Functional Expression of Gastric H\(^+\),K\(^+\)-ATPase and Site-directed Mutagenesis of the Putative Cation Binding Site and the Catalytic Center*

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Gastric H\(^+\),K\(^+\)-ATPase was functionally expressed in the human kidney HEK293 cell line. The expressed enzyme catalyzed ouabain-resistant K\(^+\)-dependent ATP hydrolysis. The K\(^+\)-ATPase activity was inhibited by SCH 28080, a specific inhibitor of gastric proton pump, in a dose-dependent manner. By using this functional expression system in combination with site-directed mutagenesis, we investigated effects of mutations in the putative cation binding site and the catalytic center of the gastric H\(^+\),K\(^+\)-ATPase. In Na\(^+\),K\(^+\)-ATPase, the glutamic acid residue in the 4th transmembrane segment is regarded as one of the residues responsible for the K\(^+\)-induced conformational change (Kuntzweiler, T. A., Wallick, E. T., Johnson, C. L., and Lingrel, J. B. (1995) J. Biol. Chem. 270, 2993-3000). When the corresponding glutamic acid (Glu-345) of H\(^+\),K\(^+\)-ATPase was mutated to aspartic acid, lysine, or valine, the SCH 28080-sensitive K\(^+\)-ATPase activity was abolished. However, when this residue was replaced by glutamine, about 50% of the activity was retained. This mutant showed a 10-fold lower affinity for K\(^+\) (K\(_m\) = 2.6 mM) compared with the wild-type enzyme (K\(_m\) = 0.24 mM). Thus, Glu-345 is important in determining the K\(^+\)-affinity of H\(^+\),K\(^+\)-ATPase. When the aspartic acid residue in the phosphorylation site was mutated to glutamic acid, this mutant showed no SCH 28080-sensitive K\(^+\)-ATPase activity. Thus, amino acid replacement of the phosphorylation site is not tolerated and a stringent structure appears to be required for enzyme activity. When the lysine residue in the fluorescein isothiocyanate binding site (part of ATP binding site) was mutated to arginine, asparagine, or glutamic acid, the SCH 28080-sensitive K\(^+\)-ATPase activity was eliminated. However, the mutant in which this residue was changed to glutamine had about 30% of the activity, suggesting that amino acid replacement of this site is tolerated to a certain extent.

H\(^+\),K\(^+\)-ATPase is the proton pump responsible for gastric acid secretion (1, 2). It consists of \(\alpha\)- and \(\beta\)-subunits. The \(\alpha\)-subunit is the catalytic subunit with a molecular mass of 114 kDa (3) and contains the phosphorylation site, the ATP binding site, and the binding sites for proton pump inhibitors (4–7). The \(\beta\)-subunit is a glycoprotein with a molecular mass of 60–80 kDa (8). One of the roles of the \(\beta\)-subunit is to stabilize the \(\alpha\)-subunit in the membrane. Although the cDNAs of both subunits of many species were cloned, there have been no reports of structure-function studies using site-directed mutagenesis because there has been no effective functional expression system. Here we report the functional expression of rabbit gastric H\(^+\),K\(^+\)-ATPase in human HEK293 cells. When the cells were co-transfected with the cDNAs of the \(\alpha\)- and \(\beta\)-subunits, ouabain-resistant K\(^+\)-dependent ATPase activity was observed. The activity was inhibited by SCH 28080 and scopadulic acid B, specific inhibitors of the gastric H\(^+\),K\(^+\)-ATPase (9, 10). By using this functional expression system, we investigated the role of amino acid residues of the putative cation binding site and the catalytic center.

