Gastric parietal cells migrate from the luminal to the basal region of the gland, and they gradually lose acid secretory activity. So far, distribution and function of K\(^+\)-Cl\(^-\) cotransporters (KCCs) in gastric parietal cells have not been reported. We found that KCC3a but not KCC3b mRNA was highly expressed, and KCC3a protein was predominantly expressed in the basolateral membrane of rat gastric parietal cells located in the luminal region of the glands. KCC3a and the Na\(^+\)/H\(^+\) cotransporter-1 (\(\alpha_1\)NaK) were coimmunoprecipitated, and both of them were highly localized in a lipid raft fraction. The ouabain-sensitive K\(^+\)-dependent ATP-hydrolyzing activity (Na\(^+\)/K\(^+\)-ATPase activity) was significantly inhibited by a KCC inhibitor (R-((+)\)(-2-n-butyl-6,7-dichloro-2-cyclopentyl-1-oxo-1H-inden-5-yl)oxy)acetic acid (DIOA)). The stable exogenous expression of KCC3a in LLC-PK1 cells resulted in association of KCC3a with endogenous \(\alpha_1\)NaK, and it recruited \(\alpha_1\)NaK in lipid rafts, accompanying increases of Na\(^+\)/K\(^+\)-ATPase activity and ouabain-sensitive Na\(^+\) transport activity that were suppressed by DIOA, whereas the total expression level of \(\alpha_1\)NaK in the cells was not significantly altered. On the other hand, the expression of KCC4 induced no association with \(\alpha_1\)NaK. In conclusion, KCC3a forms a functional complex with \(\alpha_1\)NaK in the basolateral membrane of luminal parietal cells, and it up-regulates \(\alpha_1\)NaK in lipid rafts, whereas KCC3a is absent in basal parietal cells.

The cation-chloride cotransporter gene family (SLC12) includes Na\(^+\)/Cl\(^-\) cotransporters, Na\(^+\)/K\(^+\)/2Cl\(^-\) cotransporters (NKCCs), and K\(^+\)/Cl\(^-\) cotransporters (KCCs). KCCs mediate an electroneutrally coupled transport of K\(^+\) and Cl\(^-\) and contribute to transepithelial transport and cell volume regulation (1, 2). KCCs are encoded by at least four homologues genes (KCC1–KCC4), and KCC3 has three isoforms (KCC3a–KCC3c) generated by the difference of first coding exons (3–5). KCC1 is widely expressed, whereas KCC2 and KCC3b are restricted in neuron and kidney, respectively. KCC3a and KCC4 are mainly expressed in epithelial cells (3–5).

Among the SLC12 family, Na\(^+\)/K\(^+\)/2Cl\(^-\) cotransporter-1 (NKCC1) is present in the basolateral membrane of gastric parietal cells (6). NKCC1 was reported to be not involved in the gastric acid (HCl) secretion but involved in nonacidic exogenous ion secretions in mouse parietal cells (7, 8). So far, the distribution and function of KCCs have not been reported in the gastric parietal cells.

Lipid rafts are glycosphingolipid- and cholesterol-enriched microdomains and are thought to be the functional domains involved in membrane trafficking and signal transduction (9–11). Recently, it has been reported that some transporters and channels, such as Na\(^+\)/K\(^+\)-ATPase (12, 13), Na\(^+\)/H\(^+\) exchanger 3 (14, 15), and epithelial Na\(^+\) channel (16), are localized in lipid rafts of several tissues. However, little is known about lipid rafts of gastric parietal cells.

In the present study, we studied distribution of KCC3a and association of KCC3a with Na\(^+\)/K\(^+\)-ATPase \(\alpha_1\)-subunit (\(\alpha_1\)NaK) in the gastric mucosa. In addition, we studied recruitment of \(\alpha_1\)NaK by KCC3a in lipid rafts using the cells stably expressing exogenous KCC3a.

**EXPERIMENTAL PROCEDURES**

**Materials**—Anti-rat KCC3 rabbit polyclonal antibody was generated with keyhole limpet hemocyanin-coupled peptides against 19 amino acids corresponding to the N-terminal sequence of KCC3 (KARNAYLNSNYEEGDEY). Both KCC3a and b contain this N-terminal sequence. This antigen peptide has no homology with the sequences of KCC1, 2, and 4. Anti-Na\(^+\)/K\(^+\)-ATPase \(\alpha_1\)-subunit (\(\alpha_1\)NaK) mouse monoclonal antibody was obtained from Upstate Biotechnology, Inc.(Lake Placid, NY). Anti-H\(^+\)/K\(^+\)-ATPase \(\alpha\)-subunit mouse monoclonal antibody (1H9) was from Medical & Biological Laboratories Co. (Nagoya, Japan). Anti-flotillin-2 mouse monoclonal antibody (B-6) and anti-human aquaporin-4 monio]propanesulfonic acid; DRM, detergent resistance membrane; EP, phosphoenzyme.
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(AQP4) goat polyclonal antibody (H-19) were from Santa Cruz Biotechnology (Santa Cruz, CA). Lipofectamine 2000, anti-Xpress mouse monoclonal antibody, Alexa Fluor 488/546-conjugated anti-mouse IgG antibody, Alexa Fluor 488/546-conjugated anti-rabbit IgG antibody, and Alexa Fluor 546-conjugated anti-goat IgG antibody were from Invitrogen. Ouabain, SCH 28080, methyl-\(\beta\)-cyclodextrin (M\(\beta\)CD), and R-(+-)[(2-n-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy]acetic acid (DIOA) were from Sigma-Aldrich. Protein A-agarose beads was from Pierce. Hygromycin B and blasticidin S were obtained from Wako Pure Chemical Industries (Osaka, Japan) and Kaken Pharmaceutical Co. (Tokyo, Japan), respectively. \(^{22}\)NaCl was obtained from PerkinElmer Life Sciences.

