Functional Association between $\text{K}^+-$Cl$^-$ Cotransporter-4 and $\text{H}^+,$$\text{K}^+-$ATPase in the Apical Canaliculular Membrane of Gastric Parietal Cells

We studied whether $\text{K}^+-$Cl$^-$ cotransporters (KCCs) are involved in gastric HCl secretion. We found that KCC4 is expressed in the gastric parietal cells more abundantly at the luminal region of the gland than at the basal region. KCC4 was found in the stimulation-associated vesicles (SAV) derived from the apical canaliculular membrane but not in the intracellular tubulovesicles, whereas $\text{H}^+,$$\text{K}^+-$ATPase was expressed in both of them. In contrast, KCC1, KCC2, and KCC3 were not found in either SAV or tubulovesicles. KCC4 coimmunoprecipitated with $\text{H}^+,$$\text{K}^+-$ATPase in the lysates of SAV. Interestingly the MgATP-dependent uptake of $^{36}\text{Cl}^-$ into the SAV was suppressed by either the $\text{H}^+,$$\text{K}^+-$ATPase inhibitor (SCH28080) or the KCC inhibitor ((R)-(+-)[(2-$\text{n}$-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy]acetic acid). The KCC inhibitor suppressed the $\text{H}^+$ uptake into SAV and the $\text{H}^+,$$\text{K}^+-$ATPase activity of SAV, but the inhibitor had no effects on these activities in the freeze-dried leaky SAV. These results indicate that the $\text{K}^+-$Cl$^-$ cotransport by KCC4 is tightly coupled with $\text{H}^+/$$\text{K}^+$ antiport by $\text{H}^+,$$\text{K}^+-$ATPase, resulting in HCl accumulation in SAV. In the tetracycline-regulated expression system of KCC4 in the HEK293 cells stably expressing gastric $\text{H}^+,$$\text{K}^+-$ATPase, KCC4 was coimmunoprecipitated with $\text{H}^+,$$\text{K}^+-$ATPase. The rate of recovery of intracellular pH in the KCC4-expressing cells after acid loading through an ammonium pulse was significantly faster than that in the KCC4-non-expressing cells. Our results suggest that KCC4 and $\text{H}^+,$$\text{K}^+-$ATPase are the main machineries for basal HCl secretion in the apical canaliculular membrane of the resting parietal cell. They also may contribute to part to massive acid secretion in the stimulated state.

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–5.

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H$^+$ secretion, coupled with $\text{K}^+$ uptake, is mediated by the gastric proton pump ($\text{H}^+,$$\text{K}^+-$ATPase). $\text{K}^+$ recycling across the luminal membrane is necessary to sustain $\text{H}^+,$$\text{K}^+-$ATPase activity in gastric parietal cells. Heteromeric KCNQ1/KCN2 channels (1, 2) and the Kir4.1 channel (3, 4) have been postulated as candidates for this $\text{K}^+$ transport. $\text{Cl}^-$ efflux across the luminal membrane is also necessary for gastric acid (HCl) secretion. CFTR$^*$ (5), CLIC-6 (6, 7), and SLC26A9 (8) have been postulated as candidates for this $\text{Cl}^-$ transport.

It has been assumed that $\text{Cl}^-$ moves passively down its electrochemical gradient through apical channels. Although the intracellular $\text{Cl}^-$ concentration of the parietal cell has not been reported, it is speculated to be much lower than that of the luminal secreted HCl (160 mM). Thus, $\text{Cl}^-$ transport through $\text{Cl}^-$ channels would require a large electrical potential difference across the apical membrane (e.g. 60 mV, inside negative against the luminal side). But to date the reports of this electrical potential differences have been low, such as 20–25 mV (9), hinting that the $\text{Cl}^-$ secretory mechanism may be more complex than previously assumed.

Electroneutral $\text{K}^+-$Cl$^-$ cotransporters (KCCs) belong to a cation-chloride cotransporter gene family (SLC12). KCCs contribute to transepithelial transport and to the regulation of cell volume (10–12). At least four KCC isoforms (KCC1–KCC4) have been identified to date. KCC3 has three splicing variants: KCC3a–KCC3c. KCC1 is widely expressed, whereas KCC2 is restricted to neurons. KCC3a and KCC4 are mainly expressed in epithelial-type cells (13, 14). Recently we found that KCC3a is expressed in the basolateral membrane of gastric parietal cells located at the luminal region of gastric glands and coimmunoprecipitated with Na$^+,$K$^+-$ATPase (15). Exogenous expression of KCC3a in LLC-PK1 cells up-regulates Na$^+,$K$^+-$ATPase activity in lipid rafts (15).

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$^2$ The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; KCC, $\text{K}^+-$Cl$^-$ cotransporter; AQP, aquaporin; SCH28080, 2-methyl-8-(phenylmethoxy)imidazo[1,2-alpyridine-3-ace-tonitrile; DIOA, (R)-(+-)[(2-$\text{n}$-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy]acetic acid; BICEF, 2,7-bis(carboxyethyl)-5(6)-carboxyfluorescein; BICEF-AM, acetoxyethyl ester of BICEF; SAV, stimulation-associated vesicles; TV, tubulovesicles; Tet, tetracycline; PIPES, 1,4-piperazinediethanesulfonic acid; pH; $\text{Na}^+$, intracellular pH; $\alpha 1$ NaK, Na$^+,$K$^+-$ATPase $\alpha 1$ subunit.
KCC4 Association with H\textsuperscript{+}, K\textsuperscript{+}-ATPase in Gastric Parietal Cells

If KCCs are present in the luminal membrane of gastric parietal cells, they may be involved in the electroneutral cotransport of K\textsuperscript{+}-Cl\textsuperscript{−}, which would be driven by the electrochemical gradient for K\textsuperscript{+} across the luminal membrane established by the H\textsuperscript{+}, K\textsuperscript{+}-ATPase (a very low luminal K\textsuperscript{+} concentration) and Na\textsuperscript{+}, K\textsuperscript{+}-ATPase (a very high intracellular K\textsuperscript{+} concentration). So far, there have been no reports describing the presence of any KCCs in the luminal membrane. In the present study, we found that KCC4 is predominantly expressed and associated with H\textsuperscript{+}, K\textsuperscript{+}-ATPase in the apical canalicul membrane of gastric parietal cells and that KCC4 is an important molecule for maintaining H\textsuperscript{+}, K\textsuperscript{+}-ATPase activity in the canalicul membrane.