H\(^+\),K\(^+\)-ATPase is a member of the P-type ATPase family. Sarcoplasmic and endoplasmic reticulum Ca\(^{2+}\)-ATPases and Na\(^+\),K\(^+\)-ATPase also belong to the same family. They actively transport the ions coupled with the hydrolysis of ATP. It has been considered that P-type ATPases have the common structures in the catalytic center including the phosphorylation site and the ATP binding site. On the other hand, their cation recognition sites and transport pathways are hypothesized to be common to some extent, but divergent depending on the species of transporting cations. From the site-directed mutagenesis and chemical labeling experiments, Glu-327 in the \(\alpha\)-subunit of Na\(^+\),K\(^+\)-ATPase (sheep \(\alpha\)-1) has been recognized as one of the pivotal residues for cation-induced conformational changes or for K\(^+\) occlusion (11–13). The replacement of this residue by glutamine completely reduced the affinity of the enzyme for Na\(^+\) and K\(^+\) (14). Glu-309 in sarcoplasmic Ca\(^{2+}\)-ATPase (the counterpart of Glu-327 of Na\(^+\),K\(^+\)-ATPase) has been suggested to be responsible for Ca\(^{2+}\) high-affinity binding. The replacement of this residue by glutamine completely eliminated the Ca\(^{2+}\)-transport activity and the Ca\(^{2+}\)-sensitivity in the phosphorylation reaction (15). Here we mutated the corresponding residue (Glu-345) of the H\(^+\),K\(^+\)-ATPase \(\alpha\)-subunit and compared the property of the mutant with those of Na\(^+\),K\(^+\)-ATPase and Ca\(^{2+}\)-ATPase.

The sequences around the phosphorylation site and the FITC\(^1\) binding site are well conserved in some of the P-type ATPases (16). In Na\(^+\),K\(^+\)-ATPase and Ca\(^{2+}\)-ATPase, amino acid replacement in the phosphorylation site is not tolerated (17, 18). In Ca\(^{2+}\)-ATPase, amino acid replacement at the FITC binding site is tolerated, and the structure is able to withstand basic amino acids, but not a negatively charged amino acid (18). In the present paper, we also replaced Asp-387 of the phosphorylation site and Lys-519 of the FITC binding site of the H\(^+\),K\(^+\)-ATPase \(\alpha\)-subunit, measured the enzyme activity of the

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†The abbreviations used are: FITC, fluorescein isothiocyanate; PCR, polymerase chain reaction; PBS, phosphate-buffered saline.
mutants, and compared the effects of these mutations with those of Na⁺,K⁺-ATPase and Ca²⁺-ATPase.

**EXPERIMENTAL PROCEDURES**

Materials—HEK293 cells (human embryonic kidney cell line) were a gift from Dr. J. Nathan Lyttton (Brigham & Women's Hospital, Harvard Medical School, Boston, MA). pCDNA3 vector was purchased from Invitrogen (San Diego, CA). Restriction enzymes and other DNA and RNA modifying enzymes were from Toyobo (Osaka, J. Japan), New England Biolabs, Life Technologies, Inc., or Pharmacia Biotech Inc. (Tokyo, Japan). DNA was purchased from Biozol Laboratories (Houston, TX), Oligotex-dT30 and MutanK kit were from Takara (Ohtsu, J. Japan), and ZAP-cDNA synthesis kit and Gigapack II Gold lambda packaging extract were from Stratagene. Block Ace was purchased from Dainippon Pharmaceutical Co. (Osaka, J. Japan). All other reagents were of molecular biology grade or the highest grade of purity available.

Preparation of DNA library—Total RNA was prepared from gastric mucosa of a male Japanese white rabbit using RNAzol. Poly(A) RNA was isolated using Oligotex-dT30. A gastric cDNA library was prepared using the ZAP-cDNA synthesis kit and Gigapack II Gold lambda packaging extract according to their instruction manuals.

cDNA Cloning of α- and β-Subunits of H⁺,K⁺-ATPase—cDNA was synthesized from total RNA from rabbit gastric mucosa using Moloney murine leukemia virus reverse transcriptase, primed with oligo(dT). The α-subunit cDNA between nucleotides 829 and 1412 (counted from initiation ATG as position 1), and the β-subunit cDNA between nucleotides 320 and 649 were amplified by PCR. PCR primers for the α-subunit were 5'-ATATCCGGGCGTCGGCCTC-3' and 5'-GAGAAC-ATTCAAGGAGGGCAGCG-3'. PCR primers for the β-subunit were 5'-CGCGTCGCCACCCGCTACCACCCTG-3' and 5'-CCGATTCGCACCTGACCACTCTG-3'. The sequences of these primers were from the cDNA sequences of the α- and β-subunits of H⁺,K⁺-ATPase (19, 20) except that the β-subunit primers contained SalI and BamHI restriction sites (underlined). The 583-basepair fragment of the α-subunit cDNA and the 329-base pair fragment of the β-subunit cDNA were gel purified, labeled with [γ³²P]-ATP, and used for screening the gastric cDNA library. The screening of the cDNA library was carried out as described below. Duplicate nitrocellulose filters (Hybond-C, Amersham) were prehybridized for 2 h at 41 °C in 1× NaCl, 5 mM EDTA, 4 mM sodium phosphate, 0.1% SDS, 5 (tonnes) Denhardt’s, 50% formamide, 100 μg/ml salmon sperm DNA, 50 μg/ml Tris-HCl (pH 8.0). Hybridization was carried out in the same solution supplemented with the 32P-labeled probe at 41 °C overnight. After washing under high stringency conditions, the filters were dried and exposed on an x-ray film. The positive plaques were screened by autoradiography. cDNAs in pBluescript SK(−) vector were prepared by in vivo excision from UniZAP XR as described in the instruction manuals.