Isolation of Tissues and Cells—The cell suspension rich in parietal cells and the gastric gland suspension were prepared from rabbit stomach as previously described (17). 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 the ethics committee approval. All of the patients gave informed consent.

Northern Blotting—Poly(A)\(^{+}\) RNA of the cells was prepared by using the PolyATtract mRNA isolation system II (Promega, Madison, WI). The amplified products were sequenced and used for the preparation of the \(^{32}\)P-labeled cDNA probes. The rabbit KCC3 probe was 712 bp long and corresponded to nucleotides 806–1507 of the KCC3a cDNA. The poly(A)\(^{+}\) RNA (2.5 \(\mu\)g) was separated on a 1\% agarose/formaldehyde gel and transferred onto a nylon membrane (Zeta-probe GT; Bio-Rad). The membrane was hybridized with the \(^{32}\)P-labeled cDNA fragment overnight at 65 °C in 250 mM Na\(_{2}\)HPO\(_{4}\)-Na\(_{2}\)HPO\(_{4}\), pH 7.2, 7% SDS; washed in 40 mM Na\(_{2}\)HPO\(_{4}\)-Na\(_{2}\)HPO\(_{4}\), pH 7.2, 1% SDS at 65 °C; and exposed to the Imaging Plate (Fuji Film) for 20 h.

Plasmid Construction—Full-length cDNAs encoding rat KCC3a and rat KCC4 were inserted into pcDNA4/His vector (Invitrogen) by using KpnI/NotI and EcoRI/XbaI restriction sites, respectively (KCC3a-pcDNA4/His vector and KCC4-pcDNA4/His vector), and also inserted into pcDNA5/TO vector (Invitrogen) by using AflII/NotI and AflII/XbaI restriction sites, respectively (KCC3a-pcDNA5/TO vector and KCC4-pcDNA5/TO vector).

Expression of KCC3a and KCC4 in Cultured Cells—To establish the tetracycline-regulated expression system of KCC3a or KCC4 in LLC-PK1 cells, the cells were cotransfected with pcDNA6/TR vector (Invitrogen), KCC3a-pcDNA5/TO vector, or KCC4-pcDNA5/TO vector by using Lipofectamine 2000 and cultured for 24 h in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. The transfected cells were selected in the presence of 800 units/ml hygromycin B and 5 \(\mu\)g/ml blasticidin S. For transient expression of KCC3a in HEK293 cells stably expressing gastric H\(^{+},K^{+}\)-ATPase (18), the cells were transfected with the KCC3a-pcDNA4/His vector by using Lipofectamine 2000 and cultured for 24 h in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum.

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, Buckinghamshire, UK). To quantify the chemiluminescence signals on the membranes, a FujiFilm LAS-1000 system and the MultiGauge software were used. Anti-KCC3, anti-Xpress, and anti-H\(^{+},K^{+}\)-ATPase (1H9) antibodies were used at 1:1,000 dilution, and anti-\(\alpha\)NaK antibody was used at 1:10,000 dilution. Anti-flotillin-2 antibody was used at 1:2,000 dilution. For negative control, one volume of each primary antibody was preincubated with five volumes of the corresponding blocking peptide. Horseradish peroxidase-conjugated antimouse, 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 O.C.T. compound (Sakura Finetechnical 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 the 3% bovine serum albumin for 1 h at room temperature to block nonspecific binding of antibody. Then they were incubated with anti-KCC3, anti-H\(^{+},K^{+}\)-ATPase (1H9), anti-\(\alpha\)NaK, or anti-AQP4 antibody (1:100 dilution) overnight at 4 °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 by using a Zeiss LSM 510 laser scanning confocal microscope.

Immunocytochemistry—LLC-PK1 cells were fixed with ice-cold methanol for 7 min 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-Xpress or anti-\(\alpha\)NaK antibody (1:100 dilution) overnight at 4 °C and then with the Alexa Fluor 488-conjugated or Alexa Fluor 546-conjugated anti-IgG antibody (1:100 dilution) for 1 h at room temperature. Immunofluorescence images were visualized by using a Zeiss LSM 510 laser scanning confocal microscope.

