

Mutational Analysis of Putative SCH 28080 Binding Sites of the Gastric H^+,K^+ -ATPase*

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A compound, SCH 28080 (2-methyl-8-(phenylmethoxy)-imidazo[1,2-*a*]pyridine-3-acetonitrile), reversibly inhibits gastric and renal ouabain-insensitive H^+,K^+ -ATPase, but not colonic ouabain-sensitive H^+,K^+ -ATPase. By using the functional expression system and site-directed mutagenesis, we analyzed the putative binding sites of SCH 28080 in gastric H^+,K^+ -ATPase α -subunit. It was previously reported that the binding site of SCH 28080, which is a K^+ -site inhibitor specific for gastric H^+,K^+ -ATPase, was in the first extracellular loop between the first and second transmembrane segments of the α -subunit; Phe-126 and Asp-138 were putative binding sites. However, we found that all the mutants in the first extracellular loop including Phe-126 and Asp-138 retained H^+,K^+ -ATPase activity and sensitivity to SCH 28080. Therefore, amino acid residues in the first extracellular loop are not directly involved in the SCH 28080 binding nor indispensable for the H^+,K^+ -ATPase activity. Here we propose a candidate residue that is important for the binding with SCH 28080, Glu-822 in the sixth transmembrane segment. Mutations of Glu-822 to Asp and Ala (mutants termed E822D and E822A, respectively) decreased the ATPase activity to about 45% and 35% of the wild-type enzyme, respectively, while the mutations to Gln and Leu abolished the activity. Mutant E822A showed a significantly lower affinity for K^+ than the wild-type enzyme, indicating that Glu-822 is involved in determining the affinity for K^+ . The sensitivity of mutant E822D to SCH 28080 was 8 times lower than that of the wild-type enzyme. The counterpart of Glu-822 in gastric H^+,K^+ -ATPase is Asp in Na^+,K^+ -ATPase and other colonic ouabain-sensitive H^+,K^+ -ATPase, which are insensitive to SCH 28080. These results suggest that Glu-822 is one of important sites that bind with SCH 28080.

H^+,K^+ -ATPase is the proton pump responsible for gastric acid secretion (1). It is the target molecule for irreversible proton pump inhibitors such as omeprazole (2), rabeprazole (E3810) (3), and SCH 28080¹ (4). SCH 28080 is a reversible inhibitor of gastric H^+,K^+ -ATPase, which competitively binds

to the luminal K^+ high affinity site of the enzyme (5). This inhibitor does not inhibit Na^+,K^+ -ATPase, therefore, discriminating the K^+ site of H^+,K^+ -ATPase from that of Na^+,K^+ -ATPase. Furthermore, SCH 28080 inhibits renal ouabain-insensitive H^+,K^+ -ATPase (6), but not colonic ouabain-sensitive H^+,K^+ -ATPase (7, 8). The binding site of SCH 28080 in the gastric H^+,K^+ -ATPase α -subunit was reported to be in the first extracellular loop between the M1 and M2 transmembrane segments from the study in which a photo-affinity derivative of this inhibitor was used (9). Furthermore, Phe-126 and Asp-138 (in the rabbit α -subunit) were proposed to be involved in the interaction with the derivative of SCH 28080 based on a computer-generated model (9). The corresponding loop between M1 and M2 transmembrane segments of Na^+,K^+ -ATPase is known to be important for determining ouabain sensitivity (10). Here we mutated amino acid residues in the first extracellular loop including Phe-126 and Asp-138, studied the sensitivity of the mutant H^+,K^+ -ATPase to the inhibitor, and found that all mutants studied here retained H^+,K^+ -ATPase activity and sensitivity to SCH 28080, suggesting that this loop is not directly involved in SCH 28080 binding.

Several acidic and polar amino acid residues in the M5 and M6 transmembrane segments of Na^+,K^+ -ATPase α -subunit and sarcoplasmic Ca^{2+} -ATPase are involved in the interaction with cations and are important for these ATPase activities (11–18). Here we mutated one of the acidic amino acids in the M6 transmembrane segment of rabbit H^+,K^+ -ATPase α -subunit, Glu-822, and studied the properties of the mutants. When this residue was mutated to aspartic acid and alanine, the mutants E822D and E822A partially retained the ATPase activity. E822A mutant showed significantly lower affinity for K^+ and higher affinity for ATP. E822D mutant showed significantly lower sensitivity to SCH 28080, although the affinities for K^+ and ATP were unchanged. We propose the possibility that Glu-822 is involved in determining the affinity for K^+ and the binding with SCH 28080.