Results—Cloning of Sequences—The 5′-untranslated cDNA—The 5′-untranslated cDNA between nucleotides 28 and 495 was amplified by PCR. The PCR primers were 5'-CCGTAATCAAAGGGCGCCGCGCAGCGAGGG-3' and 5'-GCTCCAGGTCCTGACATTGAGTCAGG-3'. The 540-base pair fragment was purified on a gel and digested with EcoRI and BstEII. The cDNA cassette between EcoRI and BstEII of the α-subunit cDNA construct was replaced by the PCR-derived fragment in order to remove the cDNA sequence in its 5′-noncoding region (nucleotides 68 to 29). This truncated cDNA was used as the cDNA construct for the α-subunit.

Constructs of the α- and β-subunit cDNAs in Mammalian Expression Vector pCDNA3—The α- and β-subunit cDNAs were digested with EcoRI and XhoI. The obtained fragments were each ligated into pCDNA3 vector treated with EcoRI and XhoI.

DNA Sequencing—DNA sequencing was done by the dyeodeoxy chain termination method using an Autotrace DNA sequencing kit and an ALF-11 DNA Sequencer (Pharmacia Biotech Inc.).

Site-directed Mutagenesis—Site-directed mutagenesis was carried out by the method of Kunkel (21) using a MutanK kit. Synthetic oligonucleotides long and containing one or two mutations near the center, were hybridized with the uridine-containing single strand template of the construct of the H⁺,K⁺-ATPase α-subunit. After sequencing, the appropriate fragment of mutant α-subunit cDNA was excised and ligated back into the relevant position of the wild-type construct of the α-subunit.

Cell Culture and Transfection—HEK293 cells were maintained in a humidified incubator at 37 °C under 5% CO₂ atmosphere in Dulbecco’s modified Eagle medium (high glucose) (Life Technologies, Inc.) containing 2 mM l-glutamine, 100 μM minimum essential medium nonessential amino acids, 10% fetal calf serum, penicillin G (100 units/ml), and streptomycin (100 μg/ml). DNA transfection was performed by the calcium phosphate method with 10 μg of cesium chloride-purified DNA per 10-cm dish. Cells were harvested 2 days after the DNA transfection.

Preparation of Membrane Fractions from HEK293 Cells—Membrane fractions from the transfected cells and control (mock-transfected) cells were prepared from cells scraped from five to twenty 10-cm Petri dishes. Cells were washed with 5 ml of PBS, scraped, and suspended in PBS containing 5 mM EDTA. After washing twice with PBS, cells were incubated in 2 ml of low ionic salt buffer (0.5 mM MgCl₂, 10 mM Tris-HCl, pH 7.4) at 0 °C for 10 min. Phenylmethylsulfonyl fluoride (1 mM) and aprotinin (0.09 unit/ml) were added to the suspension. The cell homogenates were then passed through a Dounce homogenizer, and the homogenate was diluted with an equal volume of a solution containing 500 μM sucrose and 10 mM Tris-HCl, pH 7.4. The cell suspension was centrifuged with 25 strokes. The homogenated suspension was centrifuged at 800 × g for 90 min, and the pellet was suspended in a solution containing 250 mM sucrose and 5 mM Tris-HCl, pH 7.4.

Gastric Vesicles—Gastric vesicles enriched in H⁺,K⁺-ATPase were prepared from mucosa in the fundic region of hog stomachs by differential and density gradient centrifugation as described elsewhere (22). SDS-Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was carried out as described elsewhere (23). Membrane preparations (30 μg of protein) or gastric vesicles (0.5 or 1 μg of protein) were incubated in a sample buffer containing 2% SDS, 2% mercaptoethanol, 10% glycerol, and 10 mM Tris-HCl, pH 6.8, at room temperature for 2 min and applied onto the SDS-polyacrylamide gel.