Isolation of Lipid Rafts—The membrane proteins were lysed with the ice-cold MBS buffer (150 mM NaCl, 25 mM MES-NaOH, pH 6.5) containing 1% CHAPS, 10 \(\mu\)g/ml phenylmethylsulfonyl fluoride, 1 \(\mu\)g/ml leupeptin, and 1 \(\mu\)g/ml pepstatin A for 30 min. The solution was mixed with equal volume of 66% sucrose in MBS buffer, the mixture was placed at the bottom of an ultracentrifuge tube, and a discontinuous gradient was formed by overlaying with the 30% sucrose and the 5% sucrose solutions. The sample was centrifuged at 261,000 \(\times\) g in SW41Ti rotor (Beckman) for 18 h at 4 °C. Ten fractions of 1 ml each were collected from the top of the gradient, and proteins were precipitated by acetone before SDS-polyacrylamide gel electrophoresis and Western blotting. For depletion of cholesterol, the cells were treated with 10 mM MBD for 30 min at 37 °C before harvesting the cells.

Immunoprecipitation—Membrane fractions of rat gastric mucosa (500 \(\mu\)g of protein) and KCC3a-expressing LLC-PK1 cells (2.5 mg of protein) were solubilized in the 500 \(\mu\)l of 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 °C. The lysate was precleared with protein A-agarose beads, and the supernatant was incubated with anti-KCC3 antibody or control rabbit IgG for 24 h at 4 °C with end-over-end rotation. The antibody-antigen complexes were incubated with protein A-agarose beads for 4 h at 4 °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.

**Measurement of Na\(^{+},K^{+}\)-ATPase Activity—Na\(^{+},K^{+}\)-ATPase activity of LLC-PK1 (30 \mu g of protein) and membrane fractions of the rabbit gastric glands (30 \mu g of protein) and rabbit kidney (3 \mu g of protein) was measured in a 1 ml solution containing 120 mM NaCl, 15 mM KCl, 3 mM MgSO\(_4\), 1 or 3 mM ATP, 50 \mu M SCH 28080, and 40 mM Tris-HCl, pH 7.4, in the presence or absence of 100 \mu M ouabain. After incubation for 10 or 30 min at 37 °C, the reaction was terminated by addition of the ice-cold stop solution containing 12% perchloric acid and 3.6% ammonium molybdate, and inorganic phosphate released was measured (20).**

The Na\(^{+},K^{+}\)-ATPase activity was calculated as the difference between the activities in the presence and absence of ouabain.

**Measurement of Phosphorylation Level of Na\(^{+},K^{+}\)-ATPase—**Ten \mu g of membrane proteins were phosphorylated in a solution comprising 2 \mu M [\gamma\(^{32}\)P]ATP (4 \times 10\(^{6}\) cpm), 20 mM NaCl, 0.43 mM MgCl\(_2\), and 25 mM imidazole-HCl (pH 6.0) for 20 s at 0 °C. The reaction was quenched by the solution containing 10% trichloroacetic acid, 10 mM inorganic phosphate, and 1 mM ATP. Then the samples were centrifuged at 13,000 \times g for 3 min at 4 °C, and the pellet was washed with ice-cold 10% trichloroacetic acid, solubilized in a sample buffer comprising 2% SDS, 2.5% dithiothreitol, 10% glycerol, and 50 mM Tris-HCl, pH 6.8, and subjected to 5% SDS-polyacrylamide gel electrophoresis under acidic conditions at pH 6.0 (21, 22). The radioactivity associated with the Na\(^{+},K^{+}\)-ATPase \alpha\,-subunit separated on the gel was visualized and quantified by a digital autoradiography of the dried gel using Bio-Imaging Analyzer BAS2000 (Fuji Film).

**Measurement of H\(^{+},K^{+}\)-ATPase Activity—**H\(^{+},K^{+}\)-ATPase activity of HEK293 cells (30 \mu g of protein) was measured in a 1-ml solution containing 15 mM KCl, 3 mM MgSO\(_4\), 1 mM ATP, 5 mM NaN\(_3\), 2 mM ouabain, and 40 mM Tris-HCl, pH 6.8, in the presence or absence of 50 \mu M SCH 28080, a specific inhibitor of H\(^{+},K^{+}\)-ATPase. After incubation for 30 min at 37 °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 (20).

