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Role of cholesterol in functional association between K^+–Cl^- cotransporter-3a and Na^+,K^+-ATPase

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ABSTRACT

K^+–Cl^- cotransporter-3a (KCC3a) is associated with Na^+,K^+-ATPase α1-subunit (α1NaK) in lipid rafts of gastric acid-secreting cells and positively regulates Na^+,K^+-ATPase activity. Here, effects of cholesterol on association of KCC3a with α1NaK in lipid rafts were studied in LLC-PK1 cells stably expressing KCC3a. In the cells, lipid rafts destructed by methyl-β-cyclodextrin (MβCD) could be reconstructed by exogenous addition of cholesterol accompanying a shift of both KCC3a and α1NaK from non-rafts to rafts. The KCC3a-increased Na^+,K^+-ATPase activity was abolished by MβCD, and recovered by repletion of cholesterol without changing expression levels of KCC3a and α1NaK in the cells. KCC3a was co-immunoprecipitated with α1NaK even after destruction of lipid rafts by MβCD, indicating that molecular association of KCC3a with α1NaK still retains in the non-raft environment. Our results suggest that cholesterol is essential for eliciting up-regulation of Na^+,K^+-ATPase activity by KCC3a in the KCC3a–α1NaK complex.

1. Introduction

K^+–Cl^- cotransporter 3 (KCC3) mediates the electroneutral transport of Cl^- coupled to K^+ across the plasma membrane of cells. In genetic studies, KCC3 has been suggested to have critical roles in keeping homeostasis of neuronal and epithelial cells [1,2]. It has two major splicing variants (KCC3a and KCC3b). They are generated by transcriptional initiation 5′ of two distinct first coding exons [3,4]. A longer isoform KCC3a is expressed in several tissues such as brain, lung and stomach, while a shorter isoform KCC3b is predominantly expressed in kidney [3].

We found that KCC3a is co-expressed with Na^+,K^+-ATPase in lipid rafts of gastric acid-secreting cells (parietal cells) [5]. Interestingly, Na^+,K^+-ATPase activity was found to be positively regulated by KCC3a in rabbit and rat stomach [5,6]. In contrast, no positive regulation of Na^+,K^+-ATPase activity by KCC3b was observed in rabbit and rat kidney [5,6]. In LLC-PK1 cells, exogenously expressed KCC3a co-immunopreciptated with endogenous Na^+,K^+-ATPase α1-subunit (α1NaK) and up-regulated the Na^+,K^+-ATPase activity, while exogenously expressed KCC3b did not co-immunoprecipitate with endogenous α1NaK inducing no change of the Na^+,K^+-ATPase activity [6].

In this study, we investigated effects of cholesterol, one of major components of lipid rafts, on the KCC3a-increased Na^+,K^+-ATPase activity and molecular association between KCC3a and α1NaK.

2. Materials and methods

2.1. Chemicals

Anti-rat KCC3 rabbit polyclonal antibody was generated with KLH-coupled peptides against 19 amino acids corresponding to the N-terminus sequence of KCC3 (KKARNAYLNNSNYEEGDEY) [5]. Anti-α1NaK mouse monoclonal antibody (C464.6) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Lipofectamine 2000 and anti-Xpress mouse monoclonal antibody were from Invitrogen (Carlsbad, CA, USA). Horse-radish peroxidase-conjugated anti-mouse IgG was from Merck Millipore (Billerica, MA, USA). Ouabain, water-soluble cholesterol, methyl-β-cyclodextrin (MβCD) 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 (St. Louis, MO, USA). 3-[(3-cholamidopropyl)dimethyl-ammonio]propanesulfonic acid (CHAPS) was from Dojin Co. (Kumamoto, Japan). Protein A-agarose beads was from Pierce (Rockford, IL, USA). Hygromycin B and blasticidin S were obtained from Wako Pure Chemical Industries (Osaka, Japan) and Kaken Pharmaceutical Co. (Tokyo, Japan), respectively.

Abbreviations: KCC, K^+–Cl^- cotransporter; α1NaK, Na^+,K^+-ATPase α1-subunit; DIOA, R-(+)-[(2-*n*-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy]acetic acid; MβCD, methyl-β-cyclodextrin; CHAPS, 3-[(3-cholamidopropyl)dimethyl-ammonio]propanesulfonic acid; DRM, detergent resistant membrane; IP, immunoprecipitation; WB, Western blotting.

