THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 21, Issue of May 23, pp. 19237–19244, 2003
Printed in U.S.A.

Electrogenicity of Na,K- and H,K-ATPase Activity and Presence of a Positively Charged Amino Acid in the Fifth Transmembrane Segment*

Received for publication, January 28, 2003, and in revised form, March 7, 2003
Published, JBC Papers in Press, March 12, 2003, DOI 10.1074/jbc.M300946200

Muriel Burnay, Gilles Crambert, Solange Kharoubi-Hess, Käthi Geering, and Jean-Daniel Horisberger‡

From the Institute of Pharmacology and Toxicology, University of Lausanne, CH-1005 Lausanne, Switzerland

The transport activity of the Na,K-ATPase (a 3 Na^+ for 2 K^+ ion exchange) is electrogenic, whereas the closely related gastric and non-gastric H,K-ATPases perform electroneutral cation exchange. We have studied the role of a highly conserved serine residue in the fifth transmembrane segment of the Na,K-ATPase, which is replaced with a lysine in all known H,K-ATPases. Ouabain-sensitive ^{86}Rb uptake and K^+-activated currents were measured in *Xenopus* oocytes expressing the *Bufo* bladder H,K-ATPase or the *Bufo* Na,K-ATPase in which these residues, Lys^{800} and Ser^{782}, respectively, were mutated. Mutants K800A and K800E of the H,K-ATPase showed K^+-stimulated and ouabain-sensitive electrogenic transport. In contrast, when the positive charge was conserved (K800R), no K^+-induced outward current could be measured, even though rubidium transport activity was present. Conversely, the S782R mutant of the Na,K-ATPase had non-electrogenic transport activity, whereas the S782A mutant was electrogenic. The K800S mutant of the H,K-ATPase had a more complex behavior, with electrogenic transport only in the absence of extracellular Na^+. Thus, a single positively charged residue in the fifth transmembrane segment of the α-subunit can determine the electrogenicity and therefore the stoichiometry of cation transport by these ATPases.

P-type ATPases form a large group of ion-motive ATPases that have an essential physiological role in the transport of various cations across cell membranes. Even though a high resolution structure of two conformations of one of these P-type ATPases, the sarcoplasmic/endoplasmic reticulum Ca-ATPase (SERCA),[1] has been determined (1, 2), the mechanism of cation translocation is not yet known. Group IIc of the P-ATPases, according to the classification proposed by Axelsen and Palmgren (3), comprises the closely related Na,K- and H,K-ATPases, which share >60% amino acid identity and provide related functions: all members of this group import K^+ ions into the cell in exchange for export of another cation, Na^+ or H^+. The activity of the Na,K-ATPases is electrogenic because of the asymmetrical exchange of 3 Na^+ ions for 2 K^+ ions, and this electrogenicity has been verified for several isoforms and species origin of the enzyme (4–6). In contrast, the gastric H,K-ATPase is known to perform an electroneutral exchange of H^+ for K^+, most probably 2 K^+ ions for 2 protons (7). We have recently shown that the "non-gastric" H,K-ATPase has electroneutral transport activity (8), but this information only indicates that a symmetrical number of cations have to be exchanged. The exact nature of the transported cations has not yet been fully determined, as both H^+ (9) and Na^+ (10, 11) seem to be transported in exchange for K^+; and thus, the transport stoichiometry of this ATPase is not known.

The intramembranous part of the group IIc P-ATPases is made up of 10 transmembrane segments of the α-subunit plus the single transmembrane segment of the associated β-subunit. The α-subunits of the Na,K- and H,K-ATPases contain between 5 and 7 negatively charges residues in the transmembrane segments (this count does not include the charged residues located at the intracellular or extracellular borders of most of the 10 transmembrane segments). Within the transmembrane segments, no positively charged amino acids are present in any of the known sequences of the Na,K-ATPase α-subunit, whereas a single positively charged residue, a highly conserved lysine, is present in all known sequences of the gastric and non-gastric H,K-ATPases (for instance, Lys^{800} in the *Bufo marinus* bladder H,K-ATPase α-subunit sequence). A similarly highly conserved serine residue (for instance, Ser^{775} in the sheep α_1-subunit sequence) is present at the corresponding position in all Na,K-ATPase α-subunit sequences. Fig. 1 shows a comparison of the highly conserved sequences of the fifth transmembrane segment of the Na,K- and H, K-ATPases α-subunits.

Because of the coincidence of electrogenic and non-electrogenic types of activity in the absence and presence, respectively, of a positively charged residue in the middle of the fifth transmembrane segment, we hypothesized that this charged amino acid has a major role in the stoichiometry of cation transport by the group IIc P-ATPases. We tested this hypothesis by studying the electrogenicity of cation transport by the Na,K- and non-gastric H,K-ATPases mutated at this single position.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—The full-length cDNA of the *B. marinus* bladder H,K-ATPase α-subunit (9) was first subcloned into the pSD5 vector containing a 130-bp poly(T) tail. Removal of 110 bp of the 5′-untranslated region containing a GC-rich region significantly improved expression of this protein in *Xenopus* oocytes. The α_1-subunit of the *B. marinus* Na,K-ATPase (12) was available in the same vector. For simplicity, the α-subunit of the *B. marinus* bladder H,K-ATPase and the α_1-subunit of the *B. marinus* Na,K-ATPase will be referred to α_{bl} H,K-ATPase and α_1 Na,K-ATPase, respectively. Point mutations were introduced by the PCR method (13). The linearized pSD5 vector containing

* This work was supported by Swiss Fonds National de la Recherche Scientifique Grant 31-65441.01. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Inst. de Pharmacologie et de Toxicologie, Université de Lausanne, Bugnon 27, CH-1005 Lausanne, Switzerland. Tel.: 41-21-692-5362; Fax: 41-21-692-5355; E-mail: Jean-Daniel.Horisberger@ipharm.unil.ch.

[1] The abbreviations used are: SERCA, sarcoplasmic/endoplasmic reticulum Ca-ATPase; α_{bl} H,K-ATPase, *B. marinus* bladder H,K-ATPase α-subunit; α_1 Na,K-ATPase, *B. marinus* Na,K-ATPase α_1-subunit; β_{bl}, *B. marinus* bladder β-subunit; TM, transmembrane domain.

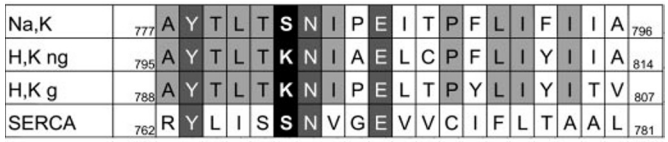

FIG. 1. **Sequence comparison of the fifth transmembrane segments of the Na,K-ATPase and gastric and non-gastric H,K-ATPase α-subunits.** The sequences shown are the α_1 Na,K-ATPase sequence (GenBank™/EBI accession number P30764; used in this study), the non-gastric (*ng*) α_{bl} H,K-ATPase sequence (accession number Q92036; used in this study), and the rat gastric (*g*) H,K-ATPase α-subunit sequence (accession number P09626). Also shown is the sequence of the fifth transmembrane segment of rabbit SERCA (accession number P04191), for which a high resolution three-dimensional structure is known (Protein Data Bank code 1EUL) (1). *Gray shading* highlights the residues that are conserved in the Na,K- and H,K-ATPase subfamily. The Tyr, Asn, and Glu residues, which are also conserved in SERCA, are indicated by *white letters on a gray background*. The positions investigated in this study are shown in *white letters on a black background*. The first and last residue positions of the segments are indicated in the first and last columns.

the cDNA of either α_1 Na,K- or α_{bl} H,K-ATPase was used as a template for the preparation of mutants. The mutated DNA fragments were introduced into the wild-type α_{bl} H,K-ATPase using unique *Nsi*I and *Pfl*MI restriction sites and into the wild-type α_1 Na,K-ATPase using unique *Bln*I and *Sun*I restriction sites. All mutations were verified by sequencing. cRNA was obtained by *in vitro* transcription.