EXPERIMENTAL PROCEDURES

Materials—Anti-human KCC4 rabbit polyclonal antibody was generated with keyhole limpet hemocyanin-coupled peptides against N-terminal amino acids corresponding to the N-terminal sequence of KCC4 (DEESRRREAKPMGCC). This antigen peptide has no homology with the sequences of KCC1, -2, and -3. Anti-mouse KCC4 rabbit polyclonal antibody was obtained from Alpha Diagnostic (San Antonio, TX). To check the expression of KCCs other than KCC4, anti-human KCC1 goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-rat KCC2 rabbit polyclonal antibody (Upstate Biotechnology, Lake Placid, NY), and anti-rat KCC3 rabbit polyclonal antibody (15) were used. Expression of gastric H\textsuperscript{+}, K\textsuperscript{+}-ATPase was checked by using anti-mouse H\textsuperscript{+}, K\textsuperscript{+}-ATPase α-subunit mouse monoclonal antibody (1H9) (Medical & Biological Laboratories Co., Nagoya, Japan), anti-mouse H\textsuperscript{+}, K\textsuperscript{+}-ATPase β-subunit mouse monoclonal antibody (2B6) (Medical & Biological Laboratories Co.), and anti-hog H\textsuperscript{+}, K\textsuperscript{+}-ATPase α-subunit rabbit polyclonal antibody (Ab1024) (16). Anti-rabbit Na\textsuperscript{+}, K\textsuperscript{+}-ATPase α1 subunit mouse monoclonal antibody was obtained from Upstate Biotechnology. Anti-human aquaporin-4 (AQP4) goat polyclonal antibody, anti-rabbit ezrin mouse monoclonal antibody, anti-human Rab11 goat polyclonal antibody, and anti-rat syntaxin-1 mouse monoclonal antibody were obtained from Santa Cruz Biotechnology. Anti-His tag mouse monoclonal antibody (2D8) was from Medical & Biological Laboratories Co. Anti-β-actin mouse monoclonal antibody, 2-methyl-8-(phenylmethoxy)imidazo[1,2-a]pyridine-3-acetonitrile (SCH28080), and (R)-(+)-(2-n-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxyacetic acid (DIOA) were from Sigma-Aldrich. Lipofectamine 2000, anti-Xpress mouse monoclonal antibody, Alexa Fluor 546-conjugated anti-mouse and anti-goat IgG antibodies, and Alexa Fluor 488-conjugated anti-rabbit IgG antibody were from Invitrogen. Protein A-agarose beads were from Pierce. Na\textsuperscript{22}Cl and ACS II scintillant were obtained from GE Healthcare. 86RbCl was from PerkinElmer Life Sciences. Hygromycin B and acridine orange were from Wako Pure Chemical Industries (Osaka, Japan). Blasticidin S was from Kaken Pharmaceutical Co. (Tokyo, Japan). 2',7'-Bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) and acetoxymethyl ester of BCECF (BCECF-AM) were from Dojindo Laboratories (Kumamoto, Japan).

Isolation of Gastric Tissues and Cells—Mice, rats, and rabbits were humanely killed in accordance with the guidelines presented by the Animal Care and Use Committee of the University of Toyama, and their gastric mucosae were isolated from the stomachs. The cell suspension rich in parietal cells was prepared from isolated rabbit gastric mucosa as described previously (17). Hog gastric mucosa was prepared from the stomach obtained from Toyama meat center (Toyama, Japan). Human gastric mucosa was obtained from surgical resection of Japanese patients at Toyama University Hospital in accordance with the recommendations of the Declaration of Helsinki and with ethics committee approval. All of the patients gave informed consent.

Preparation of Hog Gastric Vesicles (Stimulation-associated Vesicles (SAV) and Tubulovesicles (TV))—Two kinds of gastric vesicles (heavy and light vesicles) were prepared simultaneously from hog gastric mucosa as described previously (18). Briefly, the fundic region of the mucosa was scraped and homogenized in 250 mM sucrose, 1 mM EGTA, and 5 mM Tris-HCl (pH 7.4). The suspension was centrifuged at 1,000 × g for 10 min, and the supernatant was further centrifuged at 13,500 × g for 30 min. The pellet, resuspended in the buffer solution, was applied to the top of a 7% Ficoll shelf on a 12% Ficoll step gradient and centrifuged in an RPV-50T rotor (Hitachi Koki Co., Tokyo, Japan) at 132,000 × g for 1 h. Heavy vesicles were collected from the interface between the 7 and 12% Ficoll layers, and they were washed with 250 mM sucrose to remove the Ficoll. Then the sample was centrifuged at 120,000 × g in an SW41 Ti rotor (Beckman) for 20 h laying on a discontinuous sucrose gradient (10, 20, 30, and 50% sucrose), and 10 fractions of 1 ml each were collected from the top of the gradient. Fraction 5 was used as the SAV that contained the apical canalicul membranes of parietal cells (rich in H\textsuperscript{+}, K\textsuperscript{+}-ATPase and least contaminated with Na\textsuperscript{+}, K\textsuperscript{+}-ATPase) (supplemental Fig. 1). On the other hand, the supernatant after 13,500 × g centrifugation was centrifuged at 100,000 × g for 30 min. The samples were then applied to a 250 mM sucrose and 7% Ficoll step gradient and centrifuged at 132,000 × g for 1 h. Light (microsomal) vesicles were collected from the interface between the 250 mM sucrose and 7% Ficoll layers, and the sample was used as the TV that contained the intracellular microsomal membranes of parietal cells. All procedures were carried out at 4 °C. When indicated, freeze-dried SAV and TV were prepared by lyophilization, which increased leakiness of the vesicular membranes.

Northern Blotting—Poly(A)	extsuperscript{+} RNA of the cells was prepared by using PolyATtract mRNA isolation system II (Promega, Madison, WI), and 2.5 µg of it was separated on a 1% agarose, formaldehyde gel and transferred onto a nylon membrane (Zeta-probe GT, Bio-Rad). Northern blotting was performed as described previously (15) using the 32P-labeled rabbit KCC4 probe that is 713 bp long and corresponds to nucleotides 682–1394 of the KCC4 cDNA.

Western Blotting—Preparation of membrane fractions and Western blotting were carried out as described previously (19). The signals were visualized with the ECL Plus system (GE Healthcare). To quantify the chemiluminescence signals on the membranes, a FujiFilm LAS-1000 system and MultiGauge software were used. Anti-H\textsuperscript{+}, K\textsuperscript{+}-ATPase antibodies were used at 620 JOURNAL OF BIOLOGICAL CHEMISTRY

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1:5,000 (1H9 and 2B6) or 1:4,000 dilution (Ab1024). Anti-KCC1, anti-KCC2, anti-KCC3, anti-KCC4, and anti-\(\beta\)-actin antibodies were used at 1:1,000 dilution. Anti-Na\(^+\),K\(^+\)-ATPase \(\alpha\)1 subunit antibody was used at 1:10,000 dilution. Anti-Xpress antibody was used at 1:5,000 dilution. Anti-erzin and anti-synaptin-1 antibodies were used at 1:500 dilution. Anti-Rab11 antibody was used at 1:200 dilution. For the negative control, 1 volume of each primary antibody was preincubated with 5 volumes of the corresponding blocking peptide. Horseradish peroxidase-conjugated anti-mouse, anti-rabbit, or anti-goat IgG was used as a secondary antibody (1:2,500 dilution).

