Glu-857 Moderates $K^+$-dependent Stimulation and SCH 28080-dependent Inhibition of the Gastric H,K-ATPase*

(Received for publication, February 9, 1999, and in revised form, March 4, 1999)

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The rabbit H,K-ATPase α- and β-subunits were transiently expressed in HEK293 T cells. The co-expression of the H,K-ATPase α- and β-subunits was essential for the functional H,K-ATPase. The $K^+$-stimulated H,K-ATPase activity of 0.82 ± 0.2 μmol/mg/h saturated with a $K_{0.5}$ (KCl) of 0.6 ± 0.1 mM, whereas the 2-methyl-8-(phenylmethoxy)imidazo[1,2a]pyridine-3-acetonitrile (SCH 28080)-inhibited ATPase of 0.62 ± 0.07 μmol/mg/h saturated with a $K$ (SCH 28080) of 1.0 ± 0.3 μM. Site mutations were introduced at the N,N-dicyclohexylcarbodiimide-reactive residue, Glu-857, to evaluate the role of this residue in ATPase function. Variations in the side chain size and charge of this residue did not inhibit the specific activity of the H,K-ATPase, but reversal of the side chain charge by substitution of Lys or Arg for Glu produced a reciprocal change in the sensitivity of the H,K-ATPase to $K^+$ and SCH 28080. The $K_{0.5}$ for $K^+$-stimulated ATPase was decreased to 0.2 ± 0.05 and 0.2 ± 0.03 mM, respectively, in Lys-857 and Arg-857 site mutants, whereas the $K$ for SCH 28080-dependent inhibition was increased to 6.5 ± 1.4 and 5.9 ± 1.5 μM, respectively. The H,K-ATPase kinetics were unaffected by the introduction of Ala at this site, but Leu produced a modest reciprocal effect. These data indicate that Glu-857 is not an essential residue for cation-dependent activity but that the residue influences the kinetics of both $K^+$ and SCH 28080-mediated functions. This finding suggests a possible role of this residue in the conformational equilibrium of the H,K-ATPase.

The gastrin H,K-ATPase produces the acidic environment of the gastric mucosa through an electroneutral exchange of $H^+$ for $K^+$ (1, 2). It is a member of a phosphoenzyme-forming ATPase family (P2) that includes the Ca-ATPase, the Na,K-ATPase, and the $Neurospora$ H+ pump (3). This family of transporters and the more distantly related P1 and P3 ATPase isoforms (15, 16). The primary structure of the gastric α-subunit isoform is highly conserved among various mammalian species including rat (7), rabbit (8), hog (9), and man (10), but sequence conservation deteriorates in H,K-ATPase isoforms present in the urinary bladder (11), skin (12), colon (13), and kidney (14) as well as in other P2-type pump isoforms (15, 16).

The P2 family of ion pumps consists of polytopic membrane proteins generally modeled with 8–10 membrane spanning regions. A variety of evidence has yielded a consensus for the identity of the transmembrane spanning domains M1–M4 within the N-terminal half of the ATPases (17–21) but has not conclusively defined the C-terminal transmembrane spanning domains (22–25).

Although the organization of the C-terminal membrane spanning domains remains a focus of investigation, there is general consensus that charged residues within these domains participate in cation-dependent function. Cation binding to membrane-embedded proteolytic fragments is dependent upon the integrity of the ATPase from M5 to the C terminus in both the Na,K-ATPase and the H,K-ATPase (17, 19, 26, 27). The disruption of the C-terminal sequence at the cytoplasmic-M7 interface facilitates the thermal inactivation of ATPase (28, 29), release of the putative M5-M6 hairpin to the supernatant (30), and exposure of Cys-983 (31). Amino acid substitutions within membrane spanning segments of M4, M5, M6, and M8 have identified conserved residues in the expressed Na,K-ATPase, Ca-ATPase, and H,K-ATPase that are essential for ion-dependent transport or cation-dependent catalytic activity (32–39).

