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Phe^{783}, Thr^{797}, and Asp^{804} in Transmembrane Hairpin M5-M6 of Na^+,K^+-ATPase Play a Key Role in Ouabain Binding*

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Ouabain is a glycoside that binds to and inhibits the action of Na^+,K^+-ATPase. Little is known, however, about the specific requirements of the protein surface for glycoside binding. Using chimeras of gastric H^+,K^+-ATPase and Na^+,K^+-ATPase, we demonstrated previously that the combined presence of transmembrane hairpins M3-M4 and M5-M6 of Na^+,K^+-ATPase in a backbone of H^+,K^+-ATPase (HN34/56) is both required and sufficient for high affinity ouabain binding. Since replacement of transmembrane hairpin M3-M4 by the N terminus up to transmembrane segment 3 (HNN3/56) resulted in a low affinity ouabain binding, hairpin M5-M6 seems to be essential for ouabain binding. To assess which residues of M5-M6 are required for ouabain action, we divided this transmembrane hairpin in seven parts and individually replaced these parts by the corresponding sequences of H^+,K^+-ATPase in chimera HN34/56. Three of these chimeras failed to bind ouabain following expression in *Xenopus laevis* oocytes. Altogether, these three chimeras contained 7 amino acids that were specific for Na^+,K^+-ATPase. Individual replacement of these 7 amino acids by the corresponding amino acids in H^+,K^+-ATPase revealed a dramatic loss of ouabain binding for F783Y, T797C, and D804E. As a proof of principle, the Na^+,K^+-ATPase equivalents of these 3 amino acids were introduced in different combinations in chimera HN34. The presence of all 3 amino acids appeared to be required for ouabain action. Docking of ouabain onto a three-dimensional-model of Na^+,K^+-ATPase suggests that Asp^{804}, in contrast to Phe^{783} and Thr^{797}, does not actually form part of the ouabain-binding pocket. Most likely, the presence of this amino acid is required for adopting of the proper conformation for ouabain binding.

Na^+,K^+-ATPase maintains the electrochemical gradients present across the plasma membrane of mammalian cells by catalyzing ATP-dependent transport of sodium and potassium ions (1). Cardiac glycosides such as ouabain specifically inhibit Na^+,K^+-ATPase activity. The Na^+,K^+-ATPase-binding site for ouabain has not been identified yet, although many studies with this goal have been performed. One of the difficulties is that during the catalytic cycle, the enzyme is in different conformations, each of which has a different affinity for ouabain. It has been established that the highest affinity for ouabain is obtained when the enzyme is in the phosphorylated E_2P form (2). Since it is known that ouabain acts on the α-subunit from the extracellular side, most attention has been paid to the extracellular loops and the transmembrane domains of this subunit. Price and Lingrel (3) demonstrated that two polar but uncharged amino acids (Gln^{111} and Asn^{122}) at the border of the first extracellular loop are responsible for the high ouabain sensitivity of nonrodent Na^+,K^+-ATPase. In ouabain-resistant rodents, two charged amino acids are present on these positions. Although in first instance, most attention was directed to this region of the catalytic subunit, later studies showed that other parts of the catalytic subunit might also be involved in ouabain binding. Extensive random mutagenic analysis of the Na^+,K^+-ATPase α-subunit, coupled with an ouabain selection system, has been used to identify amino acid residues that alter the inhibitory potency of ouabain (4–6). Several amino acids present in or around the third extracellular loop (Phe^{786}, Leu^{793}, Thr^{797}) that are possibly involved in ouabain binding were identified. In these experiments, the apparent IC_{50} for the effect of ouabain on Na^+,K^+-ATPase activity was determined. The disadvantage of this approach is that a change in the IC_{50} value can be attributed to a direct effect on the ouabain-binding site but also to a conformational equilibrium shift.

Gastric H^+,K^+-ATPase, like Na^+,K^+-ATPase, belongs to the P_2 type ATPases and has a similar subunit composition and structure. Although the catalytic subunits of gastric H^+,K^+-ATPase and Na^+,K^+-ATPase are 63% identical, the former enzyme is not inhibited by ouabain. Thus those amino acids that differ between these two enzymes might be important for the specificity of ouabain binding.