Immunohistochemistry—The gastric mucosa isolated from rat stomach was embedded in the optimum cutting temperature compound (Sakura Finetechical Co., Tokyo, Japan) and was cut at 8 \(\mu\)m. The sections were fixed in ice-cold methanol for 7 min at room temperature and were pretreated with 1.5% bovine serum albumin for 1 h at room temperature to block nonspecific binding of antibody. Then the sections were incubated with anti-mouse KCC4, anti-H\(^+\),K\(^+\)-ATPase (1H9), anti-Na\(^+\),K\(^+\)-ATPase \(\alpha\)1 subunit, or anti-AQP4 antibody (1:100 dilution) overnight at 4 \(^\circ\)C. Alexa Fluor 488-conjugated and Alexa Fluor 546-conjugated anti-IgG antibodies (1:100 dilution) were used as secondary antibodies. Immunofluorescence images were visualized using a Zeiss LSM 510 laser scanning confocal microscope.

Immunocytochemistry—HEK293 cells were fixed with ice-cold methanol for 7 min at room temperature and permeabilized with phosphate-buffered saline containing 0.3% Triton X-100 and 0.1% bovine serum albumin for 15 min at room temperature. Nonspecific binding was blocked by 3% bovine serum albumin. The permeabilized cells were incubated with the anti-mouse KCC4 or anti-H\(^+\),K\(^+\)-ATPase (1H9) antibody (1:100 dilution) overnight at 4 \(^\circ\)C and then with the Alexa Fluor 488-conjugated and Alexa Fluor 546-conjugated anti-IgG antibodies (1:100 dilution) for 1 h at room temperature. Immunofluorescence images were visualized using a Zeiss LSM 510 laser scanning confocal microscope.

Immunoprecipitation—Membrane fractions of SAV (100 \(\mu\)g of protein) and the HEK293 cells stably expressing both KCC4 and H\(^+\),K\(^+\)-ATPase (2 \(\mu\)g of protein) were solubilized in lysis buffer (phosphate-buffered saline containing 0.5% Triton X-100, 0.1% bovine serum albumin, and 1 mM EDTA) for 30 min on ice and centrifuged at 90,000 \(\times\) g for 30 min at 4 \(^\circ\)C. The lysate was precleared with protein A-agarose beads, and the supernatant was incubated in the presence and absence of anti-KCC4 antibody (1:50 dilution) or anti-His tag antibody (1:100 dilution) for 12 h at 4 \(^\circ\)C with end-over-end rotation. Antibody-antigen complexes were incubated with protein A-agarose beads and incubated for 4 h at 4 \(^\circ\)C with end-over-end rotation. Then the beads were washed three times with the lysis buffer and suspended in SDS sample buffer. The samples were used for Western blotting to check the expression of KCC4, H\(^+\),K\(^+\)-ATPase \(\alpha\)-subunit, or \(\beta\)-actin.

Measurement of \(^{36}\)Cl\(^-\) Uptake into Hog Gastric Vesicles (SAV and TV)—SAV or TV (100 \(\mu\)g of protein) were preincubated with a solution of 150 mM KCl, 250 mM sucrose, and 40 mM Pipes-Tris (pH 6.8) for 5 min or 20 h at 4 \(^\circ\)C. Then the vesicle (SAV or TV) was incubated with a solution containing 150 mM KCl, 4 mM MgSO\(_4\), 250 mM sucrose, 2 mM ATP, 5 \(\mu\)Ci/ml Na\(^{36}\)Cl, and 40 mM Pipes-Tris (pH 6.8) for 5 min at 25 \(^\circ\)C. The reaction mixtures were rapidly filtered through a 0.45-\(\mu\)m HAWP filter. The filter was washed with a solution containing 150 mM KCl, 250 mM sucrose, and 40 mM Pipes-Tris (pH 6.8) for 5 min at 25 \(^\circ\)C. Then the radioactivity of Na\(^{36}\)Cl was counted.

Measurement of H\(^+\) Uptake into SAV and TV—H\(^+\) uptake into hog gastric vesicles was assessed by measuring the quenching of acridine orange fluorescence (20). The reaction was started by addition of ATP (350 \(\mu\)M) to the mixture containing SAV (20 \(\mu\)g/ml) or TV (5 \(\mu\)g/ml), 150 mM KCl, 2 mM MgCl\(_2\), 5 \(\mu\)M acridine orange, 10 \(\mu\)g/ml valinomycin, and 20 mM Pipes-NaOH (pH 7.4). In the experiments to test anion selectivity, 150 mM KCl was replaced with 150 mM KX (X = Br, I, H\(_2\)PO\(_4\), or glucuronate). When indicated, SCH28080 (20 \(\mu\)M), a specific inhibitor of H\(^+\),K\(^+\)-ATPase, or DIOA (10 \(\mu\)M), an inhibitor of KCCs, was added. Fluorescence of acridine orange was measured in a Shimadzu RF-5000 spectrofluorometer at 25 \(^\circ\)C (excitation, 495 nm; emission, 530 nm). The H\(^+\) uptake was expressed as change of fluorescence intensity from 0 to 3 min after addition of ATP.

Measurement of H\(^+\),K\(^+\)-ATPase Activity—H\(^+\),K\(^+\)-ATPase activities of SAV and TV were measured in a pyruvate kinase-lactate dehydrogenase-linked system where hydrolysis of ATP is coupled with oxidation of NADH (21). The reaction mixture contained TV (10 \(\mu\)g/ml) or SAV (40 \(\mu\)g/ml), 150 mM KCl, 3 mM MgSO\(_4\), 200 \(\mu\)M NADH, 1 mM ATP, 0.8 mM phosphoenolpyruvate, 11 IU/ml lactate dehydrogenase, 4 IU/ml pyruvate kinase, 10 \(\mu\)g/ml valinomycin, and 5 mM Pipes-NaOH (pH 7.4) in the presence or absence of 10 \(\mu\)M SCH28080. In the experiments to test anion selectivity, 150 mM KCl was replaced with 150 mM KX (X = Br, I, H\(_2\)PO\(_4\), or glucuronate). When indicated, DIOA (10 \(\mu\)M) was added. The decrease in the amount of NADH was measured by a Beckman spectrophotometer in a dual wavelength mode at 340 and 500 nm at 25 \(^\circ\)C.