Immunoblot—Proteins were blotted onto nitrocellulose filters in a transfer solution containing 20% methanol, 192 mM glycine, and 25 mM Tris, pH 8.3, as described elsewhere (24). The filters were soaked in 4% Block Ace solution for 20 min at room temperature. They were incubated for 2 h with the primary antibody diluted with 0.8% Block Ace solution. The blots were washed with Tris-buffered saline containing 0.1% Tween 20 and incubated with peroxidase-conjugated anti-rabbit IgG for 1 h. Finally, the blots were stained with Peroxidase Immunostain kit from Wako Pure Chemicals (Osaka, J. Japan).

Antibody—Ab1024 was previously raised against the carboxyl-terminal peptide (residues 1024–1034) of the H⁺,K⁺-ATPase α-subunit (PG-SWWDEELYY) (25).

Assay of H⁺,K⁺-ATPase Activity—The H⁺,K⁺-ATPase activity was measured in 1 ml of a solution containing 50 μg of membrane protein, 40 mM Tris-HCl, pH 7.4, 3 mM MgCl₂, 3 mM ATP, 5 mM NaN₃, 1 mM ouabain, and 15 mM KCl. After the reaction at 37 °C for 30 min, the inorganic phosphate released was measured as described elsewhere (26). The K⁺-ATPase activity was calculated as the difference between the activities in the presence and absence of KCl. Furthermore, the SCH 28080-sensitive K⁺-ATPase activity was calculated as the difference between the K⁺-ATPase activities in the presence and absence of 50 μM SCH 28080.

Protein was measured using the BCA Protein Assay Kit from Pierce with bovine serum albumin as a standard.

**RESULTS**

Expression of the α- and β-Subunits of H⁺,K⁺-ATPase—We prepared the cDNAs encoding the α- and β-subunits of rabbit gastric H⁺,K⁺-ATPase according to the cDNA sequences presented elsewhere (19, 20) with the exception that some part of the 5′-untranslated region (nucleotides 68 to 29) of the α-cDNA was removed as described under “Experimental Procedures.” These cDNAs were introduced in a mammalian expression vector, pCDNA3, which contained a strong cytomegalovirus promoter. Fig. 1 shows the immunoblot of the crude membrane fractions from HEK293 cells; mock-transfected (lane 1) and transfected with the cDNA constructs of α- (lane 2), β-subunit (lane 3) or α- plus β-subunits (lane 4). The blots were detected by using an anti-α subunit antibody. A hog gastric vesicle preparation was used as a positive control (lanes 5 and 6). In the mock-transfected cells and cells transfected with the β-subunit cDNA, no band was detected. When the cells were transfected with the α-subunit cDNA, a single weak band was detected around 95 kDa. When the cells were co-transfected with the α- and β-subunit cDNAs, a significantly denser band was observed. The α-subunit expression on the immunoblot...
Site-directed Mutagenesis of H⁺,K⁺-ATPase

**Fig. 1. Immunoblotting with Ab1024 directed against the H⁺,K⁺-ATPase α-subunit.** Thirty micrograms of HEK293 cell membrane fractions (lanes 1–4) and 0.5 and 1 μg of gastric vesicles (lanes 5 and 6) were applied to the gel. Lanes: 1, mock-transfected cells; 2, cells transfected with the α-subunit cDNA; 3, the β-subunit cDNA; 4, α- and β-subunit cDNAs; 5, gastric vesicles (0.5 μg); 6, gastric vesicles (1 μg).

from 30 μg of the crude membrane fraction of the expressed cells appears to be comparable to that of 0.5 μg of the gastric vesicle preparation.