Previously, we demonstrated that the mere introduction of transmembrane hairpins M3-M4 and M5-M6 of Na^+,K^+-ATPase into a backbone of gastric H^+,K^+-ATPase resulted in the formation of a high affinity ouabain-binding site (see Fig. 1), as measured by direct binding of [^{3}H]ouabain to the E_2P form of Na^+,K^+-ATPase (7). Neither the introduction of hairpin M3-M4 nor that of hairpin M5-M6 alone rendered the chimeric enzyme sensitive to ouabain. Ishii *et al.* (8, 9) showed, however, that a chimera containing the N-terminal 200 amino acids of Na^+,K^+-ATPase and the backbone of Ca^{2+}-ATPase was able to bind ouabain, although with a low affinity. In our previous study, we found that chimera HNN3, containing the N-terminal 279 amino acids of Na^+,K^+-ATPase and the backbone of H^+,K^+-ATPase, could not bind ouabain (7). In the present study, we show that another chimeric enzyme (HNN3/56), containing the N-terminal 279 amino acids and M5-M6 of Na^+,K^+-ATPase, was sensitive for ouabain. Taken together, these results indicate that the transmembrane hairpin M5-M6 of Na^+,K^+-ATPase plays a crucial role in ouabain binding. Therefore, this region was investigated in detail by mutating the amino acids in this hairpin that are specific for Na^+,K^+-ATPase, in order to pinpoint the individual amino acids that are important for ouabain binding. This report shows that a

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chimera in which only the M3-M4 hairpin and 3 amino acids of M5-M6 (Phe^{783}, Thr^{797}, and Asp^{804}) originate from Na^+,K^+-ATPase, whereas all other parts of the α-subunit as well as the complete β-subunit originate from H^+,K^+-ATPase, binds ouabain with a high affinity.

EXPERIMENTAL PROCEDURES

Construction of Chimeras and Mutants—The chimeras and mutants presented in this report were constructed from the rat Na^+,K^+-ATPase α_1-subunit containing the R113Q and D124N mutations (3, 7) and the rat gastric H^+,K^+-ATPase α-subunit. Chimera HN34/56 was made as described previously (7), and chimera HNN3/56 was constructed from chimeras HNN3 and HN56 (7). The rat Na^+,K^+-ATPase α_1- and β_1-subunits, the rat gastric H^+,K^+-ATPase α- and β-subunits, and the chimeras were subcloned into the pTLN vector, which is suitable for the *Xenopus Laevis* oocyte expression system (10). Site-directed mutagenesis was used to generate the mutants described in this report. All introduced mutations were verified by sequencing. For clarity reasons, we also used the numbering of the pig Na^+,K^+-ATPase for residues in part originating from H^+,K^+-ATPase.

Expression in X. laevis Oocytes—*X. laevis* were sacrificed, and parts from ovaries were removed. Oocytes were separated by incubation for 2 h in modified Ringer's solution (90 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 5 mM MOPS,[1] pH 7.4, 30 units/liter penicillin, and 30 mg/liter streptomycin) containing 2 mg/ml collagenase A. Prophase-arrested oocytes of stages V and VI were selected for injection of cRNA. cRNA synthesis was carried out with SP6-polymerase (Promega, Madison, WI) and capping analogue (Invitrogen). As a template, the HpaI-linearized pTLN vector was used. For the expression of the α- and β-subunits, oocytes were injected with 10 and 2 ng of the corresponding cRNAs. After injection, the oocytes were incubated for 3 days at 18 °C in modified Ringer's solution.

Preparation of Total Membranes—Oocytes were disrupted by passing them ±20 times through a standard 200-μl Gilson pipette in homogenization buffer (10 μl/oocyte; 250 mM sucrose, 0.5 mM EDTA, 5 mM Tris/HCl, pH 7.4, and Complete protease inhibitor (according to the instructions of the manufacturer, Roche Applied Science)). The yolk granules were removed by centrifugation of the samples ($1000 \times g$, 4 °C, 3 min), and the supernatant was collected in a new tube. This step was repeated twice. Subsequently, the membranes were pelleted by centrifugation ($16{,}000 \times g$, 4 °C, 30 min). Finally, the pellet was resuspended in homogenization buffer (4 μl/oocyte), and the samples were stored at −20 °C.

Western Blotting—The total membrane fraction of *X. laevis* oocytes was solubilized in sample buffer and separated on SDS-PAGE gels containing 10% acrylamide according to Laemmli (11). For immunoblotting, the separated proteins were transferred to Immobilon polyvinylidenedifluoride membranes (Millipore, Bedford, MA). The α-subunits of the chimeras and H^+,K^+-ATPase were detected with the polyclonal antibody HKB (12). The primary antibody was detected using an anti-rabbit secondary antibody, which was labeled with peroxidase (DAKO A/S, Glostrup, Denmark).

[^{3}H]Ouabain Binding—A total membrane fraction equivalent to 2.5 oocytes was incubated at room temperature in 50 mM Tris-acetic acid (pH 7.0), 1 mM ATP, 5.0 mM $MgCl_2$, and 250 nM (or 10 nM) [^{3}H]ouabain in a final volume of 50 μl. After 1 h, the reaction mixture was chilled for 15 min at 0 °C. The ouabain-protein complex was collected by filtration over a 0.8-μm membrane filter (Schleicher and Schuell). After washing with ice-cold water twice, the filters were analyzed by liquid scintillation analysis.