H\(^+\),K\(^+\)-ATPase activities of freeze-dried vesicles prepared from TV and SAV (10 \(\mu\)g of protein) and the membrane fraction of HEK293 cells (30 \(\mu\)g of protein) were measured in a 1-ml solution containing 15 mM KCl, 3 mM MgSO\(_4\), 1 mM ATP, 5 mM Na\(_2\)PO\(_4\), 2 \(\mu\)M ouabain, and 40 mM Tris-HCl (pH 6.8) in the presence or absence of 50 mM SCH28080. After incubation for 10 min (freeze-dried vesicles) or 30 min (HEK293 cells) at 37 \(^\circ\)C, the reaction was terminated by addition of the ice-cold stop solution containing 12% perchloric acid and 3.6% ammonium molybdate, and the inorganic phosphate released was measured (22).

Measurement of \(^{86}\)Rb\(^+\) Uptake into SAV—SAV (200 \(\mu\)g of protein) were added to the solution containing 150 mM KX (X = Cl, Br, H\(_2\)PO\(_4\), or glucuronate), 3 mM MgSO\(_4\), 1 mM ATP, 20 \(\mu\)Ci/ml \(^{86}\)RbCl, and 5 mM Pipes-Tris (pH 7.4) and incubated for 10 min at 25 \(^\circ\)C. The reaction mixtures were rapidly filtered through a 0.45-\(\mu\)m HAWP filter. The filter was washed with a solution containing 150 mM KX (X = Cl, Br, H\(_2\)PO\(_4\), or glucuronate) and 5 mM Pipes-Tris (pH 7.4), transferred to a counting vial; and solubilized with 5 ml of ACS II scintillant. Then the radioactivity of \(^{86}\)Rb\(^+\) was measured.
vial, and solubilized with 5 ml of ACS II scintillant. Then the radioactivity of $^{86}$Rb$^+$ was counted.

**Plasmid Construction**—Full-length cDNA encoding rat KCC4 was inserted into the pcDNA4/His vector (Invitrogen) by using EcoRI and XbaI restriction sites (KCC4-pcDNA4/His vector). The KCC4 cDNA (with Xpress epitope) cut from the KCC4-pcDNA4/His vector was inserted into the pcDNA5/TO vector by using AflIII and XbaI restriction sites (KCC4-pcDNA5/TO vector).

**Tetracycline-regulated Expression System of KCC4 in HEK293 Cells Stably Expressing H$^+\cdot$K$^+\cdot$ATPase—HEK293** cells stably expressing $\alpha$- and $\beta$-subunits of the gastric H$^+\cdot$K$^+\cdot$ATPase were established as described previously (23). The cells were cotransfected with the KCC4-pcDNA5/TO and pcDNA6/TR vectors (Invitrogen) using Lipofectamine 2000 and cultured for 24 h. The transfected cells were selected in the presence of 400 units/ml hygromycin B and 6 $\mu$g/ml blasticidin S.

**Measurement of Intracellular pH**—Intracellular pH (pH$_i$) of the HEK293 cells was measured by monitoring the fluorescence of BCECF as described previously (24, 25). The cells (1 $\times$ 10$^6$ cells), seeded on coverslips (13 mm; Matsunami Glass, Osaka, Japan) coated with poly-L-lysine and rat tail collagen I (Invitrogen), were cultured for 24 h. The transfected cells were selected in the presence of 400 units/ml hygromycin B and 6 $\mu$g/ml blasticidin S.

The cells were incubated with BCECF-AM (10 $\mu$m) in buffer containing 145 mM NaCl, 5 mM KCl, 1 mM CaCl$_2$, 1.2 mM MgSO$_4$, 2 mM Na$_2$HPO$_4$, 10.5 mM glucose, and 32 mM HEPES (pH 7.4) for 30 min at 37 °C. When indicated, DIOA (10 $\mu$m) or SCH28080 (10 $\mu$m) was added. The coverslip was placed in the chamber of a spectrofluorometer (RF-5000, Shimadzu) equipped with a continuous buffer flow system. Intracellular BCECF was alternately excited with 490 and 440 nm light, and the emitted light was measured at 520 nm. The cells were acid-loaded by successively incubating in the NH$_4$H$_2$PO$_4$-containing buffer composed of 125 mM NaCl, 20 mM NH$_4$Cl, 5 mM KCl, 1 mM CaCl$_2$, 1.2 mM MgSO$_4$, 2 mM Na$_2$HPO$_4$, 10.5 mM glucose, and 32 mM HEPES (pH 7.4) for 30 min at 37 °C. When indicated, DIOA (10 $\mu$m) or SCH28080 (10 $\mu$m) was added. The time course of pH$_i$ recovery after acid loading was monitored.

**Statistics**—Results are shown as means ± S.E. Differences between groups were analyzed by one-way analysis of variance, and correction for multiple comparisons was made by using Tukey's multiple comparison test. Comparison between the two groups was made by using Student's $t$ test. Statistically significant differences were assumed at $p < 0.05$.

**RESULTS**

**Expression of KCC4 in Gastric Parietal Cells**—First we examined whether KCC4 mRNA was expressed in gastric parietal cells. Northern blotting showed significant expression of KCC4 mRNA (~4.9 kb) in rabbit gastric parietal cells (Fig. 1A). Western blotting showed that anti-mouse KCC4 antibody reacted with the 165-kDa protein in mouse and rat gastric mucosa and that anti-human KCC4 antibody reacted with the 165-kDa protein in human gastric mucosa (Fig. 1B, upper panels). Both antibodies reacted with the 165-kDa bands in the membrane fraction of HEK293 cells transfected with rat KCC4 (cloned KCC4) (3 $\mu$g of protein), mouse gastric mucosa (10 $\mu$g of protein), and rat gastric mucosa (50 $\mu$g of protein) where two sets of three samples (as A-B-C-A-B-C) in a membrane were electrophoresed, and then the membrane was cut into two pieces, which were separately probed with anti-mouse KCC4 antibody (upper panel) and with the anti-mouse KCC4 antibody plus the corresponding blocking peptide (lower panel). A band of 165 kDa was observed (upper panel). The 165-kDa bands disappeared in the presence of the blocking peptide (lower panel). In the right panels, Western blotting was performed with the cloned KCC4 (15 $\mu$g of protein), hog gastric mucosa (100 $\mu$g of protein), and human gastric mucosa (100 $\mu$g of protein) where two sets of three samples in a membrane were electrophoresed, and then the membrane was cut into two pieces, which were separately probed with anti-human KCC4 antibody (upper panel) and with the anti-human KCC4 antibody plus the corresponding blocking peptide (lower panel). A band of 165 kDa was observed (upper panel). The 165-kDa bands disappeared in the presence of the blocking peptide (lower panel).