EXPERIMENTAL PROCEDURES

Materials

HEK-293 cells (human embryonic kidney cell line) were a kind gift from Dr. Jonathan Lytton (Brigham & Women's Hospital, Harvard Medical School, Boston, MA). pcDNA3 vector was obtained from Invitrogen Co. (San Diego, CA). Mutant kit was from Takara Shuzo Co. (Ohtsu, Japan). Vent DNA polymerase was obtained from New England Biolabs. Restriction enzymes and other DNA and RNA modifying enzymes were from Toyobo (Osaka, Japan), New England Biolabs, Life Technologies, Inc., or Pharmacia Biotech Inc. (Tokyo, Japan). SCH 28080 was obtained from Schering Co. (Bloomfield, NJ). All other reagents were of molecular biology grade or the highest grade of purity available.

cDNAs of α - and β -Subunits of H^+,K^+ -ATPase

cDNAs of the α - and β -subunits of H^+,K^+ -ATPase were prepared from rabbit gastric mucosae as described elsewhere (19). The α - and

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¹ The abbreviations used are: SCH 28080, 2-methyl-8-(phenylmethoxy)imidazo[1,2-*a*]pyridine-3-acetonitrile; PCR, polymerase chain reaction.

β -subunit cDNAs were digested with *Eco*RI and *Xho*I. The obtained fragments were each ligated into pcDNA3 vector treated with *Eco*RI and *Xho*I.

DNA Sequencing

DNA sequencing was done by the dideoxy chain termination method using an Autoread DNA sequencing kit and an ALF-II DNA sequencer (Pharmacia Biotech Inc.).

Site-directed Mutagenesis

Introduction of site-directed mutations in the M1-M2 domain of the H^+, K^+ -ATPase α -subunit was carried out by sequential polymerase chain reaction (PCR) steps (20), in which appropriately mutated α -subunit cDNAs (segments between *Eco*RI site (nucleotide -28) and *Bst*EII site (nucleotide 456)) were prepared. Two kinds of flanking sequence primers were prepared, one is the 5'-flanking sense primer, 5'-CCGAATTCAAGGAGGGCAGCGCAGCGAG-3' (nucleotide -28 to -9, *Eco*RI site is underlined), and the other is the 3'-flanking antisense primer, 5'-GCCTCGAGCTCGGATCACCGTGGCTTGC-3' (nucleotides 534-553, *Xho*I site is underlined). In addition, sense and antisense synthetic oligonucleotides, each 21 bases long containing one or two mutated bases near the center, were designed (referred as the sense mutating primer and the antisense mutating primer). In the first PCR amplification step, the H^+, K^+ -ATPase α -subunit cDNA subcloned in pBluescript SK(-) (19) was used as a DNA template. Two fragments were prepared in this step: one between the 5'-flanking sense primer and the antisense mutating primer, and the other between the sense mutating primer and the 3'-flanking antisense primer. Each amplified fragment was purified by gel electrophoresis, combined and incubated with the 5'-flanking sense primer and the 3'-flanking antisense primer in the second PCR amplification. The amplified fragment was purified by gel electrophoresis, subcloned in pBluescript SK(-), and sequenced. PCR was routinely carried out in the presence of 300 μ M each dNTP, 6 μ M primers, 10 mM KCl, 20 mM Tris-HCl, pH 8.8, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 100 μ g/ml bovine serum albumin, and 2 units of Vent DNA polymerase for 25 cycles. After sequencing, the amplified fragment in the second PCR was digested with *Eco*RI and *Bst*EII, and ligated back into the relevant position of the wild-type construct of the α -subunit. Site-directed mutagenesis in Glu-822 was carried out as described elsewhere by using the MutanK kit (19, 21).

Cell Culture, Transfection, and Preparation of Membrane Fractions

Cell culture of HEK-293 was carried out as described previously (19). α - and β -subunit cDNA transfection was performed by the calcium phosphate method with 10 μ g of cesium chloride-purified DNA/10-cm dish. Cells were harvested 2 days after the DNA transfection. Membrane fractions of HEK cells were prepared as described previously (19). Briefly, cells in a 10-cm Petri dish were washed with phosphate-buffered saline, and incubated in 2 ml of low ionic salt buffer (0.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4) at 0 °C for 10 min. After the addition of phenylmethylsulfonyl fluoride (1 mM) and aprotinin (0.09 units/ml) the cells were homogenized in a Dounce homogenizer, and the homogenate was diluted with an equal volume of a solution containing 500 mM sucrose and 10 mM Tris-HCl, pH 7.4. The homogenized suspension was centrifuged at $800 \times g$ for 10 min. The supernatant was centrifuged at $100,000 \times g$ for 90 min, and the pellet was suspended in a solution containing 250 mM sucrose and 5 mM Tris-HCl, pH 7.4.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblot

SDS-polyacrylamide gel electrophoresis was carried out as described elsewhere (22). Membrane preparations (30 μ g protein) were incubated in a sample buffer containing 2% SDS, 2% β -mercaptoethanol, 10% glycerol, and 10 mM Tris-HCl, pH 6.8, at room temperature for 2 min and applied to the SDS-polyacrylamide gel. Immunoblot was carried out as described previously (19).