Chemical modification studies utilizing carboxyl reagents such as DEAC (40) and DCCD (41–45) have investigated the role of hydrophobic carboxyl groups in several pumps within the P2 pump family. These studies show that protection from inhibition by the transported cation is a general characteristic of the inhibitor-dependent inactivation of these ATPases. Even though the mechanism of inactivation is controversial, the cation specificity and concentration dependence providing protection have frequently been interpreted as evidence for the modification of residues within the cation binding site. A microsequence has now been obtained for several reactive residues in or near transmembrane spanning domains within M5, M7, and M9 of the native H,K-ATPase (45) and Na,K-ATPase (44, 46). Mutational studies of the modified residue and analysis of the expressed Na,K-ATPase have provided evidence to confirm biochemical studies that Glu-799 (NPE), a DEAC-reactive site, is important for cation-dependent activity (46–48) but contradicted a similar biochemical study that Glu-959 (FEET), a [14C]DCCD-reactive site (44, 47, 49), is important for...
Na,K-ATPase activity.

Glut-857 (LVNE), a residue at the fourth putative cytosolic-seventh transmembrane spanning region interface (C4—M7) of the gastric H,K-ATPase has been identified as a K⁺-protected, [³⁵S]DCCD-reactive site (45). To clarify whether DCCD-dependent inhibition is the result of its modification of an essential residue within the cation binding site of the H,K-ATPase or whether inhibition may result from a secondary effect, such as cross-linkage or cation-dependent protection of a DCCD-reactive site at an alternative locus, a series of site mutants were prepared at this position varying the size, charge, and hydrophobicity of the side chain residue. The site mutants were transiently expressed in HEK293 T cells, and the expressed H,K-ATPase was analyzed to determine the effect of the modifications on the steady-state kinetic constants describing K⁺-stimulated activation or SCH 28080-dependent inhibition of the ATPase.

The results of this investigation demonstrate that Glut-857 is not essential for K⁺-dependent ATPase activity but do suggest that the C4—M7 domain is an important region involved in the conformational sensitivity of the molecule.

**Experimental Procedures**

**Plasmid Constructs—**Plasmids containing the α-subunit (GenBank accession number X64694) and β-subunit (GenBank accession number M35544) of the rabbit (Oryctolagus cuniculus) gastric H,K-ATPase in the vector pcDNA3 (Invitrogen) were a kind gift of Dr. George Sachs (Wadsworth Veterans Affairs Medical Center, UCLA). Bidirectional sequence analysis of the 5’ and 3’ regions verified the integrity of the ORF in the end regions of each construct. The β-subunit plasmid, pcDNA3-β, is a 6775-bp construct with its ORF flanked by original 5’- and 3’-untranslated regions (UTR) of 27 and 405 base pairs, respectively. The 5’ UTR of the β-subunit is linked to the multiple cloning site of the pcDNA3 vector through a 16-bp sequence (AAT GGG GTA C- CG AAT T), and the 3’-UTR is linked by an 11-bp sequence (TTC TTC- GAA AT). The α-subunit, pcDNA3-α, is a 8574-bp construct with its ORF flanked by original 5’- and 3’-UTR of 12 and 200 base pairs, respectively. The 5’-end UTR of the α-subunit is linked to the multiple cloning site of the pcDNA3 vector with a 21-bp linker sequence (GG-T ACC CAA TTC CTG CAG CCC), and the 3’-UTR is linked with a 24-bp linker sequence (GAT CCA CTA GGT CTA GGA GGA TCC).

**DNA Sequencing—**All site mutations were verified by DNA sequencing performed by the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University. Sequence analysis was carried out on an Applied Biosystems 373A Stretch DNA Sequencer. The sequencing reactions utilized fluorescently labeled dideoxynucleotides and Taq FS DNA polymerase in a thermal cycling protocol. The submitted samples, containing 750 ng of circularized plasmid DNA and 0.4 mM DNA polymerase in a thermal cycling protocol. The submitted samples, H,K-ATPase was analyzed to determine the effect of the modifications on the steady-state kinetic constants describing K⁺-stimulated activation or SCH 28080-dependent inhibition of the ATPase.