Expression in Xenopus Oocytes—Oocytes were obtained from *Xenopus* females anesthetized by exposure to MS222 (2 g/liter; Sandoz, Basel, Switzerland) and prepared as described previously (14). Oocytes were injected with 8 ng of wild-type or mutant α_{bl} H,K-ATPase cRNA or with 8 ng of α_1 Na,K-ATPase cRNA in combination with 1 ng of *B. marinus* bladder β-subunit cRNA (15). Expression of wild-type and mutant Na,K- and H,K-ATPases was examined by pulse (24 h)-chase (48 h) experiments using [^{35}S]methionine metabolic labeling. Microsomes were prepared as described previously (16) and loaded onto 5–13% SDS-polyacrylamide gels. For the Na,K-ATPase mutants, the α-subunit was also immunoprecipitated using anti-*Bufo* α_1-subunit antibody (17) under nondenaturing conditions as described (18), allowing co-immunoprecipitation of the associated β-subunit. The dissociated immune complexes were separated by SDS-PAGE, and labeled proteins were detected by fluorography. Quantification of immunoprecipitated bands was performed with an Amersham Biosciences Ultrascan 2202 laser densitometer.

Electrophysiological Measurements—Three days after injection, the oocytes were loaded with Na^+ by a 2-h exposure to a K^+-free solution as described (19). The incubation solution contained 0.2 μM ouabain to inhibit the endogenous *Xenopus* oocyte Na,K-ATPase; we have shown previously (6, 20) that this concentration of ouabain is sufficient to fully inhibit the endogenous *Xenopus* oocyte Na,K-ATPase and that the dissociation rate constant of ouabain from the *Xenopus* oocyte Na,K-pump is slow enough so that inhibition is maintained during the duration of the electrophysiological recording even if no ouabain is included in the measurement solutions. Currents associated with the activity of the putative H,K- and Na,K-ATPases were measured by the two-electrode voltage-clamp technique using a TEV-200 voltage-clamp apparatus (Dagan Corp., Minneapolis, MN). Current signals were filtered at 20 Hz and recorded on a Gould Model 220 paper chart recorder. The intracellular potential was held at −50 mV. Current/voltage curves were obtained by applying series of 500-ms voltage steps ranging from −150 to + 30 mV.

The Na,K-pump function was evaluated in an Na^+-rich solution (100 mM sodium gluconate, 0.82 mM $MgCl_2$, 0.41 mM $CaCl_2$, 10 mM *N*-methyl-D-glucamine/Hepes, 5 mM $BaCl_2$, and 10 mM tetraethylammonium chloride, pH 7.4) or in an Na^+-free solution (a similar solution in which 100 sodium gluconate was replaced with 160 mM sucrose) by activation of the current induced by addition of K^+ (10 mM K^+ when an Na^+-rich solution was used or 5 mM K^+ when an Na^+-free solution was used) to a previously K^+-free solution and by the effect of 2 mM ouabain in the K^+-containing solution. In another set of experiments, the kinetics of activation of the current by K^+ were studied in Na^+-rich and Na^+-free solutions by measuring the current induced by increasing concentrations of extracellular K^+ (0.3, 1, 3, and 10 mM K^+ in Na^+-rich solution and 0.02, 0.1, 0.5, and 5 mM K^+ in Na^+-free solutions) as described previously (19). The maximal K^+-activated current (I_{max}) and the half-activation constant ($k_{½}$) were determined as the best fitting parameters of the Hill equation using a Hill coefficient of 1.6 for the measurements performed in the presence of Na^+ and a Hill coefficient of 1.0 for those performed in the absence of extracellular Na^+ as described previously (19).

Whereas the K^+-induced current is a reliable indicator of the Na,K-pump activity at low K^+ concentrations, increasing the extracellular K^+ concentration above 10 mM results in sizable and variable inward currents independent of the activity of the Na,K-pump. These currents are probably due to K^+ flowing through channels that are insufficiently blocked by Ba^{2+} and tetraethylammonium; thus, for some mutants of the Na,K-ATPase showing a very low affinity for K^+, the electrogenic transport was evaluated as the current sensitive to 2 mM ouabain in the presence of 40 mM extracellular K^+ (a solution similar to the Na^+-rich solution described above, except that Na^+ was partially replaced with K^+, yielding 60 mM Na^+).

^{86}Rb Uptake Measurements—^{86}Rb uptake was performed as previously described (16). Briefly, after loading the oocytes with Na^+ (see above), oocytes were transferred to a solution containing 5 mM KCl, 90 mM NaCl, 1 mM $CaCl_2$, 5 mM $BaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, pH 7.4, 0.2 μM ouabain (to inhibit the endogenous oocyte Na,K-ATPase), and 10 μM bumetanide (to inhibit the uptake of ^{86}Rb by the Na-K-2Cl cotransporter). After addition of 5 μCi/ml ^{86}Rb (Amersham Biosciences), oocytes were incubated for 12 min at room temperature and than washed in a solution containing 90 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM Hepes, pH 7.4. Individual oocytes were then dissolved in 0.5% SDS and counted. In each experiment, a group of 10–12 oocytes injected with the β-subunit alone was used a control. The transport activity of the expressed wild-type or mutant ATPase was estimated as the ^{86}Rb uptake by an experimental oocyte minus the mean ^{86}Rb uptake measured in the same experiment by the group of oocytes injected with the β-subunit alone. As described above, some mutants had a low affinity for extracellular K^+. For these mutants, the Na,K-pump transport function was evaluated as ^{86}Rb uptake sensitive to 2 mM ouabain in 40 mM K^+ solution (sodium replacement).

Statistical Analysis—Data are presented as means ± S.E. (n = number of observations). Statistical analysis of the data was performed by paired Student's t test when pairs of measurements obtained in the same oocyte were compared or by the unpaired Student's t test when different groups of oocytes were compared.

RESULTS

Expression of the B. marinus Na,K- and H,K-ATPase Mutants—We replaced the lysine in the fifth transmembrane segment of α_{bl} H,K-ATPase (Lys^{800}) with a neutral (K800A and K800S), negatively charged (K800E), or positively charged (K800R) residue. Similarly, the serine at the corresponding position in the fifth transmembrane segment of α_1 Na,K-ATPase (Ser^{782}) was mutated to a neutral (S782A), negatively charged (S782E), or positively charged (S782R and S782K) residue.

We chose to coexpress these wild-type and mutant α-subunits with the *B. marinus* bladder isoform of the β-subunit (β_{bl}), which is the amphibian homolog of the mammalian β_2-isoform, because the α_1/β_{bl} and α_{bl}/β_{bl} dimers are well expressed at a similar level in *Xenopus* oocytes (8), and it was essential to express the various wild-type and mutant α-subunits with the same β-subunit to be able to attribute the observed differences to the mutations carried by the α-subunit.