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2.2. Expression of KCC3a in LLC-PK1 cells

Full-length cDNAs encoding rat KCC3a was inserted into pcDNA5/TO vector (Invitrogen) by using AflII/NotI restriction site (KCC3a-pcDNA5/TO). To establish the tetracycline-regulated expression system of KCC3a in LLC-PK1 cells which is a hog kidney proximal cell line, the cells were co-transfected with pcDNA6/TR vector (Invitrogen) and KCC3a-pcDNA5/TO vector by using Lipofectamine 2000 and cultured for 24 h in DMEM supplemented with 10% fetal bovine serum. The transfected cells were selected in the presence of 800 U/ml hygromycin B and 5 μg/ml blasticidin S.

2.3. Western blotting

Preparation of membrane fractions and Western blotting were carried out as described previously [5–7]. Signals were visualized with the ECL Plus or ECL advance system (GE Healthcare, Buckinghamshire, UK). To quantify the chemiluminescence signals on the membranes, a FujiFilm's LAS-1000 system and the MultiGauge software were used. Anti-Xpress antibody was used at 1:5000 dilution and anti-α1NaK antibody was used at 1:3000 dilution. Horseradish peroxidase-conjugated anti-mouse IgG was used as a secondary antibody (1:5000 dilution).

2.4. Immunoprecipitation

Membrane fractions of KCC3a-expressing LLC-PK1 cells (2.5 mg of protein) were solubilized in the 500 μl of lysis buffer (PBS containing 0.5% Triton X-100, 0.1% BSA, 1 mM EDTA and 10 μg/ml aprotinin, 10 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin and 1 μg/ml pepstatin A) for 30 min on ice, and centrifuged at 90,000×*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. To quantify intensity of bands on the membranes, a FujiFilm's LAS-1000 system and the MultiGauge software were used.

2.5. Measurement of Na^+,K^+-ATPase activity

Na^+,K^+-ATPase activity of LLC-PK1 cells (30 μg of protein) was measured in a 1 ml of solution containing 120 mM NaCl, 15 mM KCl, 3 mM $MgSO_4$, 1 mM ATP, 5 mM NaN_3 and 40 mM Tris–HCl (pH 7.4), in the presence or absence of 100 μM ouabain. 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 inorganic phosphate released was measured [8]. The Na^+,K^+-ATPase activity was calculated as the difference between the activities in the presence and absence of ouabain.