**Table I**

| Mutant primers for site mutants of Glut-857 |
|--------------------------------------------|
| E857Q 5’ | GCC TCG TGC AAC CAA CCC CTG GCT GCC |
| E857A 5’ | C TCG GTC AAC GCT CCC CTG GCT GCC TA |
| E857L 5’ | GC TCG GTC AAC CTT CCC CTG GCT GCC TA |
| E857K 5’ | C TCG GTC AAC AAA CCC CTG GCT GCC TA |
| E857R 5’ | C TCG GTC AAC CGT CCC CTG GCT GCC TAC TCC T |

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The Role of Glut-857 in the Gastric H,K-ATPase

**Cell Culture and Transient Transfection—**HEK293 T cells were cultured in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, 2.0 mM glutamine, and 0.1 mg/ml penicillin and streptomycin at 37 °C in 95% CO₂, 5% O₂ atmosphere. At confluency, the cells were released by trypsin treatment, uniformly dispersed, and cultured overnight in 100-mm tissue culture dishes. At approximately 60—80% confluency, the cells were washed with serum-free Opti-MEM I supplemented with 0.9 mM CaCl₂ and co-transfected with 9 µg of pcDNA3-α and 18 µg of pcDNA3-β. The co-transfection procedure utilized 1.3 µl of LipofectAMINE™/µg of DNA. Following overnight incubation, 20 µl of the standard culture medium was added to the serum-free culture medium, and the cells were grown for an additional 48 h. The transiently co-transfected cells were harvested for membrane preparation at 72 h post-transfection.

**Membrane Preparation—**The transiently transfected cells were washed once with PBS supplemented with 5 mM EDTA and were harvested from the plate following a brief incubation in the same medium. The cells were collected by 5 min of centrifugation at 2000 rpm and resuspended in ice-cold homogenization buffer containing 10 mM Pipes, Tris, pH 7.4, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 units/ml bovine lung aprotinin. Membranes were prepared from the cells by Dounce homogenization utilizing a 7-ml vessel and tight fitting pestle. Following a 200-stroke homogenization cycle, the membranes were diluted to a final concentration of 165 mg sucrose with 0.5 M sucrose, homogenized a final 10 strokes, and layered on a sucrose column containing 10 mM Pipes, Tris, pH 7.4, 1 mM EDTA, and 44% sucrose. Membrane fractions were resolved by 1 h of centrifugation in a Beckman SW-28 rotor centrifuged at 22,000 rpm. The membrane fraction obtained from the 44% sucrose interface was diluted 1:2 (v/v) with homogenization buffer and collected in a Beckman Ti-50.2 rotor centrifuged at 34,000 rpm for 20 min. The pellet obtained by this procedure was resuspended in 10 mM Pipes, Tris, pH 7.4, and frozen at −80 °C until assay.

**Protein Quantification and Western Analysis—**Total membrane-bound protein was quantified using the Bio-Rad assay kit with γ-globulin as the standard.

The quantitative Western analysis was performed on the AMBIS image acquisition and analysis system utilizing an XC-75/75CE CCD video camera module and AMBIS core (ver. 4)/ONE-Scan software.

**ATPase Analysis—**ATPase activity was measured in membrane preparations of nontransfected (control) and transiently co-transfected HEK293 T cells. Typically, 55 µg of membrane-bound protein was assayed by the method of Yoda and Hokin (51) using 1 µl of ATPase reaction buffer containing 2 mM ATP, 2 mM MgCl₂, 0.1 mM ouabain, 1 mM EGTA, 50 mM Tris-HCl, pH 7.4, and 1 µg of oligomycin ± 10 mM KC.