ATP Phosphorylation—ATP phosphorylation was carried out as described previously (13). A total membrane fraction equivalent to 0.45 oocyte was incubated at room temperature in 50 mM Tris-acetic acid (pH 6.0) and 1.3 mM $MgCl_2$ in a volume of 50 μl. After a 30–60 min preincubation, 10 μl of 0.6 μM [γ-^{32}P]ATP was added, and the reaction mixture was incubated for another 10 s. The reaction was stopped by adding 5% (w/v) trichloroacetic acid in 0.1 M phosphoric acid, and the phosphorylated protein was collected by filtration over a 0.8-μm membrane filter (Schleicher and Schuell). After washing twice, the filters were analyzed by liquid scintillation analysis.

Calculations—Data are presented as mean values with S.E. Differences were tested for significance by means of the Student's t test.

[1] The abbreviation used is: MOPS, 3-[*N*-morpholino]propanesulfonic acid.

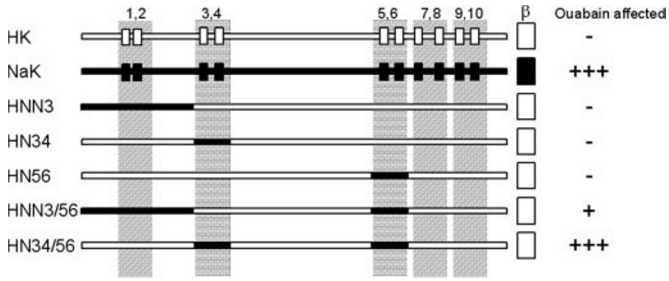

FIG. 1. **Schematic representation of the chimeras and wild type enzymes.** The *open bars* represent H^+,K^+-ATPase sequences, and the *solid bars* represent Na^+,K^+-ATPase sequences. *HK*, H^+,K^+-ATPase; *NaK*, Na^+,K^+-ATPase; *HNN3*, H^+,K^+-ATPase with amino acids Met^1-IIe^{293} replaced by those of Na^+,K^+-ATPase (Met^1-IIe^{279}); *HN34*, H^+,K^+-ATPase with amino acids IIe^{293}–Leu^{346} replaced by those of Na^+,K^+-ATPase (IIe^{279}–Leu^{332}); *HN56*, H^+,K^+-ATPase with amino acids Leu^{776}–Arg^{846} replaced by those of Na^+,K^+-ATPase (Leu^{762}–Arg^{832}); *HNN3/56*, H^+,K^+-ATPase with amino acids Met^1-IIe^{293} and Leu^{776}–Arg^{846} replaced by those of Na^+,K^+-ATPase (Met^1-IIe^{279} and Leu^{762}–Arg^{832}); *HN34/56*, H^+,K^+-ATPase with amino acids IIe^{293}–Leu^{346} and Leu^{776}–Arg^{846} replaced by those of Na^+,K^+-ATPase (IIe^{279}–Leu^{332} and Leu^{762}–Arg^{832}). The ability (+) or failure (−) of an enzyme to bind ouabain is indicated at the *right*. *Three plus signs* (+++) indicate high affinity ouabain binding, whereas *one plus sign* (+) indicates low affinity ouabain binding.

Materials—[^{3}H]Ouabain (17 Ci $mmol^{-1}$) and [γ-^{32}P]ATP (3000 Ci $mmol^{-1}$) were purchased from Amersham Biosciences.

RESULTS

In a previous study, we found that a chimera containing the backbone of the α-subunit and the complete β-subunit from gastric H^+,K^+-ATPase and only the M3-M4 and M5-M6 hairpins of Na^+,K^+-ATPase (chimera HN34/56; Fig. 1) bound [^{3}H]ouabain with a high affinity (7). We next constructed a chimera in which both the N terminus until transmembrane segment M3 (N-terminal 279 amino acids) and the hairpin M5-M6 of H^+,K^+-ATPase were exchanged by the homologous parts of Na^+,K^+-ATPase (HNN3/56). This chimera possessed no ATPase activity but could still be phosphorylated by ATP. When 1 mM ouabain was present during the phosphorylation reaction, the phosphorylation level decreased by almost 40%. The phosphorylation levels of chimeras containing only the N terminus until M3 (HNN3), M3-M4 (HN34), or M5-M6 (HN56) of Na^+,K^+-ATPase were not decreased by the same ouabain concentration. Under conditions in which chimera HN34/56 bound ouabain, chimera HNN3/56, however, did not. Thus, it can be concluded that both chimeras HNN3/56 and HN34/56 interact with ouabain, although the affinity for ouabain is much lower for HNN3/56 than for HN34/56. The common segment in these two chimeras is Na^+,K^+-ATPase hairpin M5-M6, which therefore seems to be crucial for ouabain binding. In the remainder of this study, we focused on this hairpin and identified the amino acids that are essential in ouabain binding.