In the immunohistochemistry of isolated rat gastric mucosa, KCC4 was found to be colocalized with H$^+\cdot$K$^+\cdot$ATPase, which is expressed in the intracellular tubulovesicles and the apical canalicular membrane of parietal cells (Fig. 2, A–F). The specificity of anti-KCC4 antibody for positive staining was confirmed by using the blocking peptide (Fig. 2, G–I). On the other hand, the distribution pattern of KCC4 was apparently different from that of the Na$^+\cdot$K$^+\cdot$ATPase a1 subunit (Fig. 2, J–L). It has been reported that younger parietal cells in the luminal region of the glands much more actively secrete acid than the older parietal cells in the basal region (26–28). AQP4 has been reported to be localized in the basolateral membrane of the parietal cells at the basal region of the gastric glands (29). Interestingly the present double immunostaining of KCC4 and AQP4 in the gastric mucosa showed that KCC4 is expressed in the parietal cells more abundantly at the luminal region of the glands than at the basal region. (Fig. 2, M–O).

**Expression of KCC4 in the SAV That Are Derived from the Apical Canalicular Membranes of Gastric Parietal Cells**—To determine whether KCC4 is expressed in the intracellular tubulovesicles and the apical canalicular membrane of parietal cells,
two types of gastric vesicles (SAV and TV) were prepared from hog gastric mucosa. H⁺,K⁺-ATPase α- and β-subunits were highly expressed in both TV and SAV (Fig. 3A). The expression level of the Na⁺,K⁺-ATPase α-subunit in TV and SAV was much lower than that in the gastric mucosa (Fig. 3A).

It has been reported that Rab11 is present in the H⁺,K⁺-ATPase-rich vesicular membrane and related to the vesicular trafficking machinery in gastric parietal cells (30). β-Actin has been reported to be associated with ezrin in the apical membrane of gastric parietal cells (31, 32). Here the expression level of Rab11 in TV was much higher than that in SAV. In contrast, the expression levels of β-actin and ezrin in SAV were much

![A–C show the same tissue under a microscope (as do D–F, G–I, J–L, and M–O). Double immunostaining was performed with isolated rat gastric mucosa using anti-mouse KCC4 and anti-H⁺,K⁺-ATPase α-subunit antibodies (HK) (A–F), anti-KCC4 and anti-Na⁺,K⁺-ATPase α1 subunit antibodies (NaK) (J–L), and anti-KCC4 and anti-AQP4 antibodies (M–O). A–F, colocalization of KCC4 with H⁺,K⁺-ATPase is shown. In the inset of F, an enlarged image of a parietal cell is shown. G–I, anti-KCC4 antibody is pretreated with the blocking peptide. Positive KCC4 staining disappeared. J–L, localization of KCC4 is different from that of Na⁺,K⁺-ATPase. In the inset of L, an enlarged image of a parietal cell is shown. M–O, localization of KCC4 is different from that of AQP4. Scale bars, 10 μm.](image)

![FIGURE 3. Expression of KCC4 in the SAV. A, Western blotting was performed with hog tubulovesicles (5 μg of protein) and stimulation-associated vesicles (5 μg of protein) with anti-H⁺,K⁺-ATPase α-subunit (HKα), anti-H⁺,K⁺-ATPase β-subunit (HKβ), and anti-Na⁺,K⁺-ATPase α subunit (NaK) antibodies. The specific bands for H⁺,K⁺-ATPase α-subunit, H⁺,K⁺-ATPase β-subunit, and Na⁺,K⁺-ATPase α1 subunit were observed at 95, 80, and 100 kDa, respectively. As a positive control for detecting Na⁺,K⁺-ATPase α1 subunit, hog gastric mucosa (5 μg of protein) was used (mucosa). B, expression of Rab11 (27 kDa), β-actin (45 kDa), ezrin (85 kDa), syntaxin-1 (35 kDa), KCNQ1 (70 kDa), KCNQ2 (60 kDa), and CFTR (150 kDa) was stained in SAV and TV (30 μg of protein). C, Western blotting was performed with TV, SAV, and gastric mucosa of hogs (30 μg of protein) using anti-human KCC4 antibody. A band of 165 kDa was observed in SAV but not in TV (upper panel). The 165-kDa bands disappeared in the presence of the corresponding blocking peptide (+BP; lower panel). D, Western blotting was performed with TV, SAV, and gastric mucosa of hogs (30 μg of protein) using antibodies for KCC1, KCC2, and KCC3. The 180-kDa band of KCC3 detected in the mucosa is due to KCC3a in the basolateral membrane of the gastric parietal cell (15). A membrane fraction of pig kidney LLC-PK1 cells was used as a positive control for KCC1, and that of hog brain was used as a positive control for KCC2 (control). The specific bands for KCC1, KCC2, and KCC3 were observed at 130, 138, and 180 kDa, respectively. E, immunoprecipitation (IP) was performed with the detergent extracts of SAV (100 μg of protein) using anti-KCC4 antibody and protein A-agarose in the lysate of SAV (IP:KCC4, −). In control experiments, preimmune serum instead of the antibody was used (IP:KCC4, −). The detergent extracts (input; 1/3 for KCC4) or 1/100 (for H⁺,K⁺-ATPase α-subunit (HKα) and β-actin (bottom panel). Top and middle panels were derived from a membrane blotted with two same sets of the three samples. The membrane was cut into two pieces and used for two separate immunoblotting shown in the upper and lower panels. D, Western blotting was performed with TV, SAV, and gastric mucosa of hogs (30 μg of protein) using antibodies for KCC1, KCC2, and KCC3. The 180-kDa band of KCC3 detected in the mucosa is due to KCC3a in the basolateral membrane of the gastric parietal cell (15). A membrane fraction of pig kidney LLC-PK1 cells was used as a positive control for KCC1, and that of hog brain was used as a positive control for KCC2 (control). The specific bands for KCC1, KCC2, and KCC3 were observed at 130, 138, and 180 kDa, respectively. E, immunoprecipitation (IP) was performed with the detergent extracts of SAV (100 μg of protein) using anti-KCC4 antibody and protein A-agarose in the lysate of SAV (IP:KCC4, −). In control experiments, preimmune serum instead of the antibody was used (IP:KCC4, −). The detergent extracts (input; 1/3 for KCC4) or 1/100 (for H⁺,K⁺-ATPase α-subunit (HKα) and β-actin (bottom panel). Top and middle panels were derived from a membrane blotted with two same sets of the three samples. The membrane was cut into two pieces and used for two separate immunoblotting shown in the top and middle panels. The immunoprecipitation shown is representative of three independent experiments.](image)
higher than those in TV (Fig. 3B). Syntaxin-1 was expressed in both TV and SAV as reported previously (33) (Fig. 3B). KCNQ1/KCN2 K⁺ channels and CFTR Cl⁻ channel were predominantly expressed in TV (Fig. 3B). These results confirmed that SAV and TV are derived from the apical canalicular membranes and the intracellular microsomal membranes of the gastric parietal cell, respectively.