The assay was started by the addition of ATP, incubated at 37 °C for 45 min and stopped with the addition of 1 ml of 80% ammonium molybdate, 20% perchloric acid. The expressed H,K-ATPase was differentiated from an indigenous, nonspecific phosphatase by a characteristic SCH 28080-sensitive, K⁺-stimulated activity. The reported values fitted pestle. Following a 200-stroke homogenization cycle, the membranes were diluted to a final concentration of 165 mg sucrose with 0.5 M sucrose, homogenized a final 10 strokes, and layered on a sucrose column containing 10 mM Pipes, Tris, pH 7.4, 1 mM EDTA, and 44% sucrose. Membrane fractions were resolved by 1 h of centrifugation in a Beckman SW-28 rotor centrifuged at 22,000 rpm. The membrane fraction obtained from the 44% sucrose interface was diluted 1:2 (v/v) with homogenization buffer and collected in a Beckman Ti-50.2 rotor centrifuged at 34,000 rpm for 20 min. The pellet obtained by this procedure was resuspended in 10 mM Pipes, Tris, pH 7.4, and frozen at −80 °C until assay.

The gel was resolved by a two-step electrophoresis procedure utilizing 25 mA (constant current) for 20 min and 200 V (constant voltage) for 1 h. The SDS-PAGE resolved protein was transferred to nitrocellulose for Western analysis.

The quantitative Western analysis was performed on the AMBIS image acquisition and analysis system utilizing an XC-75/75CE CCD video camera module and AMBIS core (ver. 4)/ONE-Scan software. 35–55-µg aliquots of total membrane protein were resolved by SDS-PAGE run under reducing conditions according to the method of Laemmli (50) and transferred to nitrocellulose for Western analysis.
The Role of Glu-857 in the Gastric H,K-ATPase

The concentration dependence of the \( K^+ \)-stimulated activation and the SCH 28080-dependent inhibition were measured to provide a comparison of the steady-state kinetic properties of the native gastric H,K-ATPase to that expressed in the membranes of HEK293 T cells. The data from four separate co-transfected cell populations were combined in Fig. 3. The concentration dependence of the \( K^+ \)-stimulated activity is shown in panel A, where the activity is saturable with a \( K_{m,5} \) of 0.6 ± 0.1 mM KCl. Panel B shows the concentration dependence of the SCH 28080-inhibited component, where the \( K_i \) is 0.96 ± 0.3 mM. These steady-states kinetic constants resemble those of the native gastric H,K-ATPase reported in Table III.

Structure and Function of Glu-857—To investigate the role of size and charge at Glu-857, site mutants were prepared to reverse the charge from negative to positive or vary the size of the uncharged side chain at this position. Each of these site mutants was co-transfected into HEK293 T cells with the H,K-ATPase \( \beta \)-subunit. The \( \alpha \)-subunit expressed for each site mutant is shown in Fig. 4. The Western analysis of the expressed protein confirms that all of the \( \alpha \)-subunit site mutants are expressed at approximately equivalent levels. The small distortion in lane 2 is attributable to the difference in protein

| Preparation | Subunit | \( \mu \)g subunit/mg protein | Ratio \( \beta : \alpha \) (w/w) |
|-------------|---------|----------------------------|----------------------------|
| TT2         | \( \alpha \) | 4.8                         | 1.7                        |
|             | \( \beta \) | 8.0                         |                            |
| TT3         | \( \alpha \) | 3.8                         | 1.2                        |
|             | \( \beta \) | 4.6                         |                            |
| TT4         | \( \alpha \) | 7.0                         | 0.5                        |
|             | \( \beta \) | 3.7                         |                            |
| Summary     | \( \beta \) | 5.5 ± 2.3                   |                            |

Table II

Co-expression of \( \alpha \)- and \( \beta \)-subunits in transiently transfected HK293 T cells

HK293 T cells were co-transfected with plasmid DNA constructs containing both \( \alpha \) and \( \beta \) H,K-ATPase subunits in three separate experiments. Cell membranes were prepared 72 h post-transfection and probed for subunit expression using monoclonal antibodies to each subunit.