Hairpin M5-M6 contains 21 amino acids that differ between Na^+,K^+-ATPase and H^+,K^+-ATPase (Fig. 2). To identify the essential amino acids in hairpin M5-M6, we replaced seven groups of Na^+,K^+-ATPase residues by their H^+,K^+-ATPase counterparts (Fig. 2) and measured ouabain binding. These seven mutants of chimera HN34/56 were expressed together with the H^+,K^+-ATPase β-subunit in *X. laevis* oocytes. The expression levels of the α-subunits were determined by Western blotting of total membranes by using the polyclonal antibody HKB that recognizes the M4-M5 loop of the gastric H^+,K^+-ATPase α-subunit (12). Fig. 3*A* shows that the expression levels of most mutated chimeras were similar to those of chimera HN34/56. The expression level of mutated chimera QKRQ seemed to be somewhat lower. The [^{3}H]ouabain binding levels of the mutants FF, TV, and CDG (Fig. 3*B*), as determined

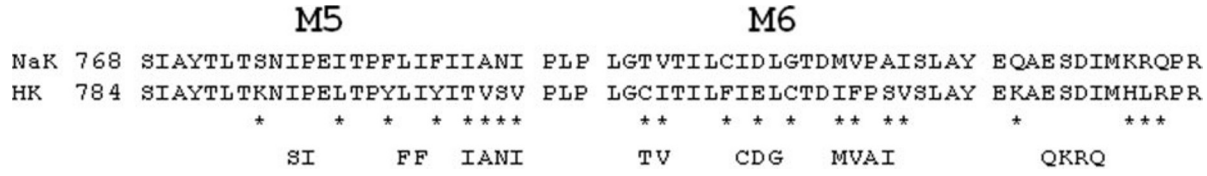

FIG. 2. **Amino acid sequence alignments between the M5-M6 region of rat Na$^+$,K$^+$-ATPase and rat gastric H$^+$,K$^+$-ATPase α_1-subunits.** The Na$^+$,K$^+$-ATPase sequence is shown *above* the corresponding H$^+$,K$^+$-ATPase sequence. Amino acid numbers are indicated on the *left side*. *Asterisks* indicate differences between both sequences. The seven mutants are indicated as *SI*, *FF*, *IANI*, *TV*, *CDG*, *MVAI*, and *QKRQ*. Note that the QKRQ mutation is outside M5-M6.

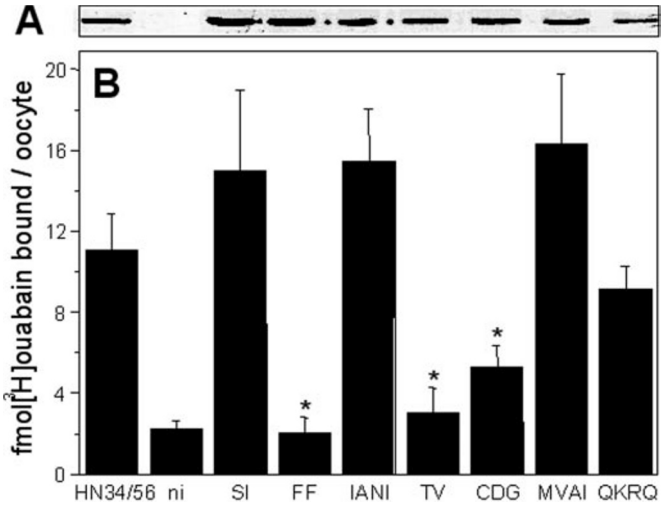

FIG. 3. **[^{3}H]Ouabain binding of HN34/56 mutants.** In *A*, membranes were blotted, and the presence of the α-subunit and was detected with antibody HKB (12), which is directed against the large intracellular loop of H$^+$,K$^+$-ATPase. In *B*, [^{3}H]ouabain binding was determined by incubating the membranes at room temperature in 5.0 mM $MgCl_2$, 50 mM Tris-acetic acid (pH 7.0), 1.0 mM ATP, and 250 nM [^{3}H]ouabain. Mean values ± S.E. of 5–8 enzyme preparations are shown. *, significantly different from HN34/56 ($p < 0.05$). The seven mutants (SI, FF, IANI, TV, CDG, MVAI, and QKRQ) are described in the legend for Fig. 2.