Expression of wild-type and mutant Na,K- and H,K-ATPases was first examined using metabolic labeling. Fig. 2*A* shows that all mutant H,K-ATPase α-subunits were expressed at similar levels as the wild-type H,K-ATPase α-subunit. All α-subunits appeared stable after a 48-h chase period, which indicates that they are associated with the β-subunit (16). The same was observed for all of the mutant Na,K-ATPase α-subunits (Fig. 2*B*). A more detailed analysis by immunoprecipitation (Fig. 2*C*) revealed that α-subunit mutants S782A and S782K, similar to the wild-type α-subunit, were associated with a fully glycosylated β-subunit after a 48-h chase period, indicating their correct routing toward the plasma membrane. In contrast, the S782E mutant and, to a lesser extent, the S782R mutant remained associated with a core glycosylated

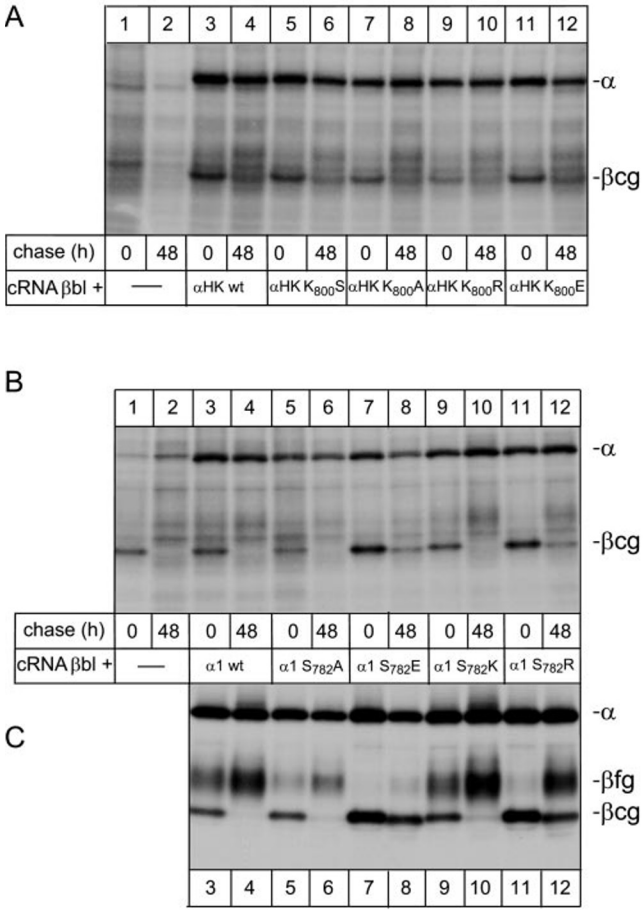

FIG. 2. **SDS-PAGE of microsomes isolated from oocytes expressing wild-type and mutant Na,K- and H,K-ATPases.** *A*, after a 24-h pulse and 48-h chase, microsomes from non-injected oocytes (*lanes 1* and *2*) or from oocytes injected with β_{bl} cRNA and the cRNA from the wild-type H,K-ATPase α-subunit (αHK *wt*; *lanes 3* and *4*), mutant K800S ($\alpha HK\ K_{800}S$; *lanes 5* and *6*), mutant K800A ($\alpha HK\ K_{800}A$; *lanes 7* and *8*), mutant K800R ($\alpha HK\ K_{800}R$; *lanes 9* and *10*), or mutant K800E ($\alpha HK\ K_{800}E$; *lanes 11* and *12*) were directly loaded onto SDS-polyacrylamide gels. *B*, after a 24-h pulse and 48-h chase, microsomes from non-injected oocytes (*lanes 1* and *2*) or from oocytes injected with β_{bl} cRNA and the cRNA from the wild-type Na,K-ATPase α_1-subunit ($\alpha 1$ *wt*; *lanes 3* and *4*), mutant S782A ($\alpha 1\ S_{782}A$; *lanes 5* and *6*), mutant S782E ($\alpha 1\ S_{782}E$; *lanes 7* and *8*), mutant S782K ($\alpha 1\ S_{782}K$; *lanes 9* and *10*), or mutant S782R ($\alpha 1\ S_{782}R$; *lanes 11* and *12*) were directly loaded onto SDS-polyacrylamide gels. *C*, the same microsomes as in *B* were immunoprecipitated as described under "Experimental Procedures." The positions of the α- and β-subunits are indicated to the right. βfg, fully glycosylated β-subunit; βcg, core glycosylated β-subunit. Due to the fuzzy appearance, the fully glycosylated β-subunit cannot clearly be distinguished in non-immunoprecipitated samples (*A* and *B*).

β-subunit, indicating their (partial) retention in the endoplasmic reticulum.

We then evaluated the functional expression of the different mutants of the Na,K- and H,K-ATPases in comparison with wild-type pumps using the ^{86}Rb uptake assay. Fig. 3*A* shows that, in the presence of 5 mM K^+, the K800S and K800A mutants of the H,K-ATPase were functionally expressed at similar levels as the wild-type Na,K- and H,K-ATPases, with rubidium transport values of ~40–60 pmol/min/oocyte above the background uptake observed in control oocytes injected with β_{bl} alone. The K800R and K800E mutants of the H,K-ATPase had smaller but significant levels of rubidium uptake (~5–20 pmol/min/oocyte). Both α_1 Na,K-ATPase and α_{bl} H,K-ATPase can be inhibited by high concentrations of ouabain (9, 12); and in another series of experiments, we tested the sensitivity of ^{86}Rb uptake to ouabain by comparing the uptake in the