FIG. 1. Subunit expression of the H,K-ATPase in HEK293 T cells. 55 \( \mu \)g of cell membrane protein per lane was resolved by SDS-PAGE and transferred to nitrocellulose. Top panel, lanes probed with H,K-ATPase \( \alpha \)-subunit antibody, mAb 12.18. Bottom panel, lanes probed with H,K-ATPase \( \beta \)-subunit antibody, mAb 2B-6. Lane 1, transfection with H,K-ATPase \( \beta \)-subunit; lane 2, transfection with H,K-ATPase \( \alpha \)-subunit; lane 3, co-transfection with \( \alpha \) and \( \beta \)-subunits; lane 4, 0.3 \( \mu \)g of the native H,K-ATPase; lane 5, untransfected HEK293 T cells.

for the \( K_{m,5} \) (KCl) and \( K_i \) (SCH 28080) were calculated from the global fit of \( n = 3 \) measurements from each of three separate transient co-transfection experiments. The steady-state kinetic constants were calculated from the nonlinear best fit to: \[ K_{m,5} = V_{max} \times [K^+] / K_{m,5} + [K^+] \] and \[ Y = 1/(1 + ([SCH 28080]/K_i) + nse (nonspecific). \]

Cell Line, Antibodies, and Materials—HEK293 T cells were a kind gift of Dr. Jamboor K. Vishwanatha. Anti-\( \beta \) mAb 2B-6 was a kind gift from Dr. A. Smolka. The sense and antisense mutant and sequencing primers were purchased from Midland Certified Reagents. LipofectAMINE

RESULTS

Transient Expression of Wild Type H,K-ATPase—The H,K-ATPase of mammalian species is organized as a \( \alpha/\beta \) heterodimer. The pore-forming \( \alpha \)-subunit resolved by SDS-PAGE and probed with H,K-ATPase \( \alpha \)-subunit antibody, mAb 12.18, is shown in Fig. 1, upper panel, lane 4. The \( \alpha \)-subunit appears as a single band at \( M_r \), 94,000. The \( \beta \)-subunit probed with H,K-ATPase \( \beta \)-subunit antibody, mAb 2B-6, is shown in lane 4 of the lower panel of Fig. 1. Because of its extensive glycosylation the \( \beta \)-subunit exhibits a mobility ranging from \( M_r \) of 70,000 to 80,000. Membrane preparations from HK293 T cells transiently transfected with the H,K-ATPase cDNA are shown in the remaining lanes of Fig. 1. As shown in lanes 1 and 2, either the \( \beta \)- or \( \alpha \)-subunit is expressed in the HK293 T cells. The cells transiently transfected with the \( \alpha \)-subunit express a subunit of comparable mobility to the native H,K-ATPase. There is no evidence of smaller protein fragments suggestive of premature termination of protein synthesis or of enhanced proteolysis of the mature subunit. In contrast to the native H,K-ATPase \( \beta \)-subunit, the expressed H,K-ATPase \( \beta \)-subunit is resolved into four bands ranging from \( M_r \), 54,000 to 77,000. This heterogeneity is most likely because of the differential glycosylation of the \( M_r \), 34,000 core protein of the \( \beta \)-subunit. As expected, because of the individual subunit expression, cells co-transfected with both \( \alpha \) and \( \beta \)-cDNA also co-express both subunits. The co-expression of the \( \alpha \) and \( \beta \)-subunits does not change the profile of \( \beta \)-subunit maturation products from that observed with the expression of the \( \beta \)-subunit alone. The membrane preparation from untransfected cells is shown in lane 5. Neither gastric H,K-ATPase \( \alpha \)- or \( \beta \)-subunits are expressed at detectable levels in the untransfected cells.

A quantitative measurement of the \( \alpha \)- and \( \beta \)-subunits co-expressed in three separate membrane preparations of co-transfected HK293 T cells is shown in Table II. The expressed \( \alpha \)-subunit ranged from 3.8 to 7.0 \( \mu \)g with a mean of 5.2 ± 1.6 \( \mu \)g/mg of total membrane protein, whereas the \( \beta \)-subunit ranged from 3.7 to 8 \( \mu \)g with a mean of 5.5 ± 2.3 \( \mu \)g of expressed protein/mg of total membrane protein. The ratio of \( \beta \)-to \( \alpha \)-subunit expressed in each membrane preparation ranged from 0.5 to 1.2 (w/w), although the molar ratio of the total \( \beta \)-subunit population was always expressed in excess of the \( \alpha \)-subunit.