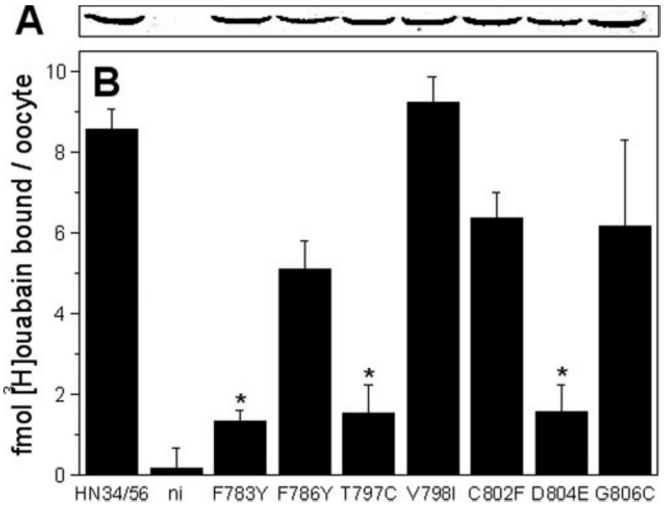

FIG. 4. **[^{3}H]Ouabain binding of HN34/56 mutants.** In *A*, membranes were blotted, and the presence of the α-subunit and was detected with antibody HKB (12), which is directed against the large intracellular loop of H$^+$,K$^+$-ATPase. In *B*, [^{3}H]ouabain binding was determined by incubating the membranes at room temperature in 5.0 mM $MgCl_2$, 50 mM Tris-acetic acid (pH 7.0), 1.0 mM ATP, and 250 nM [^{3}H]ouabain. Mean values ± S.E. of 4 enzyme preparations are shown. *, significantly different from HN34/56 ($p < 0.05$).

in the presence of 1 mM ATP and 250 nM ouabain, were significantly different from those of chimera HN34/56 ($p < 0.05$), but not from those of uninjected oocytes ($p > 0.05$). This means that these mutants lost their ability to bind [^{3}H]ouabain under these conditions. On the other hand, the [^{3}H]ouabain binding capacity of chimera HN34/56 and the mutants SI, IANI, and MVAI were similar and much higher than those of FF, TV, and CDG. Although the [^{3}H]ouabain binding capacity of mutant QKRQ was slightly lower that that of mutants SI, IANI and MVAI, it was, related to its lower expression level, not decreased. This indicates that none of the latter mutated amino acids are responsible for the ouabain specificity of Na$^+$,K$^+$-ATPase. Taken together, these findings suggest that at least 3 and maximally 7 amino acids present in hairpin M5-M6 of Na$^+$,K$^+$-ATPase, and absent in gastric H$^+$,K$^+$-ATPase, are important for ouabain binding.

To assess which of the 7 identified amino acids in M5-M6 of Na$^+$,K$^+$-ATPase are essential for high affinity ouabain binding, these amino acids of chimera HN34/56 were mutated individually into the corresponding H$^+$,K$^+$-ATPase residue. Western blotting confirmed similar expression patterns of these mutants and chimera HN34/56 (Fig. 4*A*). To exclude the possibility that a loss of ouabain binding capacity of one of these mutants is due to the impossibility of forming a phosphorylated intermediate, we tested the phosphorylation capacity of these seven mutants. Since all mutants could be phosphorylated by ATP up to a similar level (data not shown), the latter possibility could be excluded. Next, the ouabain binding level of these mutants was measured. Fig. 4*B* shows that the [^{3}H]ouabain binding levels of mutants F783Y, T797C, and D804E were not significantly different from those of uninjected oocytes ($p > 0.05$) and significantly different from those of chimera HN34/56 ($p < 0.05$), indicating that these 3 amino acids might be involved in ouabain binding. The [^{3}H]ouabain binding level of mutant F786Y was lower than that of chimera HN34/56 but still higher than that of uninjected oocytes. The ouabain binding levels of mutants V798I, C802F, and G806C were not significantly different from those of chimera HN34/56 ($p > 0.05$) and much higher than those of the mutants F783Y, T797C, and D804E. These experiments suggest that Phe^{783}, Tyr^{797}, and Asp^{804} are all important for ouabain binding. These "loss of function" experiments, however, do not exclude the possibility that mutation of these amino acids indirectly results in loss of ouabain binding capacity without involvement of these residues in direct ouabain binding.

We therefore performed "gain of function" experiments by introducing the amino acids Phe^{783}, Thr^{797}, and Asp^{804} in various combinations into chimera HN34. The latter chimera contains only hairpin M3-M4 of Na$^+$,K$^+$-ATPase and does not bind ouabain (7). Again, Western blotting (Fig. 5*A*) and ATP phosphorylation experiments (data not shown) confirmed equal expression and phosphorylation levels of all tested mutants. [^{3}H]ouabain binding measurements demonstrated that under the conditions used, none of the double mutants showed a [^{3}H]ouabain binding level significantly higher than those of uninjected oocytes (Fig. 5*A*). Only the chimera with all three mutations, HN34/FTD, was able to bind ouabain. Thus, Phe^{783}, Thr^{797}, and Asp^{804} are all essential for high affinity ouabain binding.

