Vasilyev et al., 2004). Whereas the difference between Q_{ON} and Q_{OFF} was enhanced by acidification, the amount of Q_{OFF} charges at neutral and acidic pH was equal (Fig. 5 E). This indicates that the amount of available OFF charge depends only on the number of ions in the binding sites of the pump molecules. The fact that the amount of Q_{ON} charge at hyperpolarizing potentials cannot be recovered upon OFF voltage pulses is not compatible with a reversible and exclusively extracellular Na^+ release/binding mechanism. It is conceivable that upon a hyperpolarizing voltage pulse an intermediate within the sequence $3Na^+ + E_2P \leftrightarrow E_2P(3Na^+) \leftrightarrow E_1P(3Na^+)$ is transiently accumulated, from which cations can be released intracellularly proportional to the number of enzyme molecules, which momentarily dwell in that intermediate. Such a transiently accumulating intermediate could be the Na^+-bound and -occluded E_2P state or the Na^+-occluded E_1P state, which is populated from the former. Transient accumulation of such a "leaky" intermediate could also explain the kinetically more complex transient currents of the ΔYY construct. The ON currents do not rise instantaneously (i.e., not only limited by the speed of the voltage clamp), but with a time constant of ~3 ms. Such a time course could be explained if the transient current included a significant contribution from a slowly accumulating reaction intermediate, which permits H^+/Na^+ inward leak current, in addition to the conventional extracellular Na^+-sensitive current. The contribution of such a process is conceivable because, e.g., the H^+-carried transient current of the ΔYY construct at low pH rises with the same time constant.

Influence of side chain properties of the carboxy-terminal tyrosines on function of the Na^+/K^+-ATPase

Functional analysis of the YY-FF and the YY-AA mutants showed that the simultaneous removal of the hydroxyl groups and the aromatic rings causes similar functional changes as the ΔYY deletion. In contrast, the YY-FF mutation had only mild consequences. These findings

agree with changes in apparent Na^+ affinities observed in biochemical experiments (Toustrup-Jensen et al., 2009). Toustrup-Jensen et al. (2009) have outlined the crucial importance of an arginine-π-stack interaction between the carboxy-terminal tyrosines and Arg-937 within the M8-M9 loop of the Na^+/K^+-ATPase α subunit. Because mutation of Arg-937 also decreased Na^+ affinity, it was suggested that Arg-937 represents a structural link between the C terminus and Na^+ binding site III (Toustrup-Jensen et al., 2009). Therefore, the initially proposed H bonds between the tyrosines' hydroxyl groups and Arg-937 or Lys-770 in M5 (Fig. 1 B) are less important for proper function of the Na^+/K^+-ATPase than amino-aromatic interactions between positively charged or δ^+ amino groups of Arg-937 with the δ^- of the tyrosines' π electron systems.

Compared with the ΔYY deletion, the YY-AA mutation reduced Na^+ affinity more severely, but the inward leak currents of the YY-AA mutant were smaller. If the inward leak currents reflect the extent of destabilization (leakage) of the intracellular occlusion gate, this observation indicates that the exchange of the two tyrosines for structurally unrelated amino acids partly conserves function of the intracellular gate. Thus, the changes in Na^+ affinity due to C-terminal sequence variations do not directly correlate with changes at the intracellular occlusion gate.

Concluding remarks

The ΔYY deletion at the carboxy terminus of the Na^+/K^+-ATPase α subunit apparently modifies Na^+ affinity at the unique binding site III with no significant change at the two common cation binding sites, in contrast to the ΔKETYY deletion, which also has long-range effects on the common sites. The Na^+ and pH-dependent inward leak currents of carboxy-terminally truncated pumps can be explained in analogy to the mechanism proposed for the H^+ inward currents of the WT Na^+ pump. The ΔYY deletion lowers the energy barrier for the intracellular Na^+ occlusion reaction, which destabilizes the Na^+-occluded state and in effect enables stationary inward leakage of Na^+ ions. The carboxy-terminal tyrosines are involved in amino-aromatic interactions, which are critical for structural and functional integrity. The carboxy terminus of the Na^+/K^+-ATPase α subunit might therefore represent a structural and functional relay between Na^+ binding site III and the intracellular cation occlusion gate.

APPENDIX

Scheme 1 (Fig. 8)

Holmgren and Rakowski (2006) introduced a four-state kinetic model for the Na^+ transport limb of the Na^+/K^+-ATPase to describe how binding and occlusion of Na^+ within a shallow intracellular ion well affects the availability of presteady-state charge movement resulting from the strongly electrogenic deocclusion and release of the first Na^+ ion to the extracellular space.

Within Scheme 1, the rate constant k_1 describes ADP release from the $(3Na^+)E_1P \cdot ADP$ intermediate, which is independent of Na^+_{int} and membrane voltage. K_{-1} describes reverse binding of ADP defined by $K_{-1} = k_{-1} \cdot [ADP]$. k_{-2} is a rate coefficient for reverse binding/occlusion of Na^+_{ext}, which is assumed to be voltage and $[Na^+]_{ext}$ dependent due to Na^+ passage through an extracellular ion well of fractional depth β:

$$K_{-2} = k_{-2} \cdot [Na^+]_{ext} \cdot \exp\left(-\frac{\beta \cdot F \cdot V}{R \cdot T}\right)$$

Here, k_{-2} is a voltage-independent rate coefficient that combines Na^+_{ext} rebinding/occlusion and the $E_2P \rightarrow E_1P$ conformational change. In principle, the rate coefficient k_2, which combines the $E_1P \rightarrow E_2P$ conformational change and extracellular Na^+ deocclusion/release, should be electrogenic too because it uses the same ion well. However, the voltage dependence of k_2 can be disregarded because this step is rate limited by the preceding conformational change and only becomes rate limiting by itself at very negative voltages.