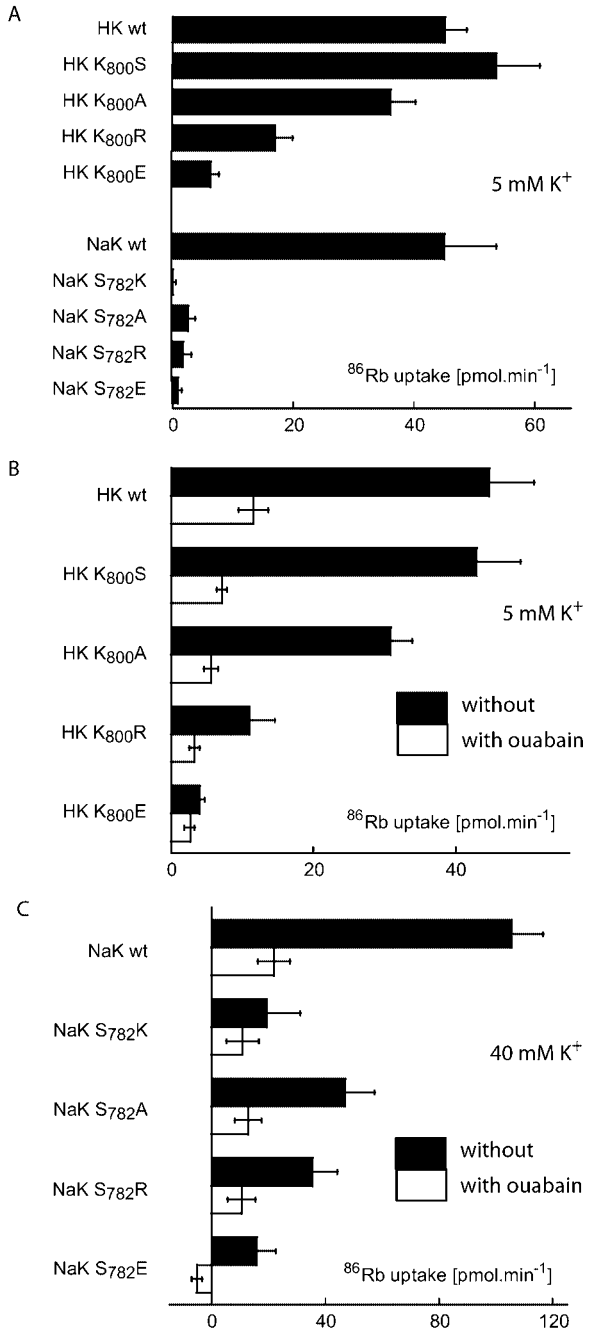

FIG. 3. **Rubidium uptake by wild-type and mutant Na,K- and H,K-ATPases.** The uptake of ^{86}Rb was evaluated in Na^+-loaded oocytes injected with the cRNA from β_{bl} alone or with an Na,K- or H,K-ATPase α-subunit. *A*, rubidium transport in 5 mM K^+ solution. In each experiment, the base-line ^{86}Rb uptake recorded in oocytes injected with the β-subunit cRNA alone was subtracted from each of the other groups; it amounted to a mean value of 8.6 ± 0.7 pmol/min (n = 34). The number of measurements was as follows: wild-type Na,K-ATPase (NaK *wt*; n = 24) and mutants S782K ($NaK\ S_{782}K$; n = 21), S782A ($NaK\ S_{782}A$; n = 22), S782R ($NaK\ S_{782}R$; n = 24), and S782E ($NaK\ S_{782}E$; n = 22); and wild-type H,K-ATPase (HK *wt*; n = 18) and mutants K800S ($HK\ K_{800}S$; n = 21), K800A ($HK\ K_{800}A$; n = 24), K800R ($HK\ K_{800}R$; n = 23), and K800E ($HK\ K_{800}E$; n = 24). *B*, ^{86}Rb uptake in 5 mM K^+ solution in the absence (*black bars*) and presence (*white bars*) of 2 mM ouabain in oocytes expressing wild-type and mutant H,K-ATPases. The number of experiments was between 19 and 42 for each group. The values shown are those obtained after subtraction of the mean value of ^{86}Rb uptake measured in a group of oocytes injected with β-subunit cRNA alone or with ouabain. *C*, ^{86}Rb uptake in 40 mM K^+ solution in the absence (*black bars*) and presence (*white bars*) of 2 mM ouabain in oocytes expressing wild-type and mutant Na,K-ATPases. The number of experiments was between 43 and 48 for each group. The values shown are those obtained after subtraction of the mean value of ^{86}Rb uptake measured in a group of oocytes injected with β-subunit cRNA alone or with ouabain.

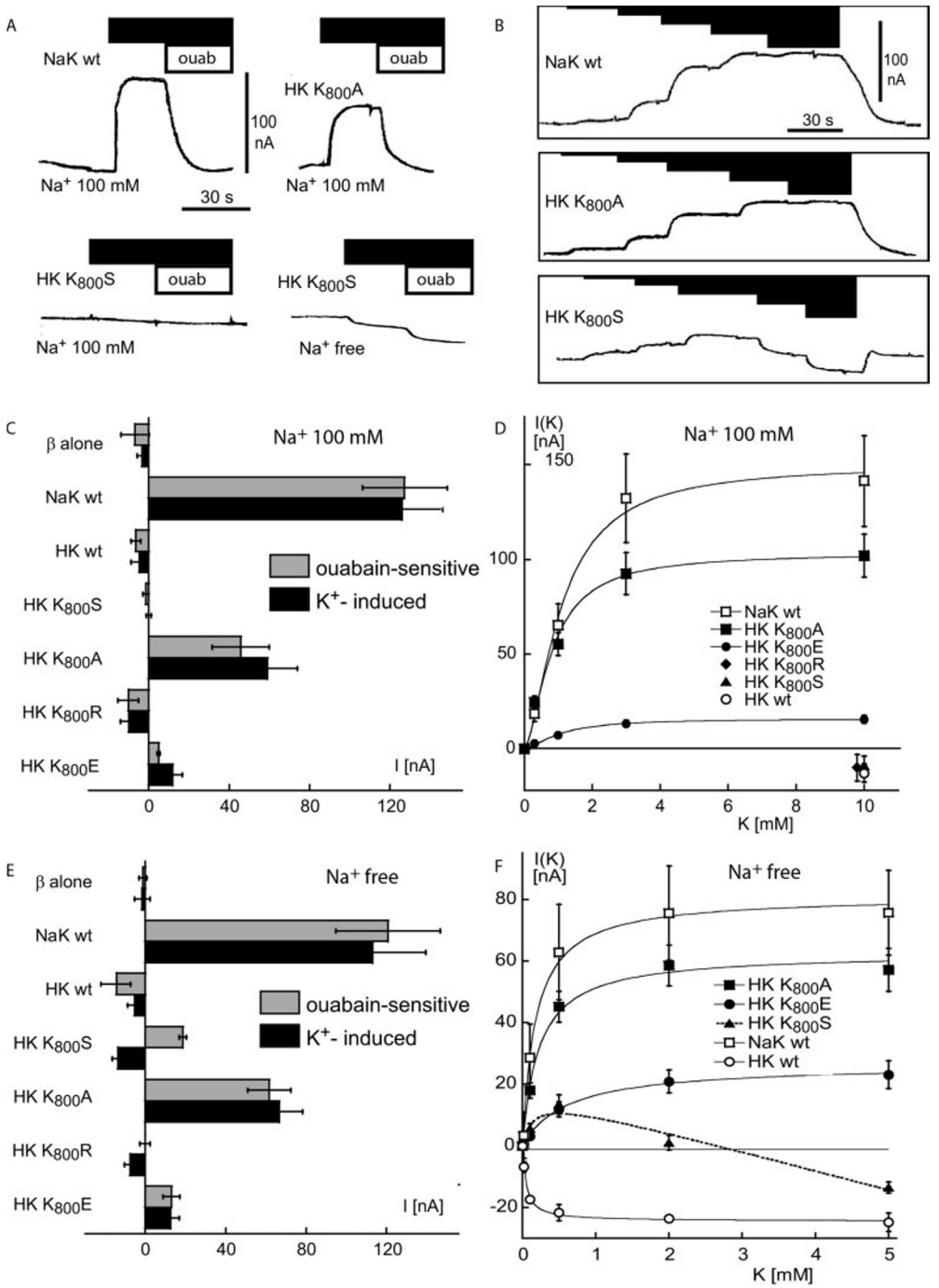

FIG. 4. **Electrogenic transport in oocytes expressing the wild-type Na,K-ATPase and wild-type and mutant H,K-ATPases.** *A*, examples of current trace recordings showing the K^+-induced (exposure to 10 mM K^+ is indicated by the *black bars* above the current traces) and ouabain-sensitive (exposure to 2 mM ouabain (*ouab*)) currents. The examples are those obtained with the wild-type Na,K-ATPase (*NaK wt*) and the K800A (*HK $K_{800}A$*) and K800S (*HK $K_{800}S$*) mutant H,K-ATPases in the presence of extracellular Na^+ and with the K800S mutant in the absence of extracellular Na^+. The holding potential was −50 mV. The indicated current and time scales are the same for the four examples. *B*, examples of current trace recordings showing the current changes induced by increasing concentrations of K^+ from K^+-free to 0.02, 0.1, 0.5, and 5 mM K^+ and back to K^+-free as indicated by the *progressively thicker black bars* above the current traces. The examples are those obtained with the wild-type Na,K-ATPase and the K800A and K800S mutant H,K-ATPases in the absence of extracellular Na^+. The indicated current and time scales are the same for the three examples. *C*, mean ± S.E. of K^+-induced and ouabain-sensitive currents in the presence of 100 mM extracellular Na^+. The current induced by 10 mM K^+ and that inhibited by 2 mM ouabain (in the presence of 10 mM K^+) were measured successively in oocytes expressing the β-subunit alone (control; $n = 6$); the wild-type Na,K-ATPase ($n = 6$); the wild-type H,K-ATPase (*HK wt*; $n = 6$); and the K800A ($n = 6$), K800S ($n = 10$), K800E (*HK $K_{800}E$*; $n = 6$), and K800R (*HK $K_{800}R$*; $n = 6$) mutant H,K-ATPases. *D*, mean ± S.E. of K^+-induced currents as a function of the extracellular K^+ concentrations in oocytes expressing the wild-type Na,K-ATPase ($n = 8$) and the K800A ($n = 12$) and K800E ($n = 8$) mutant H,K-ATPases. The measurements of the currents activated by different K^+ concentrations were recorded in the presence of 100 mM extracellular Na^+ and at a holding potential of −50 mV. The *solid lines* are the best fitting Hill equation with a Hill coefficient of 1.6 (see "Experimental Procedures"). The currents induced by 10 mM K^+ are also indicated for the K800R mutant ($n = 4$), K800S mutant ($n = 5$), and wild-type H,K-ATPases ($n = 7$); but because the amplitudes of the currents were very small in these cases, it was not possible to record reliable K^+-induced current values at lower concentration. In some cases, errors bars (S.E.) are smaller than the symbol size. *E*, K^+-induced and ouabain-sensitive currents in the absence of extracellular Na^+. The current induced by 5 mM K^+ and that inhibited by 2 mM ouabain were measured successively in oocytes expressing the β-subunit alone (control; $n = 5$); the wild-type Na,K-ATPase ($n = 6$); the wild-type H,K-ATPase ($n = 5$); and the K800A ($n = 6$), K800S ($n = 6$), K800E ($n = 8$), and K800R ($n = 6$) mutant H,K-ATPases. *F*, K^+-induced currents as a function of the