H,K-ATPase Activity in Transiently Transfected HK293 T Cells—To investigate the subunit requirement for H,K-ATPase function, the \( \alpha \)-subunit was transiently expressed in the presence or absence of the H,K-ATPase \( \beta \)-subunit. The H,K-ATPase activity associated with \( \alpha \)-subunit expression with or without the \( \beta \)-subunit is summarized in Fig. 2. As indicated in the first bar of each experimental cluster, all membrane preparations of the HK293 T cells possess a nonspecific nucleotidase activity in the presence of 0.1 mM ouabain and 1 \( \mu \)g of oligomycin (per 55 \( \mu \)g of protein). This normalized activity, reported as 1, ranged from 0.8 to 1.7 \( \mu \)mol/mg/h in four preparations. This basal nucleotidase activity was stimulated approximately 10% by 10 mM KCl and inhibited by 55 \( \mu \)M SCH 28080. This activity profile was not changed with the transient transfection and expression of the \( \alpha \)- or \( \beta \)-subunit alone. In contrast, the co-transfection and expression of both subunits significantly increased the \( K^+ \)-stimulated ATPase. The mean normalized \( K^+ \)-stimulated activity reported in Fig. 2 is approximately 50% above that of the untransfected controls. This normalized activity represents an enhanced \( K^+ \)-stimulated ATPase ranging from 0.6 to 1.1 \( \mu \)mol/mg/h in each membrane preparation. As shown, this \( K^+ \)-stimulated component is inhibited by 55 \( \mu \)M SCH 28080.
loading between the native H,K-ATPase (0.3 μg in lane 1) and the expressed H,K-ATPase (55 μg in lane 2) preparations.

The steady-state activation and inhibitory constants of each site mutant were derived from pooled data taken from three separate membrane preparations of co-transfected cells. As shown in Table III, the concentration dependence of both the K⁺-dependent stimulation of the ATPase and the SCH 28080-dependent inhibition of the co-transfected HEK293 T (wild type) cell membranes and the native hog gastric H,K-ATPase were comparable. In addition, the specific activity of the H,K-ATPase expressed in each of the site mutants was similar to that measured for the wild type activity. Thus, the site mutations at Glu-857 do not influence the level of expression of the α-subunit expression nor inhibit the specific activity of the H,K-ATPase activity.

In contrast to the specific activity of the H,K-ATPase, the steady-state kinetic constants describing the K⁺-dependent activation and the SCH 28080-dependent inhibition of the ATPase were sensitive to various site mutants. The role of the side chain was evaluated either by eliminating the charge by substituting glutamine for glutamate or reversing the charge by substituting arginine or lysine for glutamate. The removal of the charge by the conservative replacement of the side chain charge from negative to positive (Glu to Lys or Arg) and inhibitor-dependent inhibition kinetics. The reversal of the carboxyl group to a nearby residue or steric hindrance of the carboxyl activation such as the cross-linkage of the DCCD-dependent inhibition is most likely because of a secondary effect of carboxyl group to a nearby residue or steric hindrance of the inhibitor molecule inserted at this site. The former explanation has been proposed to account for carbodiimide inactivation of the Na,K-ATPase (57).

This structure-function study of Glu-857 was initiated because this residue has been identified as a K⁺-protected, [¹⁴C]DCCD-reactive site associated with the DCCD-dependent inhibition of the native, gastric H,K-ATPase (45). The data here show that various substitutions at this site do not inhibit the expressed H,K-ATPase and are inconsistent with the interpretation that Glu-857 is an essential hydrophobic residue. Thus, the interpretation of the previous report (57) of [¹⁴C]DCCD incorporation and inhibitory kinetics of the H,K-ATPase is that DCCD-dependent inhibition is most likely because of a secondary effect of carboxyl group to a nearby residue or steric hindrance of the inhibitor molecule inserted at this site. The former explanation has been proposed to account for carbodiimide inactivation of the Na,K-ATPase (57).