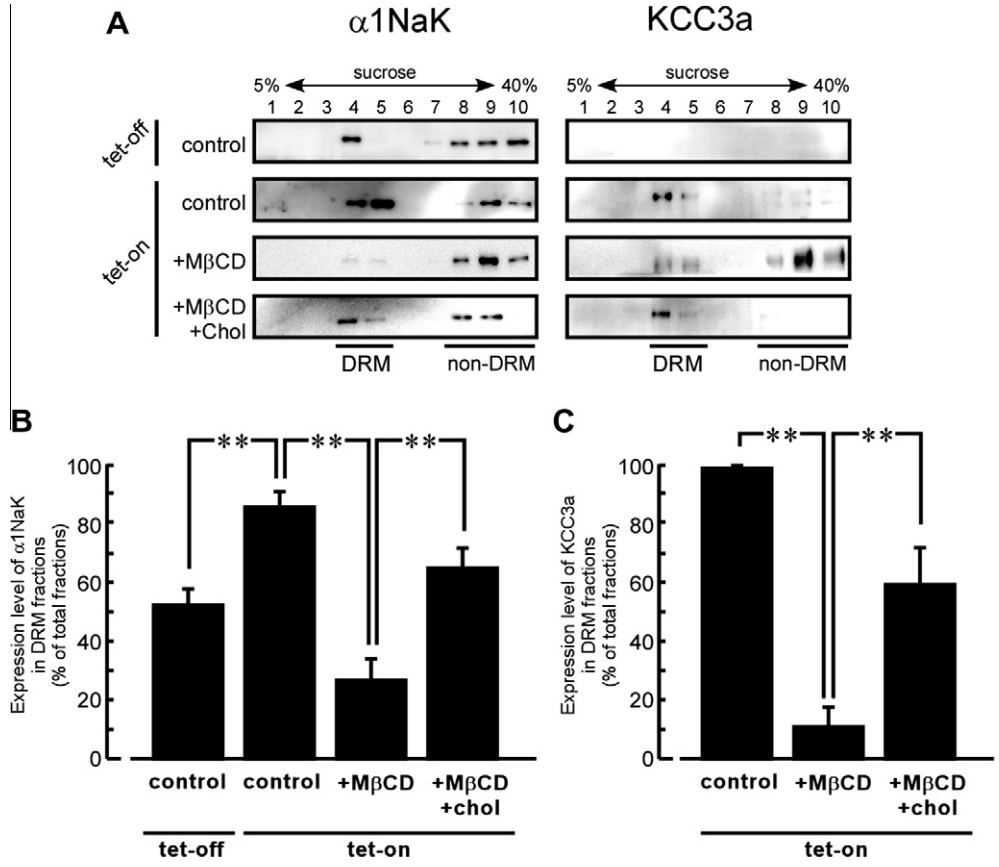

Fig. 1. Effects of MβCD and cholesterol on distribution of KCC3a and Na^+,K^+-ATPase (α1NaK) in lipid rafts. (A) The LLC-PK1 cells that were exogenously expressed KCC3a (tet-on) were treated with and without 10 mM MβCD at 37 °C for 30 min (+MβCD and control, respectively). After washing out of MβCD, the cells were treated with cholesterol (15 μg/ml) at 37 °C for 60 min (+MβCD + Chol). As control, the cells expressing no exogenous KCC3a (tet-off) were used. Lipid raft fractions (DRM) and non-raft fractions (non-DRM) were isolated from membrane fractions by sucrose gradient (5–40%) as described under Section 2. Western blotting was performed by using antibodies for KCC3a (anti-Xpress antibody) and α1NaK. (B and C) Expression level of α1NaK (B) and KCC3a (C) in the DRM fractions in the cells treated as described in (A). Intensity of the 100-kDa band of α1NaK and the 180-kDa band of KCC3a was measured by densitometry, and percentages of α1NaK and KCC3a in the DRM fraction relative to total amount of α1NaK and KCC3a in the membrane function were calculated. $n = 5$; $^{**}P < 0.01$.

2.6. Isolation of lipid rafts

Membrane proteins (200 μg) were lysed with the ice-cold MBS buffer (150 mM NaCl, 25 mM MES-NaOH, pH 6.5) containing 1% CHAPS, 10 μg/ml aprotinin, 10 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin and 1 μg/ml pepstatin A for 15 min. The solution was mixed with equal volume of 66% sucrose in MBS buffer, and 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×*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 Western blotting. The detergent resistant membrane (DRM) fractions (Fig. 1) were derived from lipid rafts [5]. For depletion of cholesterol, the cells were treated with 10 mM MβCD for 30 min at 37 °C before harvesting the cells. For repletion of cholesterol after the MβCD treatment, the cells were washed three times with DMEM to remove MβCD and then they were treated with 15 μg/ml cholesterol for 60 min at 37 °C.

2.7. Statistics

Results are shown as means ± SEM. Differences between groups were analyzed by one-way ANOVA, 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 $P < 0.05$.

3. Results

3.1. Effects of MβCD and cholesterol on distribution of KCC3a and α1NaK in lipid rafts

We found recently that exogenous expression of KCC3a up-regulates endogenous Na^+,K^+-ATPase activity in lipid rafts of LLC-PK1 cells [5,6]. Here, we examined the roles of cholesterol for the distribution of KCC3a and α1NaK in lipid rafts of the KCC3a-expressing LLC-PK1 cells (tet-on cells). No significant expression of endogenous KCC3a was observed in control LLC-PK1 cells (tet-off cells; Fig. 1A) as previously reported [5]. In the DRM fractions (corresponding to the lipid raft fraction) of the tet-off and tet-on cells, 52.9% and 85.9% of total α1NaK was present, respectively (Fig. 1A). Almost all KCC3a was present in DRM fractions of tet-on cells (Fig. 1A). Depletion of cholesterol by MβCD (10 mM) shifted a distribution of both KCC3a and α1NaK from DRM to non-DRM fractions (Fig. 1). After the MβCD treatment, cholesterol was exogenously added. Repletion of cholesterol recovered a distribution of both KCC3a and α1NaK in DRM fractions (Fig. 1).