**H⁺,K⁺-ATPase Activity of the Membrane Fractions—**The H⁺-stimulated ATPase activity was measured in the membrane fractions from the cells transfected with the α-subunit, β-subunit, or α- and β-subunit cDNAs. 1 mM ouabain and 5 mM NaN₃ were added to the reaction mixture to inhibit endogenous Na⁺,K⁺-ATPase and mitochondrial ATPase, respectively. These inhibitors did not affect the gastric H⁺,K⁺-ATPase activity (data not shown). A significant SCH 28080-sensitive K⁺-ATPase activity was detected only in the cells transfected with both the α- and β-subunit cDNAs (Table 1). This activity did not increase in the presence of the monovalent cation ionophore, gramicidin, indicating that the membranes were leaky. The expressed K⁺-ATPase was sensitive to SCH 28080, an inhibitor specific for the gastric H⁺,K⁺-ATPase. Fig. 2 shows the effect of SCH 28080 on the K⁺-ATPase activity of the membrane fraction from the αβ-transfected cells. SCH 28080 inhibited the K⁺-ATPase activity in a dose-dependent manner, with an IC₅₀ value of 2.1 μM. The inhibitory effect of SCH 28080 on the expressed H⁺,K⁺-ATPase was weaker than its effect on the hog gastric vesicles, for which the IC₅₀ value of 0.87 μM was reported (27). SCH 28080 is an inhibitor competitive with luminal K⁺. It is protonated in an acidic compartment (28). The difference in potency of SCH 28080 between our system and the gastric vesicles may be due to the facts that the K⁺ and H⁺ permeabilities of the HEK membrane were higher than those of gastric vesicles and that SCH 28080 was not well protonated. In fact, the IC₅₀ value of SCH 28080 in leaky gastric vesicles at pH 7.5 was reported to be 1.5 μM (28). The K⁺-ATPase activity was also sensitive to scopadulcic acid B, a proton pump inhibitor found in a Paraguayan medicinal herb (10). Scopadulcic acid B at 100 μM inhibited 40% of the K⁺-ATPase activity.

**Fig. 3. K⁺ dependence of the expressed SCH 28080-sensitive K⁺-ATPase activity.** The K⁺-ATPase activity was measured as a function of the K⁺ concentrations. ●, wild-type; ○, mutant E345Q. The values are mean ± S.E. for 3 observations.

**Table I**

| Cells                  | K⁺-ATPase activity (μmol Pi/mg/h) |
|------------------------|----------------------------------|
| Control (mock-transfected) | 0.06 ± 0.04 (n = 3) |
| β                      | 0.01 ± 0.08 (n = 3) |
| α                      | 0.05 ± 0.06 (n = 3) |
| α + β                  | 0.91 ± 0.11 (n = 4) |

**Fig. 2. Effects of SCH 28080 on the expressed K⁺-ATPase activity.** The K⁺-ATPase activity was measured as a function of the SCH 28080 concentrations described under “Experimental Procedures.” SCH 28080 was dissolved in ethanol. The final concentration of ethanol was below 1%. The K⁺-ATPase activity was expressed as the percentage of the control value measured in the absence of SCH 28080. The values are mean ± S.E. for 3 observations. The control value is 1.05 ± 0.09 μmol/mg/h (means ± S.E. n = 3).

**Fig. 3.** K⁺ dependence of the expressed SCH 28080-sensitive K⁺-ATPase activity. The K⁺-ATPase activity was measured as a function of the K⁺ concentrations. ●, wild-type; ○, mutant E345Q. The values are mean ± S.E. for 3 observations.

Transmembrane segment of the α-subunit (Glu-329 in rat α₁, Glu-327 in rat α₂ and sheep α₁) was reported to be involved in determining the affinity for Na⁺ and K⁺ and the cation-induced conformational changes (12, 14, 31). The mutant in which this glutamic acid was mutated to glutamine or leucine showed lower affinity for Na⁺ and K⁺ than wild-type Na⁺,K⁺-ATPase (14, 31). In sarcoplasmic Ca²⁺-ATPase, the corresponding glutamic acid residue (Glu-309) was reported to be involved in high affinity binding of Ca²⁺. This residue is critically important for the function and unalterable (15). To study whether Glu-345 of the α-subunit of the H⁺,K⁺-ATPase is essential for enzyme activity, we mutated this residue to aspartic acid, lysine, glutamine, or valine and measured the SCH...
28080-sensitive K\(^{+}\)-ATPase activity. These mutants were expressed almost in the same amount as the wild-type \(\alpha\)-subunit, judging from the immunoblotting pattern with the anti-\(\alpha\)-antibody (Fig. 4A). Mutations of Glu-345 to aspartic acid (E345D), lysine (E345K), or valine (E345V) eliminated the SCH 28080-sensitive K\(^{+}\)-ATPase activity. No activity was detected even in the presence of high concentrations of KCl (data not shown). However, the mutant having glutamine for glutamic acid (E345Q) retained 50\% of the wild-type K\(^{+}\)-ATPase activity, suggesting that there is no endogenous ATPase in the membrane fraction of the wild-type enzyme (Table II).