Interestingly KCC4 (165 kDa) was predominantly expressed in SAV but not in TV (Fig. 3C). The specificity of the antibody for the 165-kDa band was confirmed by using the corresponding blocking peptide (Fig. 3C, lower panel). No significant expression of other KCCs such as KCC1, KCC2, and KCC3 was observed in either TV or SAV (Fig. 3D).

To study whether KCC4 is associated with H⁺,K⁺-ATPase in SAV, immunoprecipitation was performed using an anti-KCC4 antibody. The subsequent Western blotting of the immune pellets with an anti-H⁺,K⁺-ATPase α-subunit antibody gave a clear band corresponding to the H⁺,K⁺-ATPase α-subunit (95 kDa), whereas blotting with an anti-β-actin antibody as a negative control gave no β-actin band (45 kDa) (Fig. 3E). These results suggest that KCC4 is associated with H⁺,K⁺-ATPase in the SAV.

Note that the apparent difference in affinity of the anti-human KCC4 antibody for KCC4 protein between Fig. 3, C and E, originated from the pretreatment of SAV with (Fig. 3E) and without (Fig. 3C) the lysis buffer. This buffer effect was confirmed by a separate experiment shown in supplemental Fig. 2.

**Inhibition of Cl⁻ Transport by the H⁺,K⁺-ATPase Inhibitor in SAV**—Here we measured Cl⁻ uptake into TV and SAV using 36Cl⁻ in a solution containing 150 mM KCl, 4 mM MgSO₄, 250 mM sucrose, 2 mM ATP, 5 μCi/ml Na36Cl, and 40 mM PIPES-Tris (pH 6.8). DIOA is known as a potent inhibitor of KCCs (34). Although high concentrations of DIOA suppressed H⁺,K⁺-ATPase activity (IC50 = 75–97 μM), no inhibitory effect of this drug on H⁺,K⁺-ATPase was observed at a lower concentration (<20–30 μM) (35). In Fig. 4, DIOA (10 μM) significantly inhibited Cl⁻ uptake in SAV (Fig. 4B) but not in TV (Fig. 4A), reflecting the presence of KCC4 in SAV and its absence from TV. SCH28080 (10 μM), a specific inhibitor of H⁺,K⁺-ATPase, significantly inhibited Cl⁻ uptake in both SAV and TV (Fig. 4). In the SAV, inhibitory effects of DIOA and SCH28080 were not significantly different from that of DIOA plus SCH28080 (Fig. 4B). These results suggest that the DIOA-sensitive Cl⁻ transport in SAV may be mediated by the H⁺,K⁺-ATPase activity.

**Inhibition of H⁺ Transport by the KCC Inhibitor in SAV**—Next the inhibitory effects of DIOA on H⁺ uptake into SAV and TV by H⁺,K⁺-ATPase were studied. SCH28080 (20 μM) markedly inhibited the H⁺ uptake in TV and SAV as expected (Fig. 5, A and B). On the other hand, no H⁺ uptake was observed in the freeze-dried TV and SAV (Fig. 5, A and B). Interestingly DIOA (10 μM) significantly inhibited the H⁺ uptake into SAV, whereas it had no significant effect in TV (Fig. 5, C and D), indicating that the inhibition of KCC4 resulted in inhibition of H⁺ uptake by H⁺,K⁺-ATPase.

**Inhibition of H⁺,K⁺-ATPase Activity by the KCC Inhibitor in SAV**—We studied whether DIOA affects the SCH28080-sensitive K⁻-ATPase activity (H⁺,K⁺-ATPase activity). DIOA (10 μM) significantly inhibited the H⁺,K⁺-ATPase activity in SAV (Fig. 6B), whereas it had no significant effects in TV (Fig. 6A). In the freeze-dried leaky TV and SAV, no significant effects of DIOA (10 μM) on the H⁺,K⁺-ATPase activity were observed (supplemental Fig. 3, A and B). These results suggest that DIOA indirectly inhibits H⁺,K⁺-ATPase activity via inhibition of the ion transport by KCC4.

**Anion Selectivity for the DIOA-sensitive H⁺ Transport and H⁺,K⁺-ATPase Activity in SAV**—To study whether the DIOA (10 μM)-sensitive H⁺ transport and H⁺,K⁺-ATPase activity in SAV depends on the species of anion, H⁺ transport and enzyme
activity were measured in various anion solutions containing 150 mM KX (where $X = \text{Cl}, \text{Br}, \text{I}, \text{H}_2\text{PO}_4$, or gluconate). The order of selectivity of various anions for $\text{H}^+$ uptake was $\text{Cl}^- > \text{Br}^- > \text{PO}_4^{3-} = \text{gluconate}$ (Fig. 7A and supplemental Fig. 4). We could not examine the effect of $\text{I}^-$ on $\text{H}^+$ uptake because KI quenches the fluorescence of acridine orange (36). The order of anion selectivity for the $\text{H}^+,\text{K}^+\text{-ATPase}$ activity was $\text{Cl}^- > \text{Br}^- > \text{I}^- > \text{PO}_4^{3-} = \text{gluconate}$ (Fig. 7B).

These orders were similar to that reported for the anion selectivity for $\text{K}^+$ transporting activity of KCC4 ($\text{Cl}^- > \text{Br}^- > \text{PO}_4^{3-} = \text{I}^- > \text{gluconate}$) (37).

**Anion Selectivity for the DIOA-sensitive $K^+$ Uptake into the SAV—** To study whether the $K^+$ uptake into SAV by $K^+,\text{Cl}^-$ cotransport depends on anion species, we measured the DIOA (10 $\mu\text{M}$)-sensitive $^{86}\text{Rb}^+$ uptake in solutions of 150 mM KX (where $X = \text{Cl}, \text{Br}, \text{H}_2\text{PO}_4$, or gluconate). The order of anion selectivity for $^{86}\text{Rb}^+$ uptake into SAV was $\text{Cl}^- > \text{Br}^- > \text{PO}_4^{3-} = \text{gluconate}$ (Fig. 7C). This selectivity is quantitatively the same as those of the DIOA-sensitive $\text{H}^+$ transport (Fig. 7A) and $\text{H}^+,\text{K}^+\text{-ATPase}$ activity (Fig. 7B), indicating that proton transport by $\text{H}^+,\text{K}^+\text{-ATPase}$ depends on ion transport by KCC4. The SCH28080 (10 $\mu\text{M}$)-sensitive $^{86}\text{Rb}^+$ uptake in solutions of 150 mM KX (where $X = \text{Cl}, \text{Br}, \text{H}_2\text{PO}_4$, or gluconate) was also assessed. The order of anion selectivity was $\text{Cl}^- > \text{Br}^- > \text{PO}_4^{3-} = \text{gluconate}$ (supplemental Fig. 5).