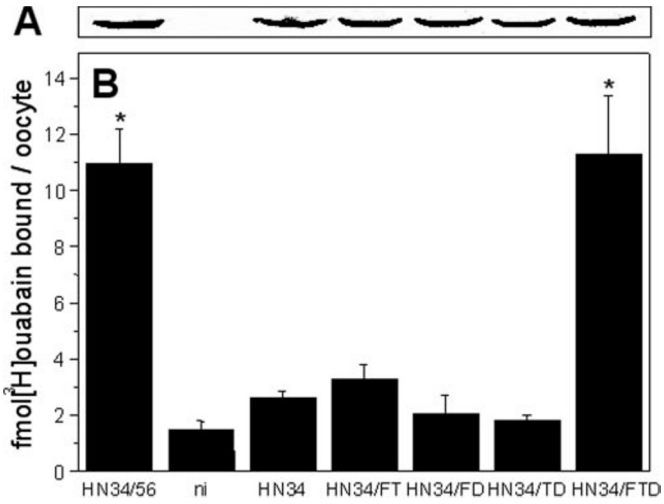

FIG. 5. **[³H]Ouabain binding of HN34 mutants.** In *A*, membranes were blotted, and the presence of the α-subunit and was detected with antibody HKB (12), that is directed against the large intracellular loop of H^+,K^+-ATPase. In *B*, [³H]ouabain binding was determined by incubating the membranes at room temperature in 5.0 mM $MgCl_2$, 50 mM Tris-acetic acid (pH 7.0), 1.0 mM ATP, and 250 nM [³H]ouabain. Mean values ± S.E. of 3–4 enzyme preparations are shown. *, significantly different from uninjected oocytes ($p < 0.05$). *F* is mutation Y783F, *T* is mutation C797T, and *D* is mutation E804D.

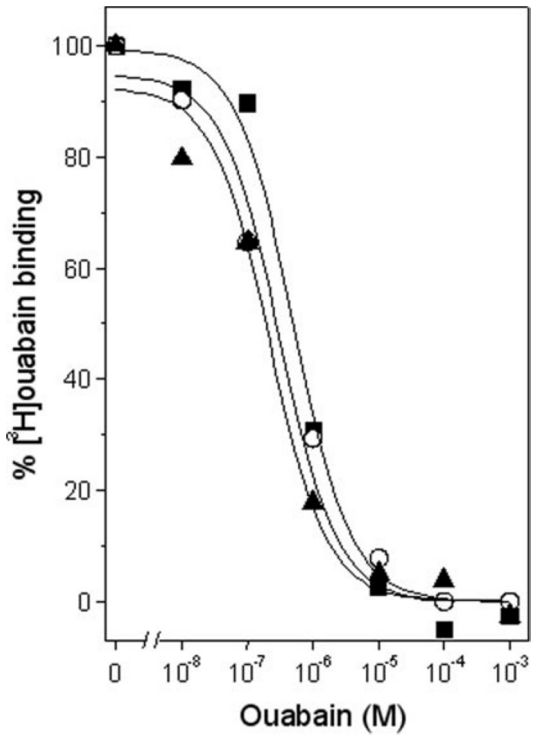

FIG. 6. **[³H]Ouabain binding to HN34/56, HN34/FTD and rat Na^+,K^+-ATPase R113Q/D124N.** [³H]Ouabain binding was determined by incubating the membranes at room temperature in 5.0 mM $MgCl_2$, 50 mM Tris-acetic acid (pH 7.0), 1.0 mM ATP, 10 nM [³H]ouabain, and varying concentrations of nonradioactive ouabain. The binding of [³H]ouabain in the absence of nonradioactive ouabain was set at 100%. ■, Na^+,K^+-ATPase; ▲, HN34/56; ○, HN34/FTD.

The mutations Y783F, C797T, and E804D were also introduced into chimera HNN3. Chimeras HNN3/FTD and HNN3/56 did not bind [³H]ouabain under our experimental conditions, but the phosphorylation levels of these chimeras decreased when 1 mM ouabain was added by 41% ± 3.5 and 38% ± 7.8 (mean ± S.E.), respectively. This indicates that both chimeras do bind ouabain, although with a lower affinity than chimera HN34/FTD.

In our previous work (7), we demonstrated that the apparent ouabain binding affinity of chimera HN34/56 was similar to that of Na^+,K^+-ATPase. In an ouabain-replacement assay (Fig. 6), we show that the apparent ouabain binding affinity of chimera HN34/FTD (0.3 ± 0.1 μM) is also similar to that of Na^+,K^+-ATPase (0.5 ± 0.1 μM) and chimera HN34/56 (0.2 ± 0.1 μM).