presence and absence of 2 mM ouabain. As shown in Fig. 3*B*, except for the K800E mutant, for which no significant ouabain-sensitive ^{86}Rb uptake could be detected, a large part of the ^{86}Rb uptake by the mutants was sensitive to a high concentration (2 mM) of ouabain, similar to wild-type α_{bl} H,K-ATPase, as expected from the known low sensitivity observed in previous measurements (9, 12).

No significant rubidium transport could be detected under similar conditions (in the presence of 5 mM K^+) in oocytes expressing the various mutants of α_1 Na,K-ATPase (Fig. 3*A*). Only the S782A mutant had a very small but significant ^{86}Rb uptake of 2.6 ± 1.1 pmol/min, whereas the wild-type Na,K-ATPase expressed a transport activity of 45.1 ± 8.6 pmol/min, similar to that of the wild-type H,K-ATPase. A low affinity for extracellular K^+ has been observed with mutants of the corresponding position (Ser^{775}) in α_1 Na,K-ATPase from other species (21–23). Assuming a similar effect of homologous mutations in the *Bufo* α_1 Na,K-pump, we studied the transport function of the Ser^{782} mutants at a higher (40 mM) concentration of K^+. Under these conditions (Fig. 3*C*), significant ouabain-sensitive ^{86}Rb uptake could be observed in the different Na,K-ATPase mutants. The rate of transport was, however, smaller than for the wild-type pump (15–45 pmol/min/oocyte for the mutants *versus* 105 pmol/min/oocyte for the wild type, n = 43–48 oocytes in each group). These results indicate that all of the mutants were indeed expressed and were able to perform rubidium uptake, although some of them with a lower affinity and at a reduced level of activity compared with the wild-type Na,K-ATPase.

Electrogenic Transport by the Lys^{800} Mutants of the H,K-ATPase—The activity of the wild-type and Lys^{800} mutant H,K-ATPases was first examined in the presence of 100 mM extracellular Na^+ as the current activated by 10 mM K^+ and as the current inhibited by 2 mM ouabain in the presence of 10 mM K^+ (Fig. 4, *A* (examples) and *C* (mean values)). When studied under these conditions, the wild-type Na,K-pump generated outward currents amounting to ~120 nA; and as reported previously (8), activation by extracellular K^+ results only in small *inward* currents in oocytes expressing the wild-type *Bufo* bladder H,K-ATPase. An K^+-activated and ouabain-sensitive outward current was observed with the K800A mutant H,K-ATPase and, with a very small amplitude, with the K800E mutant H,K-ATPase, indicating the presence of electrogenic transport activity. The smaller amplitude of the outward current expressed by the K800E mutant can be related to the lower transport activity of this mutant (Fig. 3, *A* and *B*) and might be due to a slower turnover rate of the transport cycle or to a lower level of expression of this protein at the cell surface even though the protein is synthesized and appears stable (Fig. 2).

We then examined the concentration dependence of the K^+-induced current (0.3, 1.0, 3.0, and 10.0 mM extracellular K^+) at −50 mV in the mutants that had an outward Na,K-pump current as shown in Fig. 4*D*. The $k_{½}$ values were 1.18 ± 0.10 mM for the wild-type Na,K-ATPase (n = 8), 0.75 ± 0.06 mM for the K800A mutant (n = 12), and 1.52 ± 0.23 mM for the K800E mutant (n = 8).

We also examined the electrogenic transport activity of the H,K-ATPase mutants in the absence of extracellular Na^+ (Fig. 4*B*), a condition under which the affinity for external K^+ is higher (19). Fig. 4*E* shows that ouabain-sensitive outward currents were recorded in the oocytes expressing the wild-type Na,K-ATPase and those expressing the K800A, K800S, and K800E mutants, whereas there were no such currents in the oocytes injected with the β-subunit alone (control group). The K^+-induced currents were of similar magnitude as the ouabain-sensitive currents in all these groups except for the K800S mutant, for which a small but significant inward current was induced by 5 mM K^+, whereas ouabain addition (in the presence of 5 mM K^+) caused a further inward current shift, demonstrating the existence of an ouabain-sensitive outward current.

The concentration dependence of the K^+-induced current (0.02, 0.1, 0.5, 2, and 5 mM extracellular K^+) in the absence of extracellular Na^+ and at a holding potential of −50 mV is shown in Fig. 4*F*. These results are analogous to those observed in the presence of extracellular Na^+ for the wild-type Na,K-ATPase and the K800A and K800E mutants. The K800A mutant had a slightly lower K^+-induced current amplitude, whereas the K800E mutant had a much reduced current amplitude. The apparent affinity for K^+ was 0.24 ± 0.12 mM (n = 6) for the Na,K-ATPase, a value similar to that reported earlier under similar conditions (19); 0.23 ± 0.02 mM (n = 11) for the K800A mutant; and 0.73 ± 0.14 mM (n = 6) for the K800E mutant. The K800S mutant showed a more complex K^+ concentration/current relationship with a positive (outward) current in the low concentration range, reaching a maximal value of 12.7 ± 0.6 nA (n = 15) recorded at 0.5 mM K^+, which reverted to a negative (inward) current at higher concentrations. Fig. 4*F* also shows the concentration dependence of the inward current induced by K^+ in the wild-type H,K-ATPase, a current that is related rather to the intracellular alkalization than to an electrogenic transport activity of this H,K-ATPase as shown earlier (8). The maximal K^+-induced current was −25.9 ± 1.8 nA (n = 5), and the apparent affinity for external K^+ was 0.12 ± 0.08 mM.

The voltage dependence of the activity of the wild-type Na,K- and H,K-ATPases and of the electrogenic mutants of the H,K-ATPase was also studied in Na^+-free extracellular solutions. The currents induced by 5 mM K^+ in the wild-type Na,K- and H,K-ATPases and in the K800A and K800E mutants of the H,K-ATPase are shown as a function of the membrane potential in Fig. 5*A*. For the K800S mutant of the H,K-ATPase, we chose the current induced by 0.5 mM K^+, which was the concentration giving the highest outward current. The wild-type Na,K-ATPase current/voltage curve shows little voltage dependence in the 0 to −50 mV membrane potential range and a negative slope in the high negative voltage range, similar to what has been described previously for Na,K-pumps expressed in *Xenopus* oocytes (6, 19). Potassium induced a voltage-dependent inward current in the wild-type H,K-ATPase, similar to what we have described previously (8). The K800A and K800E mutants were only weakly voltage-dependent, whereas the K800S mutant displayed stronger voltage dependence along the whole membrane potential range (a 4-fold reduction of the ouabain-sensitive current between 10 and −110 mV compared with a <30% decrease for the K800A and K800E mutants over the same voltage range).