**Measurement of \(^{22}\)Na\(^{+}\) Transport Activity—**Na\(^{+}\) transport activity of LLC-PK1 cells was estimated by measuring intracellular \(^{22}\)Na\(^{+}\) content (23, 24). In each well of 12-well culture plates, 1 \times 10\(^5\) cells equipped with the tetracycline-regulated expression system of KCC3a were seeded and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum for 2 days, followed by treatment with or without 2 \mu g/ml tetracycline for 24 h. The treated cells were incubated at 37 °C in the culture medium containing trace amounts of \(^{22}\)Na\(^{+}\) (0.5 \mu Ci/ml) for 120 min to fully equilibrate exchangeable intracellular Na\(^{+}\) with \(^{22}\)Na\(^{+}\). The \(^{22}\)Na\(^{+}\)-loaded cells were incubated at 37 °C for additional 30 min in the presence and absence of 10 \mu M ouabain. The cells were then washed three times with ice-cold solution of 100 mM MgCl\(_2\) and dissolved in 1 ml of the solution containing 1% SDS. \(^{22}\)Na\(^{+}\) contents in the samples were measured in a \gamma\,-counter (Aloka Accuspect 7001, Aloka Co., Tokyo, Japan). The Na\(^{+}\) transport activity of \(^{22}\)Na\(^{+}\)-ATPase was estimated as the difference between the intracellular \(^{22}\)Na\(^{+}\) contents in the presence and absence of ouabain.

**Statistics—**The results are shown as the means ± S.E. Differences between groups were analyzed by one-way analysis of variance, and correction for multiple comparisons was made by using Dunnett’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 KCC3a in Gastric Parietal Cells—**Northern blotting with the KCC3 cDNA probe gave a strong band of \(~8\) kb and faint band of \(~7\) kb in the preparation of rabbit gastric parietal cells (Fig. 1A). The transcript sizes of KCC3a and KCC3b were reported to be around 8 and 7 kb, respectively (25). Western blotting was performed with gastric glands of rabbits (15 \mu g of protein) and gastric mucosa of rats (50 \mu g of protein) and humans (100 \mu g of protein) by using anti-KCC3 antibody. A single band of 180 kDa was observed (upper panel). The 180-kDa band disappeared in the presence of the corresponding blocking peptide (lower panel). Western blotting was performed with gastric mucosa (50 \mu g of protein) and kidney of rats (100 \mu g of protein) by using anti-KCC3 antibody. The 180- and 150-kDa bands were observed in gastric mucosa and kidney, respectively.

**Up-regulation of Na\(^{+},K^{+}\)-ATPase by KCC3a**

![FIGURE 1. Expression of KCC3a in gastric parietal cells. A, Northern blotting was performed with poly(A\(^{+}\)) RNA (2.5 \mu g/lane) from isolated rabbit gastric parietal cells. B, Western blotting was performed with gastric glands of rabbits (15 \mu g of protein) and gastric mucosa of rats (50 \mu g of protein) and humans (100 \mu g of protein) by using anti-KCC3 antibody. A single band of 180 kDa was observed (upper panel). The 180-kDa band disappeared in the presence of the corresponding blocking peptide (lower panel). C, Western blotting was performed with gastric mucosa (50 \mu g of protein) and kidney of rats (100 \mu g of protein) by using anti-KCC3 antibody. The 180- and 150-kDa bands were observed in gastric mucosa and kidney, respectively.**

KCC3a mRNA and protein are predominantly expressed in gastric parietal cells. A 8-kb band was predominantly detected with the KCC3 cDNA probe. B, Western blotting was performed with gastric glands of rabbits (15 \mu g of protein) and gastric mucosa of rats (50 \mu g of protein) and humans (100 \mu g of protein) by using anti-KCC3 antibody. A single band of 180 kDa was observed (upper panel). The 180-kDa band disappeared in the presence of the corresponding blocking peptide (lower panel). C, Western blotting was performed with gastric mucosa (50 \mu g of protein) and kidney of rats (100 \mu g of protein) by using anti-KCC3 antibody. The 180- and 150-kDa bands were observed in gastric mucosa and kidney, respectively.
Up-regulation of Na\textsuperscript{+},K\textsuperscript{+}-ATPase by KCC3a

In the immunohistochemistry of isolated rat gastric mucosa, the distribution pattern of KCC3a (Fig. 2, A, C, D, and F) was apparently different from that of H\textsuperscript{+},K\textsuperscript{+}-ATPase (Fig. 2, B, C, E, and F). KCC3a was colocalized with Na\textsuperscript{+},K\textsuperscript{+}-ATPase \( \alpha \)-subunit (\( \alpha \)1NaK) (Fig. 2, J–L), suggesting that it is present in the basolateral membrane of gastric parietal cells. The specificity of anti-KCC3 antibody for positive staining was confirmed by using the blocking peptide (Fig. 2, G–I). AQP4 was abundantly expressed in the basal parietal cells (Fig. 2, M and N) as previously reported (26).

Previously, younger parietal cells in the luminal region of the glands have been reported to secrete acid more actively than do older parietal cells in the basal region (27–29). Interestingly, the present double immunostaining of KCC3a and AQP4 in rat gastric mucosa has shown clearly that KCC3a is predominantly expressed in the luminal parietal cells (Fig. 2M).

Association of KCC3a with \( \alpha \)1NaK in the Gastric Mucosa—To study whether KCC3a is associated with \( \alpha \)1NaK in rat gastric mucosa, immunoprecipitation was performed by using an anti-KCC3 antibody. The subsequent Western blotting of the immune pellets with an anti-\( \alpha \)1NaK antibody gave a clear band for \( \alpha \)1NaK (100 kDa), whereas that with an anti-H\textsuperscript{+},K\textsuperscript{+}-ATPase antibody gave no band for H\textsuperscript{+},K\textsuperscript{+}-ATPase (95 kDa) (Fig. 3A).