![Image](image.png)

**Fig. 4.** Immunoblotting with Ab1024 of the membrane fraction of HEK cells transfected with the mutant \(\alpha\)-subunit and wild-type \(\beta\)-subunit cDNAs. A, E345D (lane 1), E345K (lane 2), E345Q (lane 3), E345V (lane 4), and wild-type (lane 5). B, D387E (lane 1), D387H (lane 2), D387N (lane 3), and wild-type (lane 4). C, K519Q (lane 1), K519R (lane 2), K519E (lane 3), K519N (lane 4), and wild-type (lane 5).

| Table II |
| --- |
| The SCH 28080-sensitive K\(^{+}\)-ATPase activities of the membrane fractions expressing the wild-type and the mutant enzymes |

| K\(^{+}\)-ATPase activity | \(\mu\text{mol P}/(\text{mg} \cdot \text{h})\) |
| --- | --- |
| Wild-type (\(\alpha + \beta\)) | 0.89 \pm 0.06 (n = 6) |
| E345D | 0.04 \pm 0.02 (n = 3) |
| E345K | 0.05 \pm 0.06 (n = 3) |
| E345Q | 0.39 \pm 0.05 (n = 3) |
| E345V | 0.00 \pm 0.01 (n = 3) |
| D387E | 0.01 \pm 0.06 (n = 4) |
| D387H | 0.05 \pm 0.02 (n = 3) |
| D387N | 0.01 \pm 0.02 (n = 3) |
| K519E | 0.00 \pm 0.04 (n = 4) |
| K519N | 0.01 \pm 0.04 (n = 4) |
| K519Q | 0.23 \pm 0.04 (n = 4) |
| K519R | 0.01 \pm 0.01 (n = 4) |

| Table III |
| --- |
| \(K_m\) for the stimulation by K\(^{+}\) and \(V_{\text{max}}\) of wild-type and E345Q mutant H\(^{+}\), K\(^{+}\)-ATPases |

| \(K_m\) (mM) | \(V_{\text{max}}\) (\(\mu\text{mol P}/(\text{mg} \cdot \text{h})\)) |
| --- | --- |
| Wild-type | 0.24 \pm 0.03 (n = 3) |
| E345Q | 2.6 \pm 0.2 (n = 3) |