DISCUSSION

In 1953, Schatzmann (14) demonstrated that cardiac glycosides inhibit active transport of Na^+ and K^+, and a few years later, it was shown that this transport was caused by the Na^+,K^+-ATPase (24). The Na^+,K^+-ATPase-binding site for cardiac glycosides (like ouabain) has not been identified yet, but knowledge about this site might be crucial for the development of new drugs. In a previous study, we demonstrated that introduction of transmembrane hairpins M3-M4 and M5-M6 of Na^+,K^+-ATPase into H^+,K^+-ATPase resulted in the formation a high affinity ouabain-binding site, whereas the introduction of M3-M4 or M5-M6 alone did not give ouabain binding (7). In the present study, we identified 3 residues (Phe^{783}, Thr^{797}, and Asp^{804}) in M5-M6 of Na^+,K^+-ATPase that play a key role in ouabain binding.

Although the catalytic subunits of gastric H^+,K^+-ATPase and Na^+,K^+-ATPase are for 63% identical, they have different inhibitor sensitivities. Ouabain inhibits Na^+,K^+-ATPase activity but does not inhibit gastric H^+,K^+-ATPase activity. In this study, we describe chimeras HNN3/56 and HN34/56 that both interact with ouabain. However, [³H]ouabain did bind to HN34/56, but binding to HNN3/56 could not be detected under the applied assay conditions. However, the phosphorylation level of chimera HNN3/56 could be reduced by ouabain, indicating that ouabain interacts with this chimera and probably binds to it with a low affinity.

That chimeras HNN3/56 and HN34/56 both contain transmembrane hairpin M5-M6 indicates that this hairpin is essential for ouabain binding. Movement of hairpin M5-M6 during the catalytic cycle is important for active ion transport (15, 16). Binding of ouabain to this hairpin probably inhibits enzyme activity through immobilization of this hairpin. In the present study, we used chimera HN34/56 as the starting point to determine which amino acids in M5-M6 are responsible for ouabain binding. Transmembrane hairpin M5-M6 contains 21 amino acids that are different in Na^+,K^+-ATPase and H^+,K^+-ATPase.

We started with an H^+,K^+-ATPase preparation that did not bind ouabain and obtained a chimeric enzyme that could be inhibited by ouabain. All amino acids present in M5-M6 of HN34/56 were groupwise mutated into their corresponding H^+,K^+-ATPase residues. Three mutants, containing seven mutations (FF, TV, and CDG), lost their [³H]ouabain binding capacity. This suggested that between 3 and 7 of these mutated amino acids present in hairpin M5-M6 of Na^+,K^+-ATPase are involved in ouabain binding. Individual mutational analysis revealed that only mutants F783Y, T797C, and D804E completely lost the ability to bind [³H]ouabain. Finally, we introduced the mutations Y783F, C797T, and E804D into chimera HN34 (HN34/FTD). Whereas chimera HN34 did not bind ouabain, chimera HN34/FTD obtained a high affinity ouabain-binding site. The apparent affinity of this chimera is similar to that of wild type Na^+,K^+-ATPase and chimera HN34/56. These results revealed that Na^+,K^+-ATPase residues Phe^{783}, Thr^{797}, and Asp^{804} are essential for high affinity ouabain binding.

Of these amino acid residues, only a role for Thr^{797} in ouabain binding has been described. Random mutagenesis of sheep

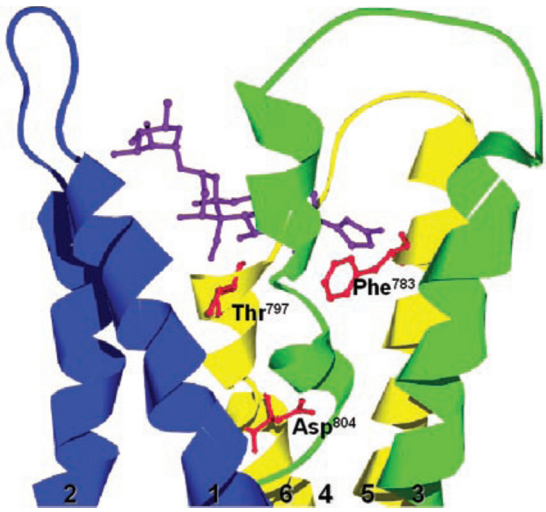

FIG. 7. **Model of transmembrane segments of Na^+,K^+-ATPase.** The model of Na,K^+-ATPase was obtained by homology modeling (22) with the E_2 structure of sarco(endo)plasmic reticulum Ca^{2+}-ATPase (21). Only transmembrane hairpin M1-M2 (*blue*), M3-M4 (*green*), and M5-M6 (*yellow*) are shown. The position of amino acids Phe^{783}, Thr^{797}, and Asp^{804} in the α-subunit of the sodium pump are indicated. The structure of ouabain (*purple*) was added according to the suggestions of Middleton *et al.* (23).