The negative slope of the Na,K-ATPase K^+-activated cur-

extracellular K^+ concentrations in an Na^+-free extracellular solution in oocytes expressing the wild-type Na,K-ATPase (n = 7); the K800A (n = 11), K800E (n = 6), and K800S (n = 13) mutant H,K-ATPases; and the wild-type H,K-ATPase (n = 5). The currents activated by different K^+ concentrations were recorded at a holding potential of −50 mV. The *solid lines* are the best fitting Hill equation with a Hill coefficient of 1 (see "Experimental Procedures"). The *dashed line* is the best fitting curve using a model in which we assume that the current is the sum of a Hill equation with a Hill coefficient of 1 plus a linear term, $I = (I_{max}/(1 + (k_{½}/[K^+]))) + k_1 \cdot [K^+]$, where I is the resulting membrane current, $[K^+]$ is the potassium concentration, I_{max} is the maximal outward current, $k_{½}$ is the apparent potassium affinity for the outward current, and k_1 is the constant defining the linear term. The best fitting parameters illustrated by the *dashed line* are I_{max} = 16.2 nA, $k_{½}$ = 0.127 mM, and k_1 = −5.9 nA/mM.

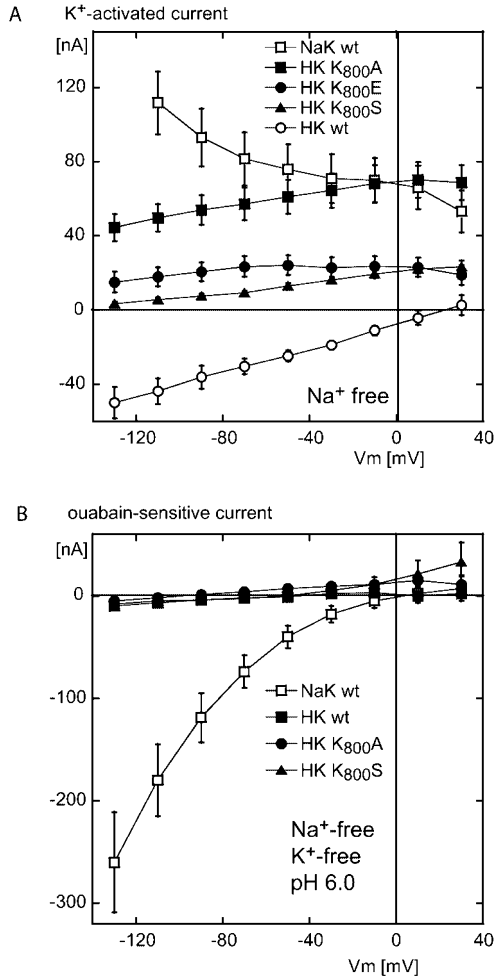

FIG. 5. **Voltage dependence of the electrogenic transport activity of the wild-type Na,K-ATPase and wild-type and mutant H,K-ATPases in the absence of extracellular Na^+.** *A*, 5 mM K^+-activated current as a function of membrane potential (V_m) in oocytes expressing the wild-type Na,K-ATPase (*NaK wt*; $n = 6$); the K800A (*HK* $K_{800}A$; $n = 8$), K800E (*HK* $K_{800}E$; $n = 5$), and K800S (*HK* $K_{800}S$; $n = 5$) mutant H,K-ATPases; and the wild-type H,K-ATPase (*HK wt*; $n = 5$). *B*, current inhibited by 2 mM ouabain in an Na^+- and K^+-free extracellular solution at pH 6.0 as a function of the membrane potential in oocytes expressing the wild-type Na,K-ATPase ($n = 5$), the K800A ($n = 7$) and K800S ($n = 6$) mutant H,K-ATPases, and the wild-type H,K-ATPase ($n = 6$).

rent/voltage curve has been attributed to inhibition by K^+ of proton leak conductance in Na^+- and K^+-free extracellular solutions (24). We examined whether the H,K-ATPase and its electrogenic mutant would present a similar "proton leak." Fig. 5*B* shows the current sensitive to 2 mM ouabain in a K^+- and Na^+-free solution at pH 6.0. As shown previously (6, 24), ouabain inhibited a large inward current in the Na,K-ATPase, but there were no such ouabain-sensitive currents in the H,K-ATPase or in any of the mutants.

Electrogenic Transport by the Ser^{782} Mutants of the Na,K-ATPase—The stimulation of electrogenic transport by extracellular K^+ was also examined in the two Na,K-ATPase mutants (S782A and S782R) that had sizable K^+ transport (^{86}Rb uptake) activity. As these mutants had a very low apparent affinity for K^+ (see above and Fig. 2), we measured the current inhibited by 2 mM ouabain in 40 mM K^+ solution. Under these conditions, as shown in Fig. 6, ouabain inhibited an outward current over the whole potential range in the wild-type Na,K-ATPase and in the S782A mutant, but not in the S782R mutant. The ouabain-sensitive current of the wild-type Na,K-

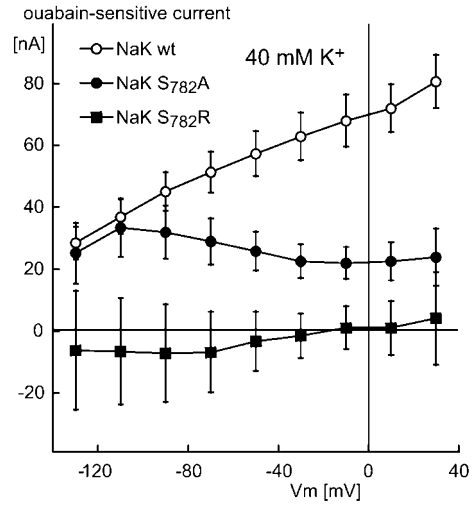

FIG. 6. **Na,K-pump currents in wild-type and mutant Na,K-ATPases at a high extracellular K^+ concentration.** The currents inhibited by 2 mM ouabain in the presence of 40 mM extracellular K^+ are shown over the −130 to +30 mV range for the wild-type Na,K-ATPase (*NaK wt*; $n = 11$), the S782A mutant (*NaK* $S_{782}A$; $n = 17$), and the S782R mutant (*NaK* $S_{782}R$; $n = 16$).

ATPase was voltage-dependent, whereas there was no clear voltage dependence for the S782A mutant over the explored potential range. As both of these mutants had significant ^{86}Rb uptake activity under similar conditions (Fig. 3*C*), these results indicate that the S782A mutant is electrogenic, but that the S782R mutant has electroneutral cation transport activity.

DISCUSSION

We have taken advantage of the functional differences and the high degree of sequence homology between the H,K- and Na,K-ATPases to attempt to identify the determinant of the electrogenicity of cation transport by the group IIc P-ATPases. In the fifth transmembrane segment, several amino acid residues have been shown to play a role in cation binding in both the H,K- and Na,K-ATPases (22, 23, 25–31) and also in SERCA (32). The middle of the fifth transmembrane segment region is highly similar between the H,K- and Na,K-ATPases (Fig. 1), except for the presence of a positively charged lysine in the H,K-ATPase instead of a non-charged serine in the Na,K-ATPase.