Antibody

Ab1024 was previously raised against the carboxyl-terminal peptide (residues 1024-1034) of the H^+, K^+ -ATPase α -subunit (PGSWWDQE-LYY) (23).

Assay of H^+, K^+ -ATPase Activity

H^+, K^+ -ATPase activity was assayed by following two different methods depending on the purpose and conditions of the experiments.

Measurement of Decrease in the Amount of NADH Coupled with Regeneration of ATP from ADP ("Coupled-enzyme Method")— H^+, K^+ -ATPase activity was measured in 1.2 ml of a reaction mixture contain-

ing 50 μ g of membrane protein, 3 mM $MgCl_2$, 160 μ M NADH, 0.8 mM phosphoenolpyruvate, 3 units/ml pyruvate kinase, 2.75 units/ml lactate dehydrogenase, 5 mM NaN_3 , 1 mM ouabain, 15 mM KCl, 40 mM Tris-HCl, pH 7.4, and various concentrations of ATP. The decrease in the amount of NADH was measured at 37 °C from the absorbance at 340 nm by a Beckman spectrophotometer as described elsewhere (24). The SCH 28080-sensitive K^+ -ATPase was calculated as the difference between the K^+ -ATPase activities in the presence and absence of 50 μ M SCH 28080.

Measurement of Inorganic Phosphate Released from ATP— H^+, K^+ -ATPase activity was measured in 1 ml of a solution containing 50 μ g of membrane protein, 3 mM $MgCl_2$, 3 mM ATP, 5 mM NaN_3 , 2 mM ouabain, and 40 mM Tris-HCl, pH 7.4, in the presence and absence of 15 mM KCl. After incubation at 37 °C for 30 min, the inorganic phosphate released was measured as described elsewhere (25). The K^+ -ATPase activity was calculated as the difference between activities in the presence and absence of KCl.

These two methods did not give qualitative differences in results, but values measured by the former method were about 20% higher than corresponding values measured by the latter method. Routinely we measured the ATPase activity by both methods. We usually showed results measured by the latter method unless indicated. When the ATPase activity was measured as a function of ATP concentrations, the former method was employed to maintain constant ATP concentrations during the incubation period.

Protein was measured using the BCA protein assay kit from Pierce with bovine serum albumin as a standard.

RESULTS

Site-directed Mutations of Phe-126 and Asp-138 in the Putative Binding Sites of SCH 28080 in the First Extracellular Loop Segment—SCH 28080 is a reversible inhibitor specific for the gastric H^+, K^+ -ATPase (4). Previously, it was proposed that Phe-126 and Asp-138 were directly involved in the interaction with SCH 28080 (9). Here we mutated these two residues into several different amino acids (F126A, F126L, F126Y, D138A, D138E, D138N, D138V and double mutants F126A/D138A and F126Y/D138N) and studied the effects on the expression levels and the ATPase activity. The expression levels of the mutant α -subunits were almost identical with that of the wild-type judging from the immunoblot pattern (Fig. 1). Table I shows the K^+ -ATPase activities of the mutants and the wild-type enzyme in the absence of SCH 28080, and the inhibition percentage in the presence of 10 and 50 μ M SCH 28080. Significant K^+ -ATPase activity was detected in all these mutants prepared here, although there were significant differences in the activities between the wild-type enzyme and some mutants such as F126L and F126A, i.e. F126L and F126A mutants showed about 40% and 61% of the activity of the wild-type enzyme, respectively. SCH 28080 at 10 and 50 μ M inhibited the K^+ -ATPase activities of these mutants by 74-98% and by 77-100%, respectively. Fig. 2 shows the effects of various concentrations of SCH 28080 on the K^+ -ATPase activity for several mutants. IC_{50} values are 2.1, 2.0, 3.3, 1.6, and 3.8 μ M for wild-type and mutants F126Y, F126L, D138E, and D138N, respectively. These results (Table I and Fig. 2) show that the affinities of the mutants for SCH 28080 were not significantly different from that of the wild-type enzyme, indicating that Phe-126 and Asp-138 are not directly involved in the binding with SCH 28080.