**Stable Coexpression of KCC4 and $\text{H}^+,\text{K}^+\text{-ATPase}$ in the HEK293 Cells—** The tetracycline-regulated expression system of KCC4 was constructed in HEK293 cells that stably expressed $\alpha$- and $\beta$-subunits of gastric $\text{H}^+,\text{K}^+\text{-ATPase}$. No significant expression of endogenous KCC4 was observed in control HEK293 cells (data not shown). In this heterologous expression system, exogenous expression of KCC4 protein was assessed by using an anti-Xpress antibody. Expression of KCC4 (165 kDa) was observed in the cells treated with tetracycline (Tet-On cells), whereas no significant expression of KCC4 was observed in the cells treated without tetracycline (Tet-Off cells) (Fig. 8A). Expression levels of $\text{H}^+,\text{K}^+\text{-ATPase}$ $\alpha$-subunit in the Tet-On cells were not significantly different from those in the Tet-Off cells (Fig. 8B). Both KCC4 and $\text{H}^+,\text{K}^+\text{-ATPase}$ were found to be present in the plasma membrane of the Tet-On cells (Fig. 8C). To check whether KCC4 is associated with $\text{H}^+,\text{K}^+\text{-ATPase}$ in the Tet-On cells as is the case in the SAV (Fig. 3E), immunoprecipitation was performed by using an anti-His tag antibody (for KCC4). The subsequent Western blotting
KCC4 Association with H⁺,K⁺-ATPase in Gastric Parietal Cells

**FIGURE 6.** Inhibition of H⁺,K⁺-ATPase activity in SAV by DIOA. SCH28080 (10 μM)-sensitive K⁺-ATPase activity (H⁺,K⁺-ATPase activity) was measured in the absence (control) and presence (DIOA) of 10 μM DIOA in TV (A) and SAV (B). n = 5. NS, not significantly different (p > 0.05); **, significantly different (p < 0.01).

of the immune pellets with an anti-H⁺,K⁺-ATPase α-subunit antibody gave a band for H⁺,K⁺-ATPase α-subunit (Fig. 8D), indicating association between KCC4 and the H⁺,K⁺-ATPase α-subunit.

**KCC4-induced Stimulation of H⁺ Transport by H⁺,K⁺-ATPase in the Heterologous Expression System**—In the membrane fractions of both Tet-Off and Tet-On cells (i.e. cell-free condition), DIOA (10 μM) did not inhibit the H⁺,K⁺-ATPase activity (supplemental Fig. 3, C and D) as found for the freeze-dried TV and SAV (supplemental Fig. 3, A and B). This would reflect the fact that the membrane samples obtained from the HEK293 cells were not tightly sealed as has been described elsewhere (38). In Fig. 9, we studied the capacity of the acid extrusion (H⁺-transport activity) in the Tet-On and Tet-Off cells. The capacity was assessed by measuring the rate of recovery of pHᵢ in the cells after acid loading through an ammonium pulse. The pHᵢ recovery was monitored in the absence of Na⁺ to exclude the contribution of the Na⁺/H⁺ exchanger. The pHᵢ recovery process in the Tet-On cells (Fig. 9, B and G) was significantly faster than that in the Tet-Off cells (Fig. 9, A and G). Interestingly DIOA (10 μM) significantly decreased the recovery rate of pHᵢ in the Tet-On cells (Fig. 9, D and G) but not in the Tet-Off cells (Fig. 9, C and G). SCH28080 (10 μM) significantly decreased the recovery rate of pHᵢ in both the Tet-On and Tet-Off cells (Fig. 9, E–G). These results suggest that KCC4 may stimulate the H⁺ transport activity of H⁺,K⁺-ATPase in the Tet-On cells.

**DISCUSSION**

In the present study, we found the following. 1) KCC4 is expressed in the apical canalicular membrane of gastric parietal cells more abundantly at the luminal region of the glands than at the basal region. In contrast, KCC1, KCC2, and KCC3 are not significantly expressed in the apical canalicular membrane. KCC4 is absent from the intracellular tubulovesicles. 2) KCC4 is associated with H⁺,K⁺-ATPase in the apical canalicular membrane. 3) In vesicles of the apical canalicular membrane (SAV), the H⁺,K⁺-ATPase inhibitor suppresses the Cl⁻ transport activity, which is sensitive to a KCC inhibitor (DIOA; 10

![Image](http://www.jbc.org/)

![Image](http://www.jbc.org/)

![Image](http://www.jbc.org/)
The KCC inhibitor suppresses the $H^+\cdotK^+$-ATPase activity in the SAV but not in the freeze-dried leaky SAV and the TV.

In the SAV, the anion selectivity of the DIOA-sensitive $H^+\cdotK^+$-ATPase activity and the DIOA-sensitive $H^+$ and $K^+$ transports are similar to that for the $K^+$ transporting activ-

FIGURE 8. Tetracycline-regulated expression system of KCC4 in the HEK293 cells stably expressing gastric $H^+\cdotK^+$-ATPase. The tetracycline-regulated expression system of KCC4 was introduced to the HEK293 cells that stably express $\alpha$- and $\beta$-subunits of gastric $H^+\cdotK^+$-ATPase. The cells were treated with (on) or without (off) 2 $\mu$g/ml tetracycline. A, the expression of KCC4 in the membrane fraction of the cells (30 $\mu$g of protein) was confirmed by Western blotting using anti-Xpress antibody. B, the expression level of $H^+\cdotK^+$-ATPase $\alpha$-subunit in the Tet-On cells (on) was compared with that in the Tet-Off cells (off). In the upper panel, a representative picture of Western blotting is shown. In the lower panel, the score for the Tet-Off cells is normalized as 1. $n = 5$. NS, $p > 0.05$. C–F show the same cells under a microscope (as do d–f and g–i). Double immunostaining was performed with the Tet-On cells (a–f) and the Tet-Off cell (g–i) using anti-KCC4 plus anti-$H^+\cdotK^+$-ATPase $\alpha$-subunit antibodies. Localizations of KCC4 (a, d, and g), $H^+\cdotK^+$-ATPase (HK) (b, e, and h), and KCC4 plus $H^+\cdotK^+$-ATPase (merged images; c, f, and i) are shown. In d–f, anti-KCC4 antibody was pretreated with the blocking peptide (+BP). Positive KCC4 staining disappeared. Scale bars, 10 $\mu$m. D, immunoprecipitation (IP) was performed with the detergent extracts of the KCC4-expressing Tet-On cells using anti-His tag antibody for KCC4 and protein A-agarose. The detergent extract (input) and the immunoprecipitation samples obtained with (IP:His(KCC4), +) and without (IP:His(KCC4), −) the antibody were detected by Western blotting (WB) using anti-Xpress antibody for detecting KCC4 (upper panel) and anti-$H^+\cdotK^+$-ATPase $\alpha$-subunit antibody (HK$\alpha$; lower panel). The immunoprecipitation shown is representatives of three independent experiments.