The results of our amino acid substitution generally support our starting hypothesis, *viz.* that the presence of the positively charged amino acid in the middle of the fifth transmembrane segment is a determinant for the stoichiometry of the cation exchange by the Na,K- and H,K-ATPases. When Ser^{782} of the Na,K-ATPase was replaced with a positively charged arginine, the mutant was able to transport ^{86}Rb, but showed no electrogenic transport activity, whereas mutation to a neutral alanine induced a reduction of the apparent affinity for K^+, but preserved an electrogenic mode of transport. Conversely, mutation of the positively charged Lys^{800} of the H,K-ATPase to alanine yielded electrogenic transport activity roughly similar in amplitude and apparent affinity for extracellular K^+ to that of the Na,K-pump, whereas replacing Lys^{800} with another positively charged residue, arginine, produced non-electrogenic transport activity. Surprisingly, replacing Lys^{800} of the H,K-ATPase with a negatively charged glutamic acid yielded significant electrogenic transport activity, which conserved an apparent affinity for extracellular K^+ not too different from that of the wild-type Na,K-pump, even though the transport activity could hardly be detected by the less sensitive ^{86}Rb transport assay. Our results do not allow us to draw conclusions about the precise stoichi-

ometry of the cation exchange. It can only be stated that the non-electrogenic H,K-ATPase and mutants must exchange a symmetrical number of cations, whereas the Na,K-ATPase and the mutant that carries an outward current must export out of the cell a larger number of cations than they import into the cell.

The only result discordant with our hypothesis was obtained with the H,K-ATPase mutant in which Lys^{800} was replaced with serine (K800S), the amino acid found at the homologous position in the Na,K-ATPase. This mutant had robust ^{86}Rb transport activity, but showed no K^+-induced or ouabain-sensitive currents at high extracellular Na^+ concentrations. However, when studied in the absence of extracellular Na^+, this mutant showed a significant ouabain-sensitive outward current at 5 mM K^+, whereas the concentration/current relationship shows a biphasic behavior: a small outward current was observed at low K^+ concentrations, but it reversed to an inward current at higher K^+ concentrations. The ouabain-sensitive outward current indicates that the oocytes injected with the K800S mutant cRNA express an electrogenic pump. The presence of a K^+-induced inward current significantly larger than that recorded in oocytes expressing the β-subunit alone shows that these oocytes have also another K^+-dependent electrogenic pathway linked to expression of the K800S mutant. However, the inward current was not ouabain-sensitive; and thus, it is not due to a normal H,K- or Na,K-pump function. We can hypothesize that this K^+-induced inward current is related to a misfolded form of the mutant protein or to any alteration of the oocyte membrane resulting from expression of this mutant, but our data do not allow us to determine the precise cause of this K^+-induced inward current. Thus, the K800S mutant seems to have a complex behavior with a stoichiometry that varies according to the presence of extracellular Na^+.

The Na,K-ATPase is responsible for an ouabain-sensitive inward current when exposed to Na^+- and K^+-free extracellular solutions, and this current is increased at acidic pH (6, 24, 33). With *Xenopus laevis* Na,K-pumps, this current has been shown to be carried, at least in part, by protons (24). This inward flow of protons has no physiological significance because the Na,K-pump is never exposed to extracellular solutions with very low concentrations of Na^+ and K^+ under physiological conditions. In contrast, the H,K-ATPases are expressed in the apical membrane of epithelial cells and can be exposed to low concentrations of Na^+ and K^+. We have shown here that neither the non-gastric H,K-ATPase nor any of its electrogenic mutants carry a pH-dependent ouabain-sensitive inward current under these circumstances. Thus, this pH-dependent ouabain-sensitive inward current is not related to the 3:2 stoichiometry of the Na,K-pump.

Taken together, our results point to the crucial role of the highly conserved Ser^{782} in the Na,K-ATPase and its homologous residue (Lys^{800}) in the H,K-ATPases. Because of the large difference in Na,K-pump transport activity at 5 and 40 mM K^+ observed in mutant S782A, our results first confirm earlier studies showing that this residue has a large influence on the apparent K^+ affinity of the Na,K-pump (21–23); more precisely, the S775A mutation of the sheep Na,K-ATPase yields a very low affinity for K^+ without changes in the voltage dependence of this affinity (21). This residue is closely associated with the cation pathway through the Na,K-pump as recently shown by its accessibility to water-soluble sulfhydryl reagents when the Na,K-ATPase has been modified to a channel by palytoxin (34).

The high resolution structure of the group IIa P-ATPase SERCA (1) includes 2 calcium ions located close to the middle of the transmembrane part of the protein, between the fourth, fifth, and sixth transmembrane segments, identifying two calcium-binding sites (sites I and II) according to Toyoshima *et al.* (1). As the Na,K-pump is known to occlude 3 Na^+ ions, the "core" of the transmembrane domain of the Na,K-pump must provide three binding sites. It is presently not known where these binding sites are located in the structure of the Na,K-ATPase, but it is reasonable to postulate that two of these three sites are homologous to the calcium sites I and II of SERCA. We propose the hypothesis that Lys^{800} in the H,K-ATPases provides a fixed positive charge that is the equivalent of the third Na^+ ion transported by the Na,K-ATPase. The location of Ser^{782} and Lys^{800} in the three-dimensional structures of the Na,K- and H,K-ATPases, respectively, can be estimated by homology from the known high resolution structure of the group IIa P-ATPase SERCA (1, 35). The rather high similarity of the sequence of the fifth transmembrane segment and, in particular, the absolute conservation of Tyr^{778}, Asn^{783}, and Glu^{786} in this segment allow an unambiguous alignment of the SERCA and Na,K- and H,K-ATPase sequences (Fig. 1). The residue homologous to Na,K-ATPase Ser^{782} and H,K-ATPase Lys^{800} is Ser^{767} in SERCA (rabbit SERCA1, GenBank™/EBI accession number P04191 and Protein Data Bank code 1EUL). This residue has been implicated in calcium binding by mutagenesis experiments (32) and is located approximately at the same level as the 2 calcium ions and next to the crucial Asn^{768}

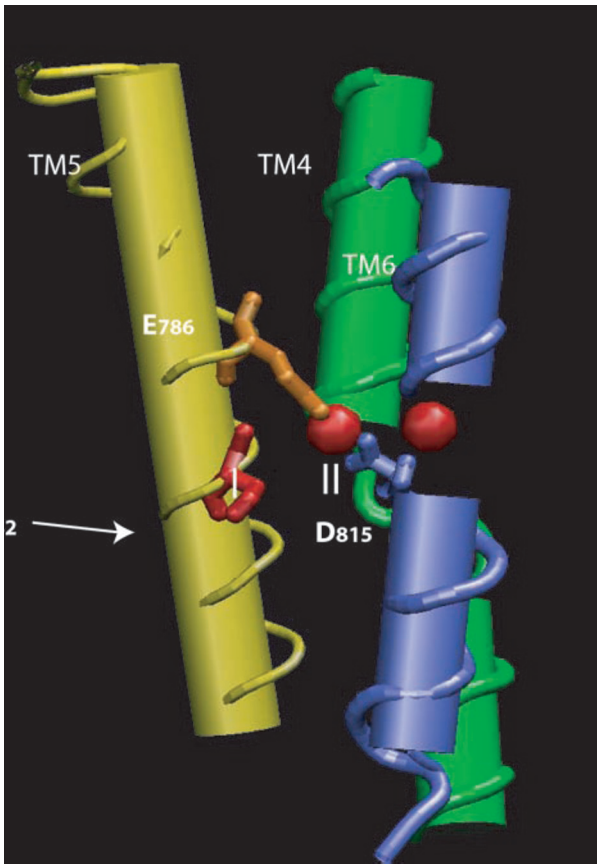

FIG. 7. **Model showing the fourth (*green*), fifth (*yellow*), and sixth (*blue*) transmembrane segments of the Na,K-ATPase and the position of Ser^{782} (*red*) in the fifth transmembrane segment with the positions of the two cations (*red spheres*) in their respective binding sites I and II, according to the equivalent structure of SERCA (1).** The positions of Glu^{786} (*orange*) and Asp^{815} (*dark blue*), which have been implicated in cation binding, are also indicated. The middle part of the sixth transmembrane segment has been removed to allow the cation-binding site to be seen. The extracellular side is at the top of the image. The model was obtained by homology with the x-ray crystal structure of SERCA (Protein Data Bank 1EUL) (1) using GeneMine/Look software, and the graphic presentation was prepared using VMD software (38).