DISCUSSION

Gastric H\(^{+}\),K\(^{+}\)-ATPase belongs to the family of P-type ATPases, which form phosphorylated intermediates in their catalytic cycles and are inhibited by vanadate (37). Sarcoplasmic and endoplasmic reticulum Ca\(^{2+}\)-ATPases and Na\(^{+}\),K\(^{+}\)-ATPase also belong to this group, and these ATPases were cloned and expressed, and their structure-function relationships have been studied extensively (14, 17, 18, 31, 33–35, 38–41). There are many reports describing the cDNA cloning of the \(\alpha\)- and \(\beta\)-subunits of the gastric H\(^{+}\),K\(^{+}\)-ATPase from rat, pig, human, rabbit, and dog (3, 19, 20, 42–47). There have been few reports, however, of the functional expression of the gastric H\(^{+}\),K\(^{+}\)-ATPase. Recently, gastric H\(^{+}\),K\(^{+}\)-ATPase subunits were expressed in renal proximal tubular epithelial cells (LCL-PK\(_1\)), but the enzyme functions were not measured (48). Very recently, Mathews et al. (49) reported the functional expression of the ATPase in Xenopus oocytes. The lack of an effective expression system slowed the study of the structure-function relationships of the gastric H\(^{+}\),K\(^{+}\)-ATPase. Here we report the functional expression of gastric H\(^{+}\),K\(^{+}\)-ATPase in HEK293 cells. The cDNAs for the \(\alpha\)- and \(\beta\)-subunits of H\(^{+}\),K\(^{+}\)-ATPase were introduced separately to pcDNA3 vectors, and the cDNAs were transfected separately or simultaneously into HEK293 cells with the calcium phosphate method. When the \(\beta\)-subunit cDNA alone was transfected, the \(\alpha\)-subunit was not detected by immunoblotting, suggesting that there is no endogenous H\(^{+}\),K\(^{+}\)-ATPase in HEK293 cells. No significant SCH 28080-sensitive K\(^{+}\)-ATPase was detected in the membrane fraction of these cells. When the \(\alpha\)-subunit cDNA was transfected without the \(\beta\)-subunit cDNA, a slight band of the \(\alpha\)-subunit was observed, but SCH 28080-sensitive K\(^{+}\)-ATPase activity was not detected. When the \(\alpha\)- and \(\beta\)-subunit cDNAs were co-transfected, the \(\alpha\)-subunit was clearly seen on immunoblot, and a significant and reproducible SCH 28080-sensitive K\(^{+}\)-ATPase activity was detected. These mutants were expressed to approximately the same extent as the wild-type \(\alpha\)-subunit (Fig. 4C). The mutations to arginine (K519R), glutamic acid (K519E), and asparagine (K519N) eliminated the ATPase activity. The mutant K519Q retained 30\% of the SCH 28080-sensitive K\(^{+}\)-ATPase activity of the wild-type enzyme (Table II).
activity could be demonstrated. Therefore, the β-subunit increases the expression of the α-subunit and is essential for the functional expression of the H⁺,K⁺-ATPase. As Na⁺,K⁺-ATPase is a ubiquitous enzyme, it is likely that endogenous Na⁺,K⁺-ATPase β-subunit exists in HEK293 cells. It would appear, however, that the α-subunit of the H⁺,K⁺-ATPase does not assemble with the β-subunit of the Na⁺,K⁺-ATPase in a functional form (50), although the possibility that a very weak ouabain-resistant K⁺-ATPase activity was manifested by the H⁺,K⁺-αNa⁺,K⁺-β hybrid molecule (Na⁺,K⁺-ATPase) cannot be excluded. So far, there has been no report that indicates the functional assembly between H⁺,K⁺-ATPase α-subunit and Na⁺,K⁺-ATPase β-subunit. The H⁺,K⁺-ATPase α-subunit seems to discriminate the H⁺,K⁺-ATPase β-subunit from the Na⁺,K⁺-ATPase β-subunit. On the other hand, there are several reports that the Na⁺,K⁺-ATPase α-subunit can assemble with the H⁺,K⁺-ATPase β-subunit in Xenopus oocytes (51, 52) and HeLa cells (53). The hybrid molecule (Na⁺K⁺-α and H⁺,K⁺-β) showed Na⁺K⁺ pump current and Rb⁺ uptake, although these activities were much smaller than those in the authentic Na⁺,K⁺-ATPase αβ complex (51). In this case, the H⁺,K⁺-ATPase β-subunit manages to act as a surrogate for the Na⁺,K⁺-ATPase β-subunit.

The expressed K⁺-ATPase activity described here did not increase in the presence of gramicidin, which stimulates K⁺-ATPase activity in gastric vesicles (54). This may be due to the leakiness of the HEK cell membrane to K⁺ and H⁺. In fact, when we measured proton transport of the membrane fraction using acridine orange, the fluorescence was not significantly quenched.

We mutated amino acid residues involved in the putative cation binding (Glu-345), the formation of the phosphorylated intermediate (Asp-387), and the ATP binding (Lys-519). We expressed these mutants in our system and compared the properties of the mutants with those of wild-type enzyme. For the Glu-345 mutants, three in four mutants we prepared (E345D, E345K, E345V) did not show the SCH 28080-sensitive K⁺-ATPase activity. The remaining mutant, E345Q, retained 50% of the K⁺-ATPase activity of the wild-type enzyme. In sarcoplasmic reticulum Ca²⁺-ATPase, Glu-309 (the counterpart of Glu-345 of the H⁺,K⁺-ATPase) is supposed to be one of the amino acid residues constituting the Ca²⁺-high affinity site, because the replacement of Glu-309 residue by glutamine resulted in complete loss of Ca²⁺-transport activity and phosphorylation from ATP, and because the phosphorylation of this mutant with inorganic phosphate was observed even in the presence of Ca²⁺ (15). This residue is well conserved in P-type ATPases, including Na⁺,K⁺-, Ca²⁺-, and H⁺,K⁺-ATPase, plasma membrane Ca²⁺-, K⁺-, and H⁺,K⁺-ATPase, yeast H⁺-ATPase, and gastric H⁺,K⁺-ATPase. However, Glu-329 in rat kidney Na⁺,K⁺-ATPase α-subunit has been shown not to be essential for active transport of Na⁺ and K⁺, because the replacement of this residue to glutamine retains the enzyme activity (14). Furthermore, mutations of Glu-327 (the counterpart of Glu-345 in Na⁺,K⁺-ATPase αβ-subunit) to glutamine and leucine allow the enzyme to retain function, whereas mutations to aspartic acid and alanine do not (31). Our results presented here suggest that Glu-345 is not absolutely essential for the ATPase function in gastric H⁺,K⁺-ATPase as is the case with Na⁺,K⁺-ATPase. Because Glu-345 can be replaced by glutamine, the negative charge of the glutamic acid residue in this site is not indispensable for the function of H⁺,K⁺-ATPase. Rather, the bulkiness of the side chain in this site appears to be important, because the glutamic acid cannot be replaced by aspartic acid. These features are also comparable to those of Na⁺,K⁺-ATPase (31).