The system of differential equations for the boxed reaction in Scheme 1 has three eigenvalues, from which the smallest nonzero one governs k_{tot}, the rate coefficient of charge relaxation:

$$k_{tot} = 0.5 \cdot \left\{ k_1 + K_{-1} + k_2 + K_{-2} - \sqrt{(k_1 + K_{-1} + K_2 + K_{-2})^2 - 4 \cdot \left(k_1 \cdot k_2 + (k_1 + K_{-1}) \cdot K_{-2}\right)} \right\} \quad (1)$$

It is assumed that Na^+ binding through the shallow intracellular ion well of fractional depth α is rapid compared with the subsequent slow occlusion step, thus enabling equilibrium binding of Na^+ to E_1ATP. The reaction steps preceding the boxed sequence in Scheme 1 can then be lumped together to an equilibrium coefficient for a certain holding potential V_h.

$$K_{m(V_h)} = K_{m(V_h=0)} \cdot \exp\left(-\frac{\alpha \cdot F \cdot V_h}{R \cdot T}\right)$$

Note that V_h indicates the voltage that was prevalent before a step to a certain test potential, which is denoted as V.

Nonsaturating $[Na^+]_{int}$ is rate limiting for the reaction denoted by k_1 in Scheme 1. Therefore, k_1 in Eq. 1 has to be replaced by an "effective" forward rate constant:

$$k_f = \frac{k_1 \cdot [Na^+]_{int}}{[Na^+]_{int} + K_{m(V_h)}}$$

k_f describes the effects of membrane voltage and $[Na^+]_{int}$ on the total charge relaxation rate constant k_{tot}. Although $K_{m(V_h)}$ is dependent on the holding potential, it does not affect the voltage dependence of k_{tot} because it is a constant for each set of voltage pulses. Scheme 1 assumes that contributions from the electrogenic intracellular Na^+ binding are not observed because binding is followed by a slow occlusion step, which equilibrates at V_h but, in effect, kinetically isolates the intracellular binding steps from the more rapid extracellular Na^+ release steps. Thus, the voltage dependence of intracellular Na^+ binding does not affect the observed rate constants k_{tot}, and it is mainly the term K_2 in front of the square root that is responsible for the pronounced increase of k_{tot} at hyperpolarizing potentials.

Scheme 2 (Fig. 8)

Vasilyev et al. (2004) used a simplified kinetic model to describe the effects of extracellular pH on ouabain-sensitive presteady-state currents. Within the kinetic relationship given by Scheme 2, $E(Na^+)$ represents a Na^+-occluded state, which can be reached from the intracellular as well as from the extracellular side. E denotes all nonoccluded states, and $E_{tot} = E(Na^+) + E$. It is assumed that the rate constants k_1, k_{-1}, k_2, and k_{-2} are voltage independent, and the principle of microscopic reversibility imposes the constraint $k_{-1} \cdot k_{-2} = k_1 \cdot k_2$. Voltage dependence is introduced by defining pseudo first-order rate coefficients K_1 and K_{-2} for the Na^+ binding/occlusion reactions, and the local concentrations of Na^+ at the binding site can be expressed according to a Boltzmann equation:

$$K_1 = k_1 \cdot \left[Na^+\right]_{\text{int}} \exp\left(\frac{\alpha \cdot F \cdot V}{RT}\right);\ K_{-2} = k_2 \cdot \left[Na^+\right]_{ext} \exp\left(-\frac{\beta \cdot F \cdot V}{RT}\right)$$

Here, α and β are the fraction of the transmembrane field crossed by Na^+ ions to reach the occlusion site along an intracellular or extracellular access channel, respectively (with $\alpha + \beta = 1$).

Using these rate coefficients, the differential equation for $E(Na^+)$ can be solved yielding a total relaxation rate constant:

$$k_{tot} = K_1 + k_{-1} + k_2 + K_{-2}$$

Inserting the derived $E(Na^+)$ and E as a function of time into the formula of the net current yields an expression for the time dependence of the total current. Notably, the total current I is composed of a transient and a steady-state component (I_{ss}). The steady-state current is given by:

$$I_{ss} = F \cdot E_{tot} \cdot \frac{K_1 \cdot k_2 - K_{-2} \cdot k_{-1}}{K_1 + k_{-1} + k_2 + K_{-2}}$$

Here, F is Faraday constant.
I_{ss} is limited to

$$I_{ss(V \to -\infty)} = -F \cdot E_{tot} \cdot k_{-1}$$

at extremely hyperpolarizing potentials.

The transient current decays with the total rate:

$$k_{tot} = k_1 \cdot \left[Na^+\right]_{\text{int}} \exp\left(\frac{\alpha \cdot F \cdot V}{RT}\right) + \frac{k_1 \cdot k_2}{k_{-2}} + k_3 + k_2 \cdot \left[Na^+\right]_{ext} \exp\left(-\frac{\beta \cdot F \cdot V}{RT}\right) \quad (2)$$

Notably, this rate increases upon hyperpolarization and depolarization. This is a consequence of the assumption that the occluded state is accessible from the extracellular as well as the intracellular medium.

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