Fig. 3 shows effects of K^+ concentrations on the SCH 28080-sensitive K^+ -ATPase activity of the mutants (F126A, F126Y, D138A, and D138N). The K^+ sensitivity of the mutants at Phe-126 (F126A and F126Y) was not significantly different from that of the wild-type enzyme. K_m values for K^+ obtained from the least-squares curve fitting in the range of the low K^+ concentrations were 0.23 mM for mutant F126A and 0.14 mM for mutant F126Y, which were comparable with the values obtained for the wild-type H^+, K^+ -ATPase expressed in HEK-293 cells ($K_m = 0.24$ mM) (19) and the H^+, K^+ -ATPase in gastric vesicles ($K_m = 0.2 \sim 0.4$ mM) (26, 27). On the other hand,

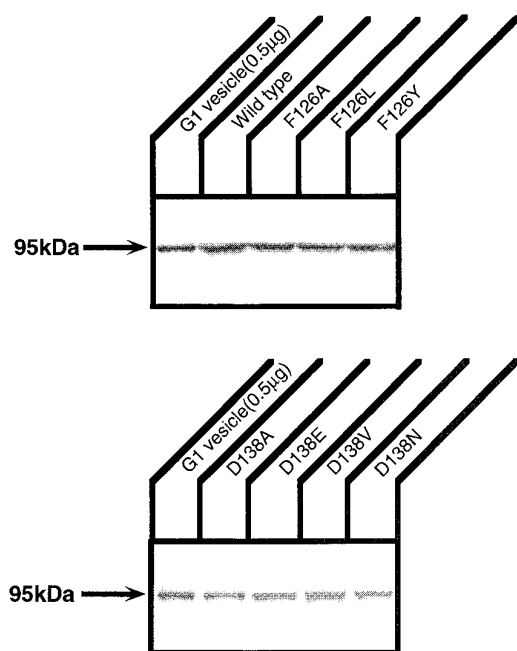


FIG. 1. Immunoblotting with Ab1024 of the membrane fraction of HEK cells transfected with the mutant α -subunit and wild-type β -subunit cDNAs. Upper panel, gastric vesicles (0.5 μ g) (lane 1) and HEK-293 cell membrane fractions (30 μ g) transfected with wild-type α -subunit (lane 2), F126A (lane 3), F126L (lane 4), or F126Y (lane 5) were applied on the gel and blotted with Ab1024. Bottom panel, gastric vesicles (0.5 μ g) (lane 1) and HEK-293 cell membrane fractions (30 μ g) transfected with D138A (lane 2), D138E (lane 3), D138V (lane 4), or D138N mutants (lane 5) were applied on the gel and blotted.

TABLE I

K^+ -ATPase activity of the mutants and the inhibition percentage of the K^+ -ATPase activity in the presence of 10 and 50 μ M SCH 28080

	K^+ -ATPase activity μ mol/mg/h	Inhibition percentage	
		10 μ M SCH	50 μ M SCH
Wild-type	1.19 \pm 0.02 (n = 5)	80	100
F126A	0.72 \pm 0.04 (n = 4)	98	100
F126L	0.48 \pm 0.05 (n = 5)	80	84
F126Y	0.91 \pm 0.07 (n = 3)	74	77
D138A	0.79 \pm 0.04 (n = 6)	75	94
D138E	0.82 \pm 0.05 (n = 5)	82	83
D138N	0.94 \pm 0.07 (n = 6)	78	79
D138V	0.89 \pm 0.05 (n = 8)	85	90
F126A/D138A	0.76 \pm 0.07 (n = 3)	76	85
F126Y/D138N	0.83 \pm 0.05 (n = 4)	81	95
E132A	0.96 \pm 0.08 (n = 5)	70	93
G133A	0.80 \pm 0.03 (n = 6)	62	100
G133E	0.81 \pm 0.08 (n = 5)	77	88
D134A	0.61 \pm 0.06 (n = 4)	56	83
L135A	0.65 \pm 0.06 (n = 3)	85	95
T136A	1.27 \pm 0.03 (n = 3)	74	94
T137A	1.18 \pm 0.06 (n = 4)	78	97

mutations at Asp-138 (D138A and D138N) slightly decreased the affinity for K^+ . K_m values were 0.94 mM for mutant D138A and 0.48 mM for mutant D138N, which were 2–4-fold higher than that of the wild-type enzyme.

Site-directed Mutations in Other Amino Acid Residues in the First Extracellular Loop Segment—Amino acid sequences in the M1 and M2 transmembrane segments are considerably well conserved between members of H^+,K^+ -ATPase family (gastric, colonic, and urinary bladder) and Na^+,K^+ -ATPase, whereas amino acids in the loop segment between M1 and M2 are not conserved (28–31) (Fig. 4). Here we prepared mutants E132A, G133A, G133E, D134A, L135A, T136A, and T137A. K^+ -ATPase activity was detected in all these mutants pre-