that participate directly in the structure of calcium site I and indirectly in the structure of site II (1). The position of Ser^{782} is illustrated in Fig. 7. According to this hypothesis, the Na,K-ATPase would load 3 Na^+ ions from the intracellular solution into three distinct binding sites; two of these sites would have positions analogous to those of calcium sites I and II in SERCA, and the third one would be close to Ser^{782}. In contrast, the H,K-ATPases would load only 2 cations from the intracellular side, the third cation-binding site being occupied by the charge provided by the butyl ammonium side chain of Lys^{800} acting as a "tethered cation," as has been proposed for the lysine residue of the DEKA motif of the pore of the voltage-gated Na^+ channel (36). Removing the Lys^{800} side chain in the H,K-ATPase would allow it to work similarly to the Na,K-ATPase. Using homology modeling with SERCA, Ogawa and Toyoshima (37) have recently proposed a position for the third Na^+ site at a location between TM5, TM6, TM8, and TM9, at about the same level in the membrane as the two other binding sites that were assumed to be homologous to sites I and II of the Ca-ATPase in the E1 (Ca^{2+}-bound) conformation. From TM5, the side chain of Tyr^{778} (homologous to Tyr^{796} in the *Bufo* non-gastric H,K-ATPase; see Fig. 1) participates in the composition of this binding site. The positions of Ser^{782} and Lys^{800} studied in the present work are just slightly more than one helix turn deeper in the membrane, and the long side chain of Lys^{800} could reach the location predicted by Ogawa and Toyoshima (37) for the third Na^+ site and place its charged terminal ammonium there. Thus, our present observations and the tethered cation hypothesis are at least compatible with the present knowledge of the structure of the transmembrane part of the Na,K-ATPase.

Acknowledgment—We are grateful to Laurent Schild for careful reading of the manuscript and helpful suggestions.

REFERENCES

1. Toyoshima, C., Nakasako, M., Nomura, H., and Ogawa, H. (2000) *Nature* **405,** 647–655
2. Toyoshima, C., and Nomura, H. (2002) *Nature* **418,** 605–611
3. Axelsen, K. B., and Palmgren, M. G. (1998) *J. Mol. Evol.* **46,** 84–101
4. De Weer, P. (1985) in *The Kidney: Physiology and Pathophysiology* (Seldin, D. W., and Giebisch, G., eds) pp. 31–48, Raven Press, New York
5. Apell, H.-J. (1989) *J. Membr. Biol.* **110,** 103–114
6. Horisberger, J.-D., and Kharoubi-Hess, S. (2002) *J. Physiol.* (*Lond.*) **539,** 669–680
7. Rabon, E. C., and Reuben, M. A. (1990) *Annu. Rev. Physiol.* **52,** 321–344
8. Burnay, M., Geering, K., and Horisberger, J.-D. (2001) *Am. J. Physiol.* **281,** F869–F874
9. Jaisser, F., Horisberger, J.-D., Geering, K., and Rossier, B. C. (1993) *J. Cell Biol.* **123,** 1421–1429
10. Cougnon, M., Bouyer, P., Planelles, G., and Jaisser, F. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 6516–6520
11. Grishin, A. V., and Caplan, M. J. (1998) *J. Biol. Chem.* **273,** 27772–27778
12. Jaisser, F., Canessa, C. M., Horisberger, J.-D., and Rossier, B. C. (1992) *J. Biol. Chem.* **267,** 16895–16903
13. Nelson, R. M., and Long, G. L. (1989) *Anal. Biochem.* **180,** 147–151
14. Geering, K., Theulaz, I., Verrey, F., Häuptle, M. T., and Rossier, B. C. (1989) *Am. J. Physiol.* **257,** C851–C858
15. Jaisser, F., Horisberger, J.-D., and Rossier, B. C. (1993) *Pfluegers Arch. Eur. J. Physiol.* **425,** 446–452
16. Jaunin, P., Horisberger, J.-D., Richter, K., Good, P. J., Rossier, B. C., and Geering, K. (1992) *J. Biol. Chem.* **267,** 577–585
17. Girardet, M., Geering, K., Frantes, J. M., Geser, D., Rossier, B. C., and Kraehenbühl, J.-P. (1981) *Biochemistry* **20,** 6684–6691
18. Geering, K., Beggah, A., Good, P., Girardet, S., Roy, S., Schaer, D., and Jaunin, P. (1996) *J. Cell Biol.* **133,** 1193–1204
19. Jaisser, F., Jaunin, P., Geering, K., Rossier, B. C., and Horisberger, J.-D. (1994) *J. Gen. Physiol.* **103,** 605–623
20. Wang, X., Jaisser, F., and Horisberger, J.-D. (1996) *J. Physiol.* (*Lond.*) **491,** 579–594
21. Peluffo, R. D., Argüello, J. M., and Berlin, J. R. (2000) *J. Gen. Physiol.* **116,** 47–59
22. Pedersen, P. A., Nielsen, J. M., Rasmussen, J. H., and Jorgensen, P. L. (1998) *Biochemistry* **37,** 17818–17827
23. Jorgensen, P. L., and Pedersen, P. A. (2001) *Biochim. Biophys. Acta* **1505,** 57–74
24. Wang, X., and Horisberger, J.-D. (1995) *Am. J. Physiol.* **37,** C590–C595
25. Argüello, J. M., Whitis, J., Lingrel, J. B. (1999) *Arch. Biochem. Biophys.* **367,** 341–347
26. Blostein, R., Wilczynska, A., Karlish, S. J. D., Argüello, J. M., and Lingrel, J. B. (1997) *J. Biol. Chem.* **272,** 24987–24993
27. Hermsen, H. P. H., Koenderink, J. B., Swarts, H. G. P., and De Pont, J. J. H. H. (2000) *Biochemistry* **39,** 1330–1337
28. Swarts, H. G. P., Koenderink, J. B., Hermsen, H. P. H., Willems, P. H. G. M., and De Pont, J. J. H. H. (2001) *J. Biol. Chem.* **276,** 36909–36916
29. Vagin, O., Munson, K., Lambrecht, N., Karlish, S. J. D., and Sachs, G. (2001) *Biochemistry* **40,** 7480–7490
30. Vilsen, B. (1999) *Biochemistry* **38,** 11389–11400
31. Zillikens, S., Gisselmann, G., and Glitsch, H. G. (2000) *Biochim. Biophys. Acta* **1509,** 155–166
32. Rice, W. J., and Maclennan, D. H. (1996) *J. Biol. Chem.* **271,** 31412–31419
33. Efthymiadis, A., Rettinger, J., and Schwarz, W. (1993) *Cell Biol. Int.* **17,** 1107–1116
34. Guennoun, S., and Horisberger, J.-D. (2000) *FEBS Lett.* **482,** 144–148
35. Sweadner, K. J., and Donnet, C. (2001) *Biochem. J.* **356,** 685–704
36. Favre, I., Moczydlowski, E., and Schild, L. (1996) *Biophys. J.* **71,** 3110–3125
37. Ogawa, H., and Toyoshima, C. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 15977–15982
38. Humphrey, W., Dalke, A., and Schulten, K. (1996) *J. Mol. Graphics* **14,** 33–38