This investigation does show that Glu-857 is important for the concentration dependence of cation-dependent activation and inhibitor-dependent inhibition kinetics. The reversal of the side chain charge from negative to positive (Glu to Lys or Arg) altered the kinetic profile by decreasing the Kᵢ for K⁺-dependent activation and increasing the Kᵢ for SCH 28080-dependent inhibition of the fully active H,K-ATPase. A partial explanation consistent with previous fluorescein isothiocyanate quench studies of the native H,K-ATPase is that the residue is involved in setting the conformational equilibrium, Kᵢ, between the E₁ and Eᵢᵢ enzyme conformations. The apparent dissociation constant, Kᵢ, derived from the Kᵢ concentration dependence of steady-state measurements of fluorescein isothiocyanate quench, was related to Kᵢ and the intrinsic K⁺ dissociation constant, Kᵢ, through the relationship Kᵢ ≈ Kᵢ/Kᵢ + 1. This

**FIG. 2.** Subunit dependence of the functional H,K-ATPase. ATPase activity in n = 3 replicates was measured in the standard ATPase buffer containing 2 mM MgCl₂ (norm. profiles in each preparation were normalized (norm.) to the activity measured in the presence of Mg⁺/K⁺/Kᵢ/α/β 0.04. The specific activity in μmol/mg/h obtained in co-transfected cell membranes was: Mg⁺/K⁺, 1.6 ± 0.4; Mg⁺/K⁺, 2.3 ± 0.7; Mg⁺/K⁺ + SCH 28080, 1.7 ± 0.04.

**DISCUSSION**

This report provides evidence of the functional expression of the gastric H,K-ATPase in a mammalian HEK293 T cell line. The finding that both the α- and β-subunits were required for expression of the functional H,K-ATPase confirms several biochemical investigations suggesting that the H,K-ATPase is an essential heterodimer (4–6). It is interesting that whereas the expression of the H,K-ATPase β-subunit was essential for H,K-ATPase function, the mature α-subunit, free of degradation products, was expressed in the absence of the H,K-ATPase β-subunit. This steady-state observation is somewhat unexpected because the co-translation of the α- and β-subunits are necessary to stabilize the Na,K-ATPase α-subunit expressed in *Xenopus laevis* oocytes (52, 53). It is possible that the indigenous Na,K-ATPase β-subunit present in the HEK293 T cells could stabilize the H,K-ATPase α-subunit, although it would then be inadequate to properly fold the functional ATPase. The data also suggest that the role of the H,K-ATPase β-subunit in ATPase function may be fulfilled by the immature β-subunit because several glycosylation variants of the β-subunit were resolved by SDS-PAGE. Expression studies of the Na,K-ATPase in *Xenopus* oocytes have shown that the glycosylated β-subunit is important in assembly efficiency but is not required for the functional maturation of the catalytic Na,K-ATPase α-subunit (54). The importance of β-subunit glycosylation for H,K-ATPase function is uncertain because it has been shown that glycosylation inhibitors inactivate the H,K-ATPase expressed in SF9 cells (55, 56). The present study does not eliminate the possibility that a sufficient quantity of a complex glycosylated species is present.

The H,K-ATPase activity of cell membranes is displayed within an activity profile containing a nonspecific nucleotidase. The expressed H,K-ATPase is differentiated from the nonspecific nucleotidase by an enhanced K⁺-stimulated component of the ATPase that is sensitive to SCH 28080, a competitive inhibitor of the gastric H,K-ATPase. The Kᵢ-dependence of the expressed H,K-ATPase is comparable with that of the native gastric H,K-ATPase with a Kᵢ (KCl) of 0.6 ± 0.1 mM and 0.5 ± 0.2 mM, respectively. Similarly, SCH 28080 inhibits the K⁺-stimulated ATPase of each with a Kᵢ (SCH 28080) = 1.0 ± 0.3 μM and 0.7 ± 0.1 μM, respectively. Overall, the specific activity of the H,K-ATPase normalized for the quantity of expressed protein within the total membrane protein population is comparable with that of the native H,K-ATPase with a specific activity of 158 μmol/mg/h.