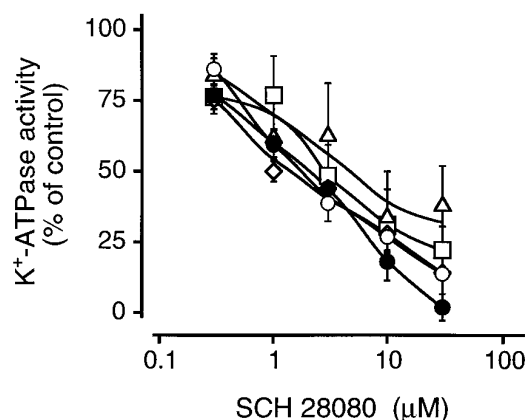


FIG. 2. Effects of SCH 28080 concentrations on the expressed K^+ -ATPase activities of the Phe-126 and Asp-138 mutants. The K^+ -ATPase activities of the wild-type (\bullet), F126Y (\circ), F126L (Δ), D138E (\diamond), and D138N mutants (\square) were measured as a function of the SCH 28080 concentrations. The K^+ -ATPase activities are expressed as the percentage of the control values measured in the absence of SCH 28080. The values are mean \pm S.E. for three observations in three transfections. The K^+ -ATPase activities in the absence of SCH 28080 were 1.05 ± 0.09 μ mol/mg/h for the wild-type, 0.91 ± 0.07 μ mol/mg/h for F126Y, 0.46 ± 0.08 μ mol/mg/h for F126L, 0.87 ± 0.12 μ mol/mg/h for D138E, and 0.98 ± 0.13 μ mol/mg/h for D138N.

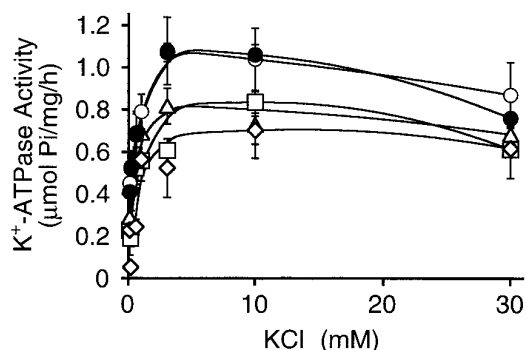
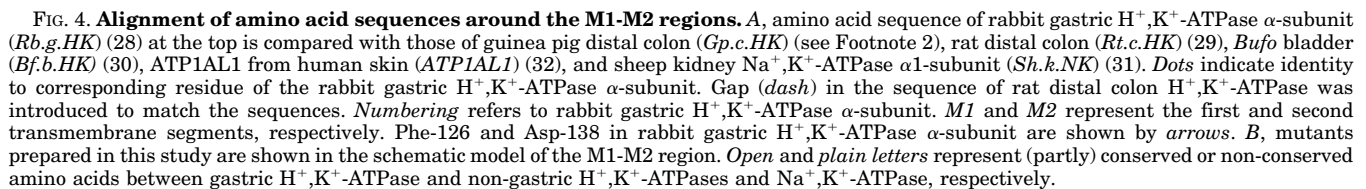


FIG. 3. Effects of K^+ concentrations on the expressed H^+,K^+ -ATPase activities of the Phe-126 and Asp-138 mutants. The K^+ -ATPase activities of the wild-type (\bullet), F126Y (\circ), F126L (Δ), D138N (\diamond), and D138A mutants (\square) were measured as a function of the K^+ concentrations. The activity is the difference between the activities measured in the presence and absence of 50 μ M SCH 28080 at the indicated K^+ concentration. The values are mean \pm S.E. for three observations in three transfections.

pared, and the activities of mutants D134A and L135A were significantly lower than that of the wild-type as shown in Table I. SCH 28080 at 10 and 50 μ M inhibited the K^+ -ATPase activity of these mutants by 56–85% and 83–100%, respectively. The sensitivity of mutant D134A to SCH 28080 was slightly lower than that of the wild-type enzyme; however, the sensitivity of this mutant was still much higher than those of Na^+,K^+ -ATPase and ouabain-sensitive colonic H^+,K^+ -ATPases. Therefore, we conclude that the first extracellular loop between the M1 and M2 segments is not an exclusive (or major) determinant of SCH 28080 sensitivity nor directly involved in the binding with SCH 28080.

In the following, we propose that Glu-822 in the M6 transmembrane segment is one of the candidates involved in the binding with SCH 28080.

Site-directed Mutations in the Putative Cation Binding Site in the M6 Transmembrane Segment—Several amino acid residues responsible for the cation binding or indispensable for the enzyme activity are present in the M5 and M6 transmembrane segments of the Ca^{2+} -ATPase and the Na^+,K^+ -ATPase α -subunit (11–18). We mutated Glu-822 in the M6 transmembrane



Western blot analysis showing protein levels for E822A, E822D, E822L, E822Q, and wild-type. The blot indicates a band at 95kDa for all variants.