To determine whether KCC3a is associated with \( \alpha \)1NaK in lipid rafts, the detergent extracts of rat gastric mucosa were fractionated by sucrose density gradient centrifugation and immunoprecipitated by using an anti-KCC3 antibody. The subsequent Western blotting using antibodies for H\textsuperscript{+},K\textsuperscript{+}-ATPase \( \alpha \)-subunit (\( \alpha \)HK; bottom panel). A, lipid raft fractions (DRM) and nonraft fractions (non-DRM) were isolated from membrane fractions of rat gastric mucosa by sucrose gradient (5–40%) as described under “Experimental Procedures.” Western blotting was performed by using antibodies for KCC3, \( \alpha \)1NaK, and flotillin-2.

Previously, younger parietal cells in the luminal region of the glands have been reported to secrete acid more actively than do older parietal cells in the basal region (27–29). Interestingly, the present double immunostaining of KCC3a and AQP4 in rat gastric mucosa has shown clearly that KCC3a is predominantly expressed in the luminal parietal cells (Fig. 2M).

Association of KCC3a with \( \alpha \)1NaK in the Gastric Mucosa—To study whether KCC3a is associated with \( \alpha \)1NaK in rat gastric mucosa, immunoprecipitation was performed by using an anti-KCC3 antibody. The subsequent Western blotting of the immune pellets with an anti-\( \alpha \)1NaK antibody gave a clear band for \( \alpha \)1NaK (100 kDa), whereas that with an anti-H\textsuperscript{+},K\textsuperscript{+}-ATPase antibody gave no band for H\textsuperscript{+},K\textsuperscript{+}-ATPase (95 kDa) (Fig. 3A). These results suggest that KCC3a is associated with \( \alpha \)1NaK in gastric parietal cells.

Distribution of KCC3a and \( \alpha \)1NaK in Lipid Rafts in Rat Gastric Mucosa—Lipid rafts are known to be insoluble for treatment with nonionic detergents such as Triton X-100 and CHAPS at low temperature and form detergent resistance membrane (DRM) fractions with low density (9). In the present study, lipid rafts were isolated from rat gastric mucosa by using CHAPS and sucrose gradient. Flotillin-2 was used as a marker for lipid rafts (30, 31). As shown in Fig. 3B, most of KCC3a and \( \alpha \)1NaK were distributed in the DRM fractions, in which flotillin-2 was also expressed (Fig. 3B).

Tetracycline-regulated Stable Expression of KCC3a in LLC-PK1 Cells—Next, we constructed the tetracycline-regulated stable expression system of KCC3a in LLC-PK1 cells, a hog
kidney proximal tubular cell line (Fig. 4). No significant expression of endogenous KCC3a was observed in control LLC-PK1 cells (data not shown). In this heterologous expression system, exogenous expression of KCC3a protein was assessed by using an anti-Xpress antibody. Expression of KCC3a (180 kDa) was observed in the cells treated with tetracycline (Tet-on cells), whereas no significant expression of KCC3a was observed in the cells treated without tetracycline (Tet-off cells) (Fig. 4A). Both KCC3a and α1NaK were found to be present in the basolateral membrane of the Tet-on cells (Fig. 4, F and G). The expression pattern of α1NaK in the Tet-on cells was similar to that in the Tet-off cells (Fig. 4, D and E).

**Distribution of KCC3a and α1NaK in Lipid Rafts in the Heterologous Expression System**—Lipid rafts were isolated from the Tet-on and Tet-off cells (Fig. 5). In the Tet-off cells, 33.0 ± 4.1% of total α1NaK was in DRM fractions in which flotillin-2 was localized, and 67.0 ± 4.1% of total α1NaK was in non-DRM fractions (Fig. 5, A and C). In the Tet-on cells, KCC3a was predominantly distributed in the DRM fractions, and an increased amount of α1NaK (76.9 ± 2.1%) was localized in the DRM fractions (Fig. 5, B and C). These findings suggest that the presence of KCC3a induces a significant recruitment of α1NaK from non-DRM fractions into the DRM fractions. Furthermore, depletion of cholesterol with MβCD (10 mM) shifted the distributions of both KCC3a and α1NaK from the DRM fractions to the non-DRM fractions (Fig. 5, B and D).

**Up-regulation of Na⁺,K⁺-ATPase Activity by KCC3a**—To study whether KCC3a is associated with α1NaK in the Tet-on cells as is the case in the rat gastric mucosa (Fig. 3A), immunoprecipitation was performed by using an anti-KCC3 antibody.