This structure-function study of Glu-857 was initiated because this residue has been identified as a K⁺-protected, [¹⁴C]DCCD-reactive site associated with the DCCD-dependent inhibition of the native, gastric H,K-ATPase (45). The data here show that various substitutions at this site do not inhibit the expressed H,K-ATPase and are inconsistent with the interpretation that Glu-857 is an essential hydrophobic residue. Thus, the interpretation of the previous report (57) of [¹⁴C]DCCD incorporation and inhibitory kinetics of the H,K-ATPase is that DCCD-dependent inhibition is most likely because of a secondary effect of carboxyl activation such as the cross-linkage of the carboxyl group to a nearby residue or steric hindrance of the inhibitor molecule inserted at this site. The former explanation has been proposed to account for carbodiimide inactivation of the Na,K-ATPase (57). This investigation does show that Glu-857 is important for the concentration dependence of cation-dependent activation and inhibitor-dependent inhibition kinetics. The reversal of the side chain charge from negative to positive (Glu to Lys or Arg) altered the kinetic profile by decreasing the Kᵢ for K⁺-dependent activation and increasing the Kᵢ for SCH 28080-dependent inhibition of the fully active H,K-ATPase. A partial explanation consistent with previous fluorescein isothiocyanate quench studies of the native H,K-ATPase is that the residue is involved in setting the conformational equilibrium, Kᵢ, between the E₁ and Eᵢᵢ enzyme conformations. The apparent dissociation constant, Kᵢ, derived from the Kᵢ concentration dependence of steady-state measurements of fluorescein isothiocyanate quench, was related to Kᵢ and the intrinsic K⁺ dissociation constant, Kᵢ, through the relationship Kᵢ ≈ Kᵢ/Kᵢ + 1. This
model predicts that the K\textsuperscript{+}-dependent concentration profile will shift from 0.6 to 0.2 mM K\textsuperscript{+} in response to a 3-fold increase in the conformational equilibrium of K\textsubscript{d} toward E\textsubscript{2} (58). It is also likely that the structural consequence of the introduced mutations is more complex than a shift in the E\textsubscript{1}/E\textsubscript{2} conformational equilibrium, because the reciprocal pattern of the concentrations dependence for the activating and inhibiting ligands necessarily implies that the reactivity of the catalytic intermediates, E\textsubscript{1}P, is shifted to favor K\textsuperscript{+} over SCH 28080. A further analysis of the functional consequences of these site modifications, including peptide mapping of ligand-stabilized proteolytic digests, the analysis of noncompetitive inhibitor kinetics, and the analysis of partial reactions, is beyond the scope of the present investigation.

The fourth cytoplasmic domain (C4) defined by the transmembrane spanning domains M6–M7 is a stretch of 23 amino acids beginning at Glu-835 (LAYE) and extending to Glu-857 (LVNE). Both the N- and C-terminal stretches of the domain exhibit a probability of α-helix formation by Chou-Fasman indices. Residues Glu-835 (LAYE), Glu-838 (KAES), and Asp-840 (DIMH) within the predicted helix of the N-terminal stretch of this domain are essential for the expression of the SCH 28080-sensitive phosphoenzyme in Sf-9 cells (59). The present investigation shows that Glu-857 in the second predicted helix of the C-terminal stretch is not essential but is an effector of the inhibitor-dependent and, to a lesser extent, the ligand-dependent kinetics of ATPase activity. The C4 domain is well conserved in the Na,K-ATPase (74%) but is nonconserved in the Ca-ATPase (1 in 23 residues). One could speculate from the kinetic signature of ATPase function and the limitation of sequence conservation to the heterodimeric isomers of the P2 ATPase family that this domain is of special significance for the relay of information between the α- and β-subunits. It seems likely that this domain is one of a complex set of structural elements that contribute to the conformational equilibrium of this ATPase.

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