Sensitivity of Glu-822 Mutants to SCH 28080—Fig. 8 shows effects of SCH 28080 concentrations on the K^+ -ATPase activity of mutants E822A and E822D. Mutant E822D showed a significantly lower sensitivity to SCH 28080 than the wild-type.

TABLE II
 H^+,K^+ -ATPase activity of the Glu-822 mutants

	H^+,K^+ -ATPase activity $\mu\text{mol}/\text{mg}/\text{h}$
Wild-type	0.76 ± 0.05 ($n = 10$)
E822A	0.23 ± 0.05 ($n = 6$)
E822D	0.31 ± 0.05 ($n = 4$)
E822L	-0.13 ± 0.04 ($n = 5$)
E822O	0.02 ± 0.01 ($n = 4$)

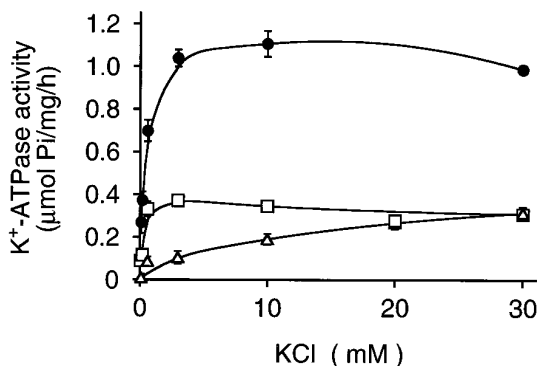


FIG. 6. Effects of K^+ concentrations on the expressed H^+,K^+ -ATPase activity of the Glu-822 mutants. H^+,K^+ -ATPase activities of the wild-type (●) and mutants E822D (□) and E822A (△) were measured as a function of the K^+ concentration. H^+,K^+ -ATPase activity was calculated as the difference between the ATPase activities in the presence and absence of KCl. The values are mean \pm S.E. for three transfections.

whereas E822A showed a significantly higher affinity. IC_{50} values are 2.1, 15, and $0.58 \mu\text{M}$ for the wild-type, mutants E822D and E822A, respectively (*i.e.* these mutations did not induce parallel changes in affinities for K^+ and SCH 28080). Taking the molecular shape and size difference between K^+ and SCH 28080 into consideration, it is considered that these mutations induced some conformational changes, resulting in complicated changes in the affinities. Therefore, Glu-822 is suggested to be involved in bindings with K^+ and SCH 28080.

DISCUSSION

H^+,K^+ -ATPase was originally found in gastric mucosae. Recently new members of the H^+,K^+ -ATPase α -subunit were found and cloned from distal colons of rat (29) and guinea pig,² toad urinary bladder (30), and human skin (32). The ouabain-sensitive K^+ -ATPase activity in the membrane preparation from the guinea pig colon was insensitive to $100 \mu\text{M}$ SCH 28080 (7). The cDNAs of rat colonic H^+,K^+ -ATPase (8), urinary bladder H^+,K^+ -ATPase (30), and ATP1A1 from human skin library (33) were expressed in *Xenopus levis* oocytes. When the α - and β -subunit cRNAs were co-injected (for colonic H^+,K^+ -ATPase and ATP1A1, these α -subunit cRNAs were co-injected with the β -subunit cRNA of rabbit gastric H^+,K^+ -ATPase), the expressed ATPases transported rubidium inward, and proton outward. The K^+ -ATPase activities of urinary bladder ATPase and ATP1A1 were sensitive to both SCH 28080 and ouabain (30, 33). However, they were much less sensitive to SCH 28080 than the gastric H^+,K^+ -ATPase, the K_i value of the urinary bladder H^+,K^+ -ATPase being $230 \mu\text{M}$ (30). The Rb^+ transport by ATP1A1 was only partly inhibited by $500 \mu\text{M}$ SCH 28080 (33). The rat colonic H^+,K^+ -ATPase expressed in *Xenopus* oocytes was sensitive to ouabain, but insensitive to SCH 28080 (8), while the same ATPase expressed in Sf9 cells was slightly inhibited by SCH 28080 (18% inhibition with $100 \mu\text{M}$ SCH

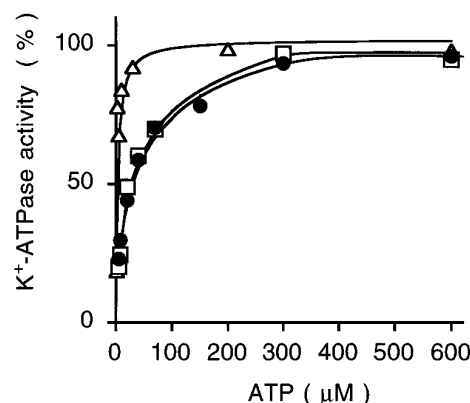


FIG. 7. Effects of ATP concentrations on the expressed H^+,K^+ -ATPase activity of the Glu-822 mutants. H^+,K^+ -ATPase activities of the wild-type (●) and mutants E822D (□) and E822A (△) were measured as a function of the ATP concentrations. H^+,K^+ -ATPase activity was calculated as the difference between the ATPase activities in the presence and absence of KCl. The values are mean \pm S.E. for three transfections.