involved in determining the K⁺-affinity. The role of this glutamic acid residue in H⁺,K⁺-ATPase is also comparable to that in Na⁺,K⁺-ATPase, whose affinity for Na⁺ and K⁺ was reduced by the replacement of glutamic acid by glutamine (14, 31). The difference in the manner by which this glutamic acid residue contributes to the functioning of H⁺,K⁺-ATPase, Na⁺,K⁺-ATPase, and Ca²⁺-ATPase might reflect the difference in the structures of the ion sensors or the difference in the manner by which K⁺ participates in the reaction cycles. Both H⁺,K⁺-ATPase and Na⁺,K⁺-ATPase actively translocate K⁺, while K⁺ functions as an accelerator from one side of the membrane in Ca²⁺-ATPase (55). Recently, Kuntzweiler et al. (12) studied the effects of cations on [³H]ouabain binding and have shown that Glu-327 in sheep Na⁺,K⁺-ATPase α₂-subunit stabilizes the K⁺-induced conformation in the reaction cycle. Until now there has been no direct evidence as to whether the K⁺ recognition system is common between H⁺,K⁺-ATPase and Na⁺,K⁺-ATPase. The present result suggests that the structure and mechanism for K⁺ recognition is similar (or partly identical) between Na⁺,K⁺-ATPase and H⁺,K⁺-ATPase. However, there are striking functional differences between Na⁺,K⁺-ATPase and H⁺,K⁺-ATPase; the former electromagnetically transports Na⁺ and K⁺, and the latter non-electromagnetically transports H⁺ and K⁺.

As to the Asp-387 mutants, all the mutants prepared (D387E, D387H, and D387N) were inactive, although they were expressed in sufficient quantities. The experimental results on the mutations to asparagine and histidine have shown that the existence of phosphate acceptor moieties at this site is indispensable for the enzyme function. Because the aspartic acid cannot be replaced by glutamic acid, the bulkiness of the side chain in the phosphorylation site appears to be very strict. These results are in agreement with the results obtained with sarcoplasmic Ca²⁺-ATPase and Na⁺,K⁺-ATPase (17, 18). The primary structure around the phosphorylation site is well conserved in the P-type ATPases. The result presented here suggests the common three-dimensional structure around the phosphorylation site among these three P-type ion-transport ATPases, and the requirement of a stringent structure for their functions.

As to the Lys-519 mutants, three in four mutants we prepared (K519E, K519N, and K519R) were inactive. The K519Q mutant retained 30% of the K⁺-ATPase activity of the wild-type enzyme. In sarcoplasmic Ca²⁺-ATPase, mutation of the corresponding lysine residue to arginine, glutamine, and glutamic acid led to activities of 60%, 25%, and 5% of the activity of the wild-type enzyme, respectively (18), indicating that this site is tolerant of amino acid replacement, although it cannot withstand a negative charge. This is also the case with H⁺,K⁺-ATPase. However, it was surprising that the effect of mutation to a basic amino acid, arginine, was quite different between Ca²⁺-ATPase and H⁺,K⁺-ATPase; the replacement did not bring severe damage to Ca²⁺-ATPase, whereas H⁺,K⁺-ATPase could not withstand the replacement. Although the amino acid sequence around the FITC binding site is well conserved between H⁺,K⁺-ATPase and Ca²⁺-ATPase (16), some steric difference must exist between the two FITC binding sites.

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