Na^+,K^+-ATPase α_1-subunit showed that mutants T797N, T797A, and T797V lost their ouabain sensitivity (66–80-fold decrease), indicating that Thr^{797} was involved in the interaction with ouabain (17, 18). Asano *et al.* (19) showed that a chimera of Na^+,K^+-ATPase and H^+,K^+-ATPase that also contained Thr^{797} obtained ouabain sensitivity, although the apparent affinity in the ATPase activity assay was more than 1000-fold lower than that of the wild type Na^+,K^+-ATPase. We confirmed the importance of Thr^{797} and showed that it is one of the amino acids in M5-M6 that is essential for high affinity ouabain binding.

Phe^{783} has never been found to be involved in ouabain binding. In contrast, such a role had been attributed to Phe^{786}. Mutating Phe^{786} to Asn or Ile increased the resistance with 11- or 19-fold, respectively (4). We also observed a decreased ouabain binding capacity for mutant F786Y (with 250 nM [^{3}H]ouabain in the assay) but demonstrated that this amino acid is not directly involved in ouabain binding: chimera HN34/FTD does not contain Phe^{786} but still has a similar high affinity ouabain-binding site as the wild type Na^+,K^+-ATPase and HN34/56 that both contain Phe^{786}.

Mutation of Asp^{804} of Na^+,K^+-ATPase into an Ala modifies the cation-binding pocket, but the resulting mutant is still able to interact with ouabain (20). Together with the present data, this indicates that Asp^{804} is involved in ouabain binding but that it is not essential. It is unclear why mutation of Asp^{804} in a Glu residue, as present in gastric H^+,K^+-ATPase, completely prevents ouabain binding.

In Fig. 7, the relative positions of Phe^{783}, Thr^{797}, and Asp^{804} in transmembrane segments M5 and M6 of the Na^+,K^+-ATPase α-subunit are given. The model was obtained by homology modeling of the Na^+,K^+-ATPase amino acid sequence into the published E_2 structure of sarco(endo)plasmic reticulum Ca^{2+}-ATPase pump (21) with the Swiss model (22) suite. The structure of ouabain is added to this figure in a position that has been suggested by Middleton *et al.* (23). This figure shows that indeed Asp^{804} is buried deep into the membrane, making a direct interaction with ouabain less likely. One has to keep in mind that the boundaries to M5 and M6 are not well established, so that the precise size of the loop and its length are not known.

Ishii and coworkers (8, 9) found that the region between Ala^{70} and Asp^{200} of Na^+,K^+-ATPase was sufficient for donating ouabain binding capacity to Ca^{2+}-ATPase, although the affinity was much less than that of the wild type Na^+,K^+-ATPase. In our previous study (17), we did not observe any interaction with ouabain when this region (Met^1–Ile^{279}) was introduced in gastric H^+,K^+-ATPase (HNN3). Here we report that chimera HNN3/FTD interacts with ouabain, although with a low affinity. However, the amino acids in the M5-M6 region of Ca^{2+}-ATPase are rather different from those of Na^+,K^+-ATPase, and the 3 amino acids we demonstrated as being important for ouabain binding are not present in Ca^{2+}-ATPase.

The approach chosen by us to identify amino acids that are involved in ouabain binding has one disadvantage. The method cannot give information on a possible role of amino acids that are similar in Na^+,K^+-ATPase and gastric H^+,K^+-ATPase. At the extracellular side of the M5/M6 hairpin, there are a few, mainly hydrophobic, amino acid residues that might be involved in ouabain binding. One of these is Leu^{793}, which is located between two conserved proline residues that are likely to be involved in the hairpin turn. This residue was found to be essential for ouabain binding by random mutagenesis studies (6).

Solid-state NMR revealed that the sugar group was only loosely associated with the binding site, facing away from the surface of the membrane, whereas the steroid group was more constrained, probably because of hydrogen bonding to residues around the K^+ channel region (23). These results favor a model for inhibition in which ouabain lies across the surface of the Na^+,K^+-ATPase α-subunit (23). Combining the NMR solid-state data with our results indicates that the steroid group might interact with the Phe^{783} and Thr^{797} amino acids of hairpin M5-M6.

In conclusion, this study showed that the 3 amino acids Phe^{783}, Thr^{797}, and Asp^{804} present in transmembrane hairpin M5-M6 of Na^+,K^+-ATPase are essential for high affinity ouabain binding. Future experiments will resolve which amino acids of M3-M4 are involved in ouabain binding.

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