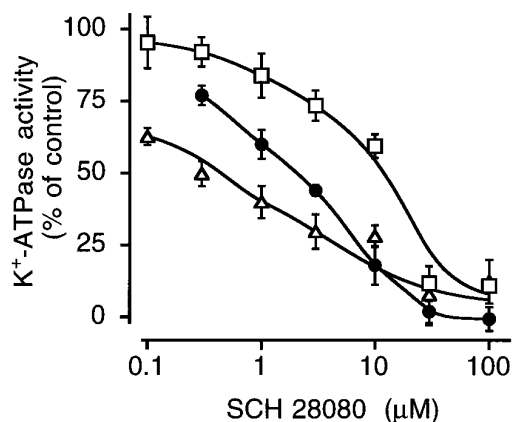


FIG. 8. Effects of SCH 28080 concentrations on the expressed K^+ -ATPase activities of the Glu-822 mutants. The K^+ -ATPase activities of the wild-type (●) and mutants E822D (□) and E822A (△) were measured as a function of the SCH 28080 concentrations. The K^+ -ATPase activities are expressed as the percentage of the control values measured in the absence of SCH 28080. The values are mean \pm S.E. for three observations in three transfections. The K^+ -ATPase activities in the absence of SCH 28080 were $1.05 \pm 0.09 \mu\text{mol}/\text{mg}/\text{h}$ for the wild-type, $0.43 \pm 0.05 \mu\text{mol}/\text{mg}/\text{h}$ for E822D, and $0.36 \pm 0.02 \mu\text{mol}/\text{mg}/\text{h}$ for E822A.

28080) and was insensitive to ouabain (34). The amino acid sequences around the first extracellular loop are well conserved in gastric H^+,K^+ -ATPases obtained from various species, *i.e.* rat (35), pig (36), human (37), rabbit (28), dog (38), *Xenopus*, and mouse (39). In Fig. 4, amino acid sequences around the first extracellular loop segment of the α -subunit are compared in the members of H^+,K^+ -ATPase and Na^+,K^+ -ATPase. The amino acid at the edge of the M1 transmembrane segment is phenylalanine (Phe-126 in rabbit gastric H^+,K^+ -ATPase) and conserved in these gastric H^+,K^+ -ATPases and rat colonic H^+,K^+ -ATPase (29), whereas the corresponding residue is tyrosine in the guinea pig colonic H^+,K^+ -ATPase, urinary bladder H^+,K^+ -ATPase (30), and sheep kidney Na^+,K^+ -ATPase (31), which are all insensitive to SCH 28080. Therefore, we initially considered that mutation at Phe-126 in the gastric H^+,K^+ -ATPases would cause some change in the sensitivity to SCH 28080, if SCH 28080 binds with this loop as suggested previously (9). Surprisingly, when the residue was replaced by tyrosine (F126Y) or alanine (F126A), the K^+ -ATPase activity of the mutants still remained sensitive to SCH 28080, and the

² T. Watanabe, M. Satoh, and Y. Suzuki, personal communication (GSDB/DBJ/EMBL/NCBI accession no. D21854).

sensitivity of these mutants to SCH 28080 was not significantly different from that of the wild-type enzyme. Because mutants F126A and F126Y are able to interact with SCH 28080, the phenyl group in the side chain of Phe-126 is not supposed to be directly involved in the interaction with the inhibitor, and the side chain of the tyrosine residue does not interrupt the binding of SCH 28080. Therefore, the low sensitivity to SCH 28080 in the guinea pig colonic H^+,K^+ -ATPase and the urinary bladder H^+,K^+ -ATPase is not due to the amino acid substitution to Tyr on this site (7, 30). The aspartic acid residue (Asp-138 in rabbit gastric H^+,K^+ -ATPase) at the edge of the M2 transmembrane segment is also well conserved in gastric H^+,K^+ -ATPases from different species, but divergent among Na^+,K^+ -ATPase and colonic and urinary H^+,K^+ -ATPases. In the Na^+,K^+ -ATPase, the corresponding amino acid residue is asparagine. In the colonic and urinary bladder H^+,K^+ -ATPases this residue is leucine and arginine, respectively. When this residue of gastric H^+,K^+ -ATPase was replaced by asparagine (D138N), alanine (D138A), glutamic acid (D138E), and valine (D138V), the K^+ -ATPase activity remained sensitive to SCH 28080, and the sensitivity of the mutants to SCH 28080 was not significantly different from that of the wild-type enzyme. Therefore, Asp-138 is not directly involved in the interaction with SCH 28080. To exclude the possibility that the interaction between H^+,K^+ -ATPase and SCH 28080 remains unaffected when only one of the two binding determinants (Phe-126 and Asp-138) is mutated, we prepared double mutants in Phe-126 and Asp-138 (F126Y/D138N and F126A/D138A). These double mutants are also active and have similar sensitivity to SCH 28080 compared with the wild-type enzyme. Our results altogether suggest that the putative SCH 28080 binding sites, Phe-126 and Asp-138, are, at least, not major determinants of SCH 28080 sensitivity nor directly involved in binding with SCH 28080.