**FIGURE 4. Tetracycline-regulated expression system of KCC3a in LLC-PK1 cells.** The tetracycline-regulated expression system of KCC3a was introduced to LLC-PK1 cells. The cells were treated with (Tet-on) or without (Tet-off) 2 μg/ml tetracycline. The expression of KCC3a was confirmed by Western blotting (A) and immunocytochemistry (B and C) using anti-Xpress antibody. In the cells, α1NaK was localized in the basolateral membrane (D, E, and G). In the Tet-on cells, KCC3a was localized in the basolateral membrane (F). Scale bars, 10 μm.

**FIGURE 5. Lipid raft analysis of the tetracycline-regulated expression system of KCC3a.** A and B, lipid raft fractions (DRM) and nonraft fractions (non-DRM) were isolated from membrane fractions of the LLC-PK1 cells (A, Tet-off cells; B, Tet-on cells) by sucrose gradient (5–40%) as described under “Experimental Procedures.” Western blotting was performed by using antibodies for KCC3a (anti-Xpress antibody), α1NaK, and flotillin-2. C, expression level of α1NaK in the DRM and non-DRM fractions in the Tet-on and Tet-off cells. Intensity of the 100-kDa band of α1NaK was measured by densitometry, and percentages of α1NaK in the DRM and non-DRM fractions relative to total amount of α1NaK in the membrane fractions were calculated. n = 3. ***, significantly different (p < 0.01). D, the membrane fractions of the Tet-on cells were treated with 10 mM MβCD for 30 min at 37 °C.
Up-regulation of Na\(^{+},K^{+}\)-ATPase by KCC3a

The subsequent Western blotting of the immune pellets with an anti-α1NaK antibody gave a single band for α1NaK (100 kDa) (Fig. 6A). The anti-α1NaK antibody gave no band in the immune pellets obtained with the rabbit IgG used as a negative control (Fig. 6A).

Next, we examined the functional relationship between KCC3a and α1NaK. Although the total expression level of α1NaK in the Tet-on cells was not significantly different from that in the Tet-off cells (Fig. 6B), the ouabain-sensitive K\(^{+}\)-ATPase activity (Na\(^{+},K^{+}\)-ATPase activity) in the Tet-on cells was significantly greater than that in the Tet-off cells (Fig. 6C).

Effects of KCC3a on H\(^{+},K^{+}\)-ATPase Activity—Here, we used HEK293 cells that stably express exogenous H\(^{+},K^{+}\)-ATPase α- and β-subunits (18). Additional exogenous expression of KCC3a changed neither the expression level of H\(^{+},K^{+}\)-ATPase nor the SCH 28080-sensitive K\(^{+}\)-ATPase activity (H\(^{+},K^{+}\)-ATPase activity) in the cells (supplemental Fig. 1).

Elevation of Phosphorylation Level of Na\(^{+},K^{+}\)-ATPase by KCC3a—Na\(^{+},K^{+}\)-ATPase belongs to a P-type ATPase family and forms phosphorylated intermediates (EP) in the catalytic cycle (32, 33). We studied whether the association of KCC3a with α1NaK affects the phosphorylation level of Na\(^{+},K^{+}\)-ATPase. In Fig. 7A, membrane fractions of the Tet-on cells were phosphorylated with [γ\(^{32}\)P]ATP under the different ionic conditions. The phosphorylated 100-kDa band was detected in the presence of 20 mM NaCl, and this band was diminished by the addition of 20 mM KCl. No phosphorylated band was observed in the presence of 20 mM KCl or 20 mM choline-Cl instead of 20 mM NaCl (Fig. 7A). The phosphorylated level of the 100-kDa band was elevated by increasing the NaCl concentration, the maximal phosphorylation being observed at 100 mM (Fig. 7B). Fig. 7C shows that phosphorylation level of the 100-kDa band in the Tet-on cells that express KCC3a is significantly greater than that in the Tet-off cells that do not express KCC3a. These results suggest that the phosphorylation level of α1NaK is affected by the expression of KCC3a.
Effects of DIOA, an Inhibitor of KCC, on Na⁺,K⁺-ATPase Activity—DIOA is known as a potent inhibitor of KCCs (IC₅₀ = 10 μM) (34). Although high concentrations of DIOA (>30 μM) suppressed Na⁺,K⁺-ATPase activity (IC₅₀ = 53–95 μM), no inhibitory effect of this drug on Na⁺,K⁺-ATPase was observed at a lower concentration of 10 μM (19). Here, DIOA (10 μM) significantly inhibited the Na⁺,K⁺-ATPase activity of rabbit gastric glands (Fig. 8A) in which KCC3a is expressed (Fig. 1B), although it had no significant effect on the Na⁺,K⁺-ATPase activity of rabbit kidney (Fig. 8B) in which KCC3b but not KCC3a is expressed (Fig. 1C). These results suggest that Na⁺,K⁺-ATPase activity is positively regulated by KCC3a in the gastric parietal cells. Furthermore, DIOA (10 μM) inhibited the KCC3a-increased Na⁺,K⁺-ATPase activity in the Tet-on cells, whereas it had no significant effects on the Na⁺,K⁺-ATPase activity in the Tet-off cells (Fig. 8C).