FIGURE 9. pH recovery of the Tet-On and Tet-Off cells after acid loading through an ammonium pulse. A–F, the pH recovery was monitored in the absence of Na$^+$ after an ammonium pulse of the Tet-Off cells (A, C, and E) and Tet-On cells (B, D, and F). Representative traces are shown. Effects of 10 $\mu$M DIOA (C and D) and 10 $\mu$M SCH28080 (SCH) (E and F) on the pH recovery were examined. As control experiments, the pH recovery was measured in the absence of DIOA and SCH28080 (A and B). The red bars show the fitted slope of pH change from 0 to 5 min (blue bar) after introducing the Na$^+$-free buffer. G, the averaged rates of the pH recovery ($\Delta$pH/5 min) are shown. $n = 4–5$. NS, $p > 0.05$; **, $p < 0.01$. $K^+$ transports are similar to that for the $K^+$ transporting activ-
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The expression of KCC4 stimulates \( H^+ \) transport across the HEK293 cell membranes that coexpress \( H^+,K^+ \)-ATPase.

Recently, we found that KCC3a is expressed in the basolateral membrane of gastric parietal cells, that KCC3a is associated with Na\(^+\),K\(^+\)-ATPase \( \alpha 1 \) subunit (\( \alpha 1 \)NaK), and that KCC3a up-regulates \( \alpha 1 \)NaK activity in the membrane fraction of the KCC3a-expressing LLC-PK1 cells and rabbit gastric mucosa. KCC3a may directly activate the \( \alpha 1 \)NaK activity in cell-free conditions (15). On the other hand, in this study, the functional association of KCC4 with \( H^+,K^+ \)-ATPase could be seen only in intact tightly sealed SAV and HEK293 cells and not in the freeze-dried leaky SAV and the leaky membrane fraction of HEK293 cells. Our present results suggest that KCC4 indirectly increases \( H^+,K^+ \)-ATPase activity by effectively supplying \( K^+ \) to the luminal surface of this ATPase.

Gastric acid secretion by the parietal cells is accompanied by dramatic morphological changes. In resting parietal cells, tubulovesicles are present in intracellular compartments underlying the apical canalicular membrane and form a reticulated meshwork. Upon stimulation, the tubulovesicles translocate and connect with the canalicular membrane, resulting in massive acid secretion (39–41). So far, several \( Cl^- \) and \( K^+ \) channels have been identified in gastric parietal cells (7, 42). CFTR (5), CLIC-6 (parchinor) (6, 7), and SLC26A9 (8) are candidates that could be involved in the luminal \( Cl^- \) efflux for gastric acid (HCl) secretion. KCNQ1/KCNE2 (1, 2) and Kir4.1 (3, 4) are candidates that could be involved in the luminal \( K^+ \) efflux for the \( K^+ \) recycling. In the present study, CFTR was confirmed to be localized predominantly in the tubulovesicles (Fig. 3B). KCNQ1 was found to be expressed mainly in the tubulovesicles (Fig. 3B) as reported previously (4, 43). It has been reported that CLIC-6 is distributed throughout the cytosol (6) and that Kir4.1 is localized in the tubulovesicles (4). Therefore, the distribution pattern of these \( K^+ \) and \( Cl^- \) channels is apparently different from that of KCC4 that is predominantly expressed in the apical canalicular membrane.

On the basis of our present findings, we suggest that these two membranes may not mix but remain separate and distinct when the tubulovesicular membrane is connected with the apical canalicular membrane upon stimulation. Fig. 10 summarizes the putative mechanisms of HCl secretion in these two membranes. In the resting state, KCC4 together with \( H^+,K^+ \)-ATPase that is present in the apical canalicular membrane is involved in the basolateral acid secretion. Upon stimulation, CFTR, CLIC-6, SLC26A9, KCNQ1/KCNE2, Kir4.1, and KCC4 are involved in the KCl transport for massive gastric acid secretion.

Interestingly, it has been reported that KCC1, KCC2, and KCC3 are inhibited at pH <7.5 when these are expressed in Xenopus oocytes, whereas KCC4 is activated at acidic pH (44). This finding suggests that KCC4 may be specialized to operate in an acidic environment. In fact, we found that KCC4 is expressed in the apical canalicular membranes, which face the gastric acid.

In the kidney, KCC4 has been reported to be crucial for \( Cl^- \) extrusion across the basolateral membrane of acid-secreting \( \alpha \)-intercalated cells and that the loss of KCC4 leads to deafness associated with renal tubular acidosis (45). In contrast to this basolateral localization in renal \( \alpha \)-intercalated cells, KCC4 is localized in the apical membrane of gastric parietal cells. It will be necessary to clarify the mechanism of the recruitment of KCC4 in the apical canalicular membrane of the parietal cells.

Gastric parietal cells migrate from the luminal to the basal region of the glands, and the luminal parietal cells more actively secrete acid than do the basal parietal cells (26–28). KCC4 is expressed in the parietal cells more abundantly at the luminal region of the glands than at the basal region. It is noted that KCC3a is located in the luminal region (15). KCC3a in the basolateral membrane of the parietal cell increases the \( Na^+,K^+ \)-ATPase activity. The electrochemical potential gradient for \( K^+ \) across the apical membrane drives the ion transport by KCC4 and is established by mainly Na\(^+\),K\(^+\)-ATPase and \( H^+,K^+ \)-ATPase. Thus, both KCC3a and KCC4 are involved in acid secretion.

In conclusion, KCC4 is functionally expressed in the apical canalicular membrane of gastric parietal cells, and its \( K^+\)-\( Cl^- \) cotransport is coupled with \( H^+,K^+ \)-ATPase activity in the canalicular membrane. KCC4 may function as a \( K^+ \)-supplying molecule for \( H^+,K^+ \)-ATPase and also as a \( Cl^- \)-transporting molecule for HCl secretion. In the resting state of the parietal
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cells, KCC4 and H\(^+\),K\(^+\)-ATPase in the apical canalicular membrane of the parietal cell are the main machineries for the basal gastric acid secretion. In the stimulated state, they also contribute to acid secretion together with other K\(^+\) and Cl\(^-\) channels and H\(^+\),K\(^+\)-ATPase that are present in tubulovesicles.

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Functional Association between K⁺-Cl⁻ Cotransporter-4 and H⁺,K⁺-ATPase in the Apical Canaliculular Membrane of Gastric Parietal Cells

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