Next we introduced mutations in other residues in the first extracellular loop segment. Amino acid residues between Glu-132 and Thr-137 were replaced with alanine to study the role of the side chain in binding with SCH 28080 (mutants termed E132A, G133A, D134A, L135A, T136A, and T137A). In addition, Gly-133 was replaced with glutamic acid, a counterpart residue in sheep kidney Na^+,K^+ -ATPase (mutant termed G133E). All these mutants prepared were active as H^+,K^+ -ATPase, suggesting that the side chains of amino acids in this segment are not indispensable for the ATPase function. Sensitivity of the mutants to SCH 28080 was not significantly different from that of the wild-type enzyme. Therefore, it is concluded that every single amino acid residue in the first extracellular loop is not a major binding site of SCH 28080.

We found that Glu-822 is one of the amino acids that determine the affinity for SCH 28080. When Glu-822 was mutated with Gln and Leu, H^+,K^+ -ATPase activity of the mutants was completely abolished. For Ala and Asp mutants, H^+,K^+ -ATPase activity was partially preserved. The affinities of mutant E822D for K^+ and ATP were not changed; however, those of mutant E822A were significantly lower for K^+ and higher for ATP than the corresponding affinities of the wild-type enzyme. These results suggest that this site is important for the function of H^+,K^+ -ATPase, especially for determining the affinity for K^+ , but that the carboxyl moiety of Glu-822 is not indispensable for the ATPase function. Very recently Swarts *et al.* (40) reported that Glu-820 of rat gastric H^+,K^+ -ATPase α -subunit (counterpart of Glu-822 of rabbit gastric H^+,K^+ -ATPase) is involved in K^+ binding, and that E820Q mutant expressed in Sf9 cells showed no K^+ -ATPase activity, which is in good agreement with our findings. The corresponding residues in rabbit sarcoplasmic Ca^{2+} -ATPase and rat Na^+,K^+ -ATPase $\alpha 2$ subunit are Asn-796 and Asp-803, respectively. Asn-796 in the

Ca^{2+} -ATPase is involved in the high-affinity binding with Ca^{2+} (17). Asp-803 in Na^+,K^+ -ATPase is indispensable for the ATPase function and unchangeable (12). Therefore, the roles of Glu-822 and the corresponding amino acid residues in the ATPase function are different among H^+,K^+ -ATPase, Ca^{2+} -ATPase, and Na^+,K^+ -ATPase. Mutant E822D showed 8 times lower sensitivity to SCH 28080, whereas the E822A showed a higher sensitivity than the wild-type enzyme. These results suggest that Glu-822 is the site involved (directly or indirectly) in binding with SCH 28080. It is noteworthy that the corresponding amino acid residue of Glu-822 is an aspartic acid in Na^+,K^+ -ATPases and non-gastric type H^+,K^+ -ATPases that are insensitive to SCH 28080 (28–30). Furthermore, the sensitivity of mutant E822D to SCH 28080 is lower than that of any mutant in the first extracellular loop segment, but still higher than those of Na^+,K^+ -ATPase and non-gastric H^+,K^+ -ATPases.

Our present results do not exclude the possibility that the binding sites of SCH 28080 are on the multiple domains in the H^+,K^+ -ATPase. In fact, amino acid residues that are involved in determining the affinity for ouabain (or cardiac glycosides) in Na^+,K^+ -ATPase are on the segments between M1 and M2 (10), M3 and M4 (41), M5 and M6 (42, 43), and M7 and M8 transmembrane segments (44). Blostein *et al.* (45) reported that the SCH 28080 binding site is in the N-terminal half of the H^+,K^+ -ATPase from the study using chimeric ATPases between H^+,K^+ -ATPase and Na^+,K^+ -ATPase.

In conclusion, we have reported here that the amino acid residues in the first extracellular loop of the H^+,K^+ -ATPase α -subunit including the putative SCH 28080 binding sites, Phe-126 and Asp-138, are in fact not exclusively involved in the interaction with this inhibitor, and that Glu-822 is involved in determining the affinity for SCH 28080.

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