Ouabain-sensitive ²²Na⁺ Transport Activity—To estimate Na⁺ transport activity of Na⁺,K⁺-ATPase in the Tet-on and Tet-off cells, the ouabain-sensitive change in the intracellular ²²Na⁺ level was measured. Ouabain-sensitive ²²Na⁺ transport activity in the Tet-on cells was significantly greater than that in the Tet-off cells (Fig. 9). DIOA (10 μM) inhibited the KCC3a-increased Na⁺ transport activity in the Tet-on cells, although it had no significant effects on the Na⁺ transport activity in the Tet-off cells (Fig. 9).

Effect of KCC4 on Na⁺,K⁺-ATPase—Finally, we studied the effects of the expression of KCC4, which has 66% of amino acid

**FIGURE 9. Up-regulation of the ouabain-sensitive ²²Na⁺ transport activity by KCC3a and inhibition of the KCC3a-increased transport activity by DIOA.** Ouabain-sensitive ²²Na⁺ transport activity in the absence and presence of 10 μM DIOA were measured in the KCC3a-expressing cells (Tet-on) and the KCC3a nonexpressing cells (Tet-off). The ouabain-sensitive Na⁺ transport activity in the Tet-off cells in the absence of DIOA was calculated to be 4.92 ± 0.05 nmol/10⁵ cells/min (n = 5). The score for Tet-off cells in the absence of DIOA is normalized as 1 (open bar), n = 5, *p < 0.05; **p < 0.01; NS, not significant (p > 0.05).

**FIGURE 10. Up-regulation of Na⁺,K⁺-ATPase by KCC3a.** The tetracycline-regulated expression system of KCC4 was introduced into LLC-PK1 cells. The cells were treated with (Tet-on) or without (Tet-off) 2 μg/ml tetracycline. A, the expression of KCC4 was confirmed by Western blotting using anti-Xpress antibody. B and C, lipid raft fractions (DRM) and nonraft fractions (non-DRM) were isolated from membrane fractions of the KCC4 nonexpressing cells (Tet-off; B) and the KCC4-expressing cells (Tet-on; C) by sucrose gradient (5–40%). Western blotting was performed by using antibodies for KCC4 (anti-Xpress antibody), α1NaK, and flotillin-2. D, E, and F, expression level of α1NaK in the DRM and non-DRM fractions in the Tet-on and Tet-off cells. The intensity of the 100-kDa band of α1NaK (B and C) was measured by densitometry, and percentages of α1NaK in the DRM and non-DRM fractions relative to total amount of α1NaK in the membrane fractions were calculated. n = 3, NS, not significant (p > 0.05). **F, Na⁺,K⁺-ATPase activity in the Tet-on and Tet-off cells. The score for Tet-off cells is normalized as 1. n = 6, NS, not significant (p > 0.05).
Up-regulation of Na\textsuperscript{+},K\textsuperscript{+}-ATPase by KCC3a

The absence of KCC4 did not significantly affect the distribution of α1NaK and the Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity. For this purpose, the tetracycline-regulated expression system of KCC4 was constructed in LLC-PK1 cells. No significant expression of endogenous KCC4 was observed in control LLC-PK1 cells (data not shown). KCC4 expression (165 kDa) was observed in the cells treated with tetracycline (Tet-on cells), whereas no significant KCC4 expression was observed in the cells treated without tetracycline (Tet-off cells) (Fig. 10A). In the Tet-off cells, 35.8 ± 1.5% of total α1NaK was localized in the DRM fractions (Fig. 10, B and D). In the Tet-on cells, KCC4 was highly distributed in the DRM fraction as well as the case for KCC3a (Fig. 10C), and 29.8 ± 4.3% of total α1NaK was localized in the DRM fractions (Fig. 10, C and D). These findings indicate that the presence and absence of KCC4 did not significantly affect the distribution of α1NaK between the DRM and non-DRM fractions (Fig. 10D). Furthermore, both the total expression level and the activity of Na\textsuperscript{+},K\textsuperscript{+}-ATPase in the Tet-off cells were not significantly different from those in the Tet-off cells (Fig. 10, E and F), respectively. These results suggest that KCC4 is not functionally coupled to α1NaK.

**DISCUSSION**

In the present study, we have found following facts: 1) KCC3a, a longest form of KCC3, is expressed in the basolateral membrane of gastric parietal cells. Interestingly KCC3a is expressed in the luminal region of the glands and absent in the basal region of the glands. 2) KCC3a is associated with α1NaK, and both of them are highly localized in lipid rafts. 3) The ouabain-sensitive Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity in rabbit gastric mucosa is significantly inhibited by a KCC inhibitor (10 μM DIOA). 4) In LLC-PK1 cells, the exogenous expression of KCC3a recruits α1NaK into lipid rafts, accompanying increases of Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity and ouabain-sensitive Na\textsuperscript{+} transport activity, which are suppressed by DIOA. Furthermore, the expression of KCC3a affects the phosphorylation level of Na\textsuperscript{+},K\textsuperscript{+}-ATPase. But the expression of KCC4 does not recruit α1NaK into lipid rafts. 5) In HEK293 cells stably expressing exogenous H\textsuperscript{+},K\textsuperscript{+}-ATPase, H\textsuperscript{+},K\textsuperscript{+}-ATPase activity was not affected by additional expression of KCC3a.

It has been suggested that the luminal parietal cells more actively secrete acid than do the basal parietal cells, because the luminal parietal cells have more transcriptional synthesizing activities for H\textsuperscript{+},K\textsuperscript{+}-ATPase compared with the basal parietal cells (27, 28). For example, the luminal and basal parietal cells were assumed to secrete acid with pH of about 1 and 3, respectively, for the calculation of acid-activated quantity of gastric proton pump inhibitors (29). Furthermore, the secretory surface of luminal parietal cells was found to be greater than that of the basal parietal cells (35). Anion exchanger 2, a Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchanger, was reported to be highly expressed in the basolateral membrane of luminal parietal cells (6) as found here for KCC3a. Anion exchanger 2 is essential for gastric acid secretion in the parietal cells of rabbits (36) and mice (37) and for normal development of secretory canalicular and tubulovesicular membranes of mouse parietal cells (37). We have found that the role of KCC3a in the luminal parietal cells resides in recruitment of α1NaK into the lipid rafts.

In contrast, NKCC1 and AQP4 have been reported to be predominantly localized in the basolateral membrane of basal parietal cells (6, 26). In the mice lacking NKCC1 (7) or AQP4 (38), gastric acid secretion was found to be normal. Thus, downward migration of gastric parietal cells from the generating region to the basal region of the gland is associated with expression of NKCC1 and AQP4 along with diminution of KCC3a and anion exchanger 2, reflecting a process that is necessary for establishing different cell functions along the gastric glands. Previously, the parietal cells were proposed to undergo a programmed conversion of their principal function from acidic chloride (HCl) secretion to nonacidic chloride (NaCl or KCl) secretion on their migration (6, 8).

Furthermore, we have found that association of KCC3a with α1NaK in lipid rafts of LLC-PK1 cells increases Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity, the increment of which was inhibited by DIOA. These findings are interesting from the point of view that the ion transport by the secondary active transporter (KCC3a) up-regulates the primary active pump activity (α1NaK). There is another example that shows a functional coupling between KCC and Na\textsuperscript{+},K\textsuperscript{+}-ATPase in mouse brain, Na\textsuperscript{+},K\textsuperscript{+}-ATPase α2-subunit and neuron-specific KCC2 were reported to be coimmunoprecipitated in purified synaptic membrane and functionally coupled to exclude Cl\textsuperscript{−} from the cytosol in respiratory center neurons (39).

Recently, other examples have been reported that Na\textsuperscript{+},K\textsuperscript{+}-ATPase plays different roles depending on locations in membrane such as between caveolae and noncaveolae and between different types of lipid rafts. In LLC-PK1 cells, pumping and nonpumping Na\textsuperscript{+},K\textsuperscript{+}-ATPase are present. The nonpumping Na\textsuperscript{+},K\textsuperscript{+}-ATPase is associated with Src and/or extracellular signal-regulated kinase and present in caveolae of the cells (24, 40). Once ouabain binds to the nonpumping Na\textsuperscript{+},K\textsuperscript{+}-ATPase, Src is activated, and subsequent tyrosine phosphorylation of multiple effectors occurs (40). The pumping Na\textsuperscript{+},K\textsuperscript{+}-ATPase is present in noncaveolae domains (24, 40). Over half of Na\textsuperscript{+},K\textsuperscript{+}-ATPase is estimated to be nonpumping Na\textsuperscript{+},K\textsuperscript{+}-ATPase (24). Depletion of cholesterol with MβCD in the cells increases the activity of Na\textsuperscript{+},K\textsuperscript{+}-ATPase because of the conversion of the nonpumping pump into the pumping pump (41). In rainbow trout gill epithelium, Na\textsuperscript{+},K\textsuperscript{+}-ATPase in the lipid raft enriched in sulfogalactosylceramide (SGC-enriched raft) acts as a normal pump, whereas the non-SGC/raft-associated Na\textsuperscript{+},K\textsuperscript{+}-ATPase acts as a Na\textsuperscript{+} pump (12). These findings suggest that caveolae and lipid rafts are the microdomains that give multiple functions to Na\textsuperscript{+},K\textsuperscript{+}-ATPase in the basolateral membrane.

In conclusion, we have found that KCC3a is abundantly expressed in the basolateral membrane of gastric luminal parietal cells and up-regulates the α1NaK activity in lipid rafts. The disappearance of KCC3a in the basal parietal cells may indicate that KCC3a is involved in the morphological and functional transitions of gastric parietal cells, which occur when the cells migrate away from their generative region.

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K⁺-Cl⁻ Cotransporter-3a Up-regulates Na⁺,K⁺-ATPase in Lipid Rafts of Gastric Luminal Parietal Cells

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