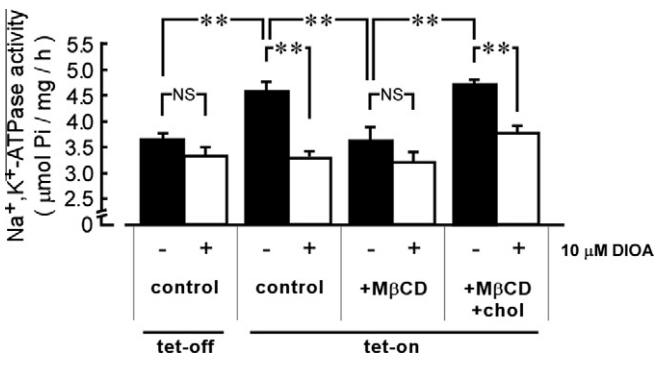

Fig. 2. Inhibition of the KCC3a-increased Na^+,K^+-ATPase activity by cholesterol depletion. The cells were treated as described in Fig. 1A. Na^+,K^+-ATPase activity in the absence and presence of 10 μM DIOA were measured in the membrane fraction of the cells. $n = 5$; $^{**}P < 0.01$; NS, not significant ($P > 0.05$).

3.2. Inhibition of the KCC3a-increased Na^+,K^+-ATPase activity by MβCD

Exogenous expression of KCC3a significantly increased the endogenous Na^+,K^+-ATPase activity and the increased activity was almost completely inhibited by DIOA, an inhibitor of KCC [9] (Fig. 2; control). Interestingly, depletion of cholesterol by MβCD significantly inhibited the KCC3a-increased and DIOA-sensitive Na^+,K^+-ATPase activity (Fig. 2; MβCD). Repletion of cholesterol after the MβCD treatment significantly rescued the Na^+,K^+-ATPase activity inhibited by MβCD (Fig. 2; MβCD + chol). Exogenous addition of cholesterol had no effects on the Na^+,K^+-ATPase activity of the cells that were not treated with MβCD ($n = 5$; data not shown). Expression levels of KCC3a and α1NaK in the cells were not significantly altered by treatment with MβCD and/or cholesterol (Fig. 3).

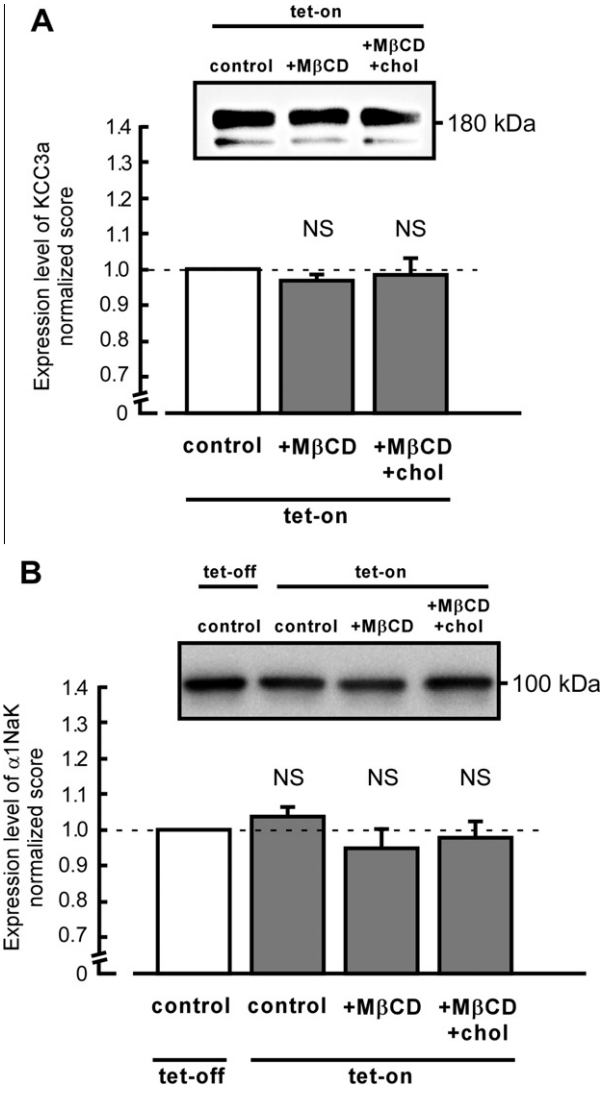

Fig. 3. No effects of MβCD and cholesterol on expression level of KCC3a and α1NaK in LLC-PK1 cells. (A and B) The cells were treated as described in Fig. 1A. Total expression level of KCC3a (A) and α1NaK (B) in the membrane fraction was assessed by Western blotting using antibodies for KCC3a (anti-Xpress antibody; A) and α1NaK (B). In insets, representative pictures of Western blotting are shown. The score for tet-on (A) and tet-off cells (B) treated without MβCD and cholesterol is normalized as 1. $n = 5$; NS, not significant ($P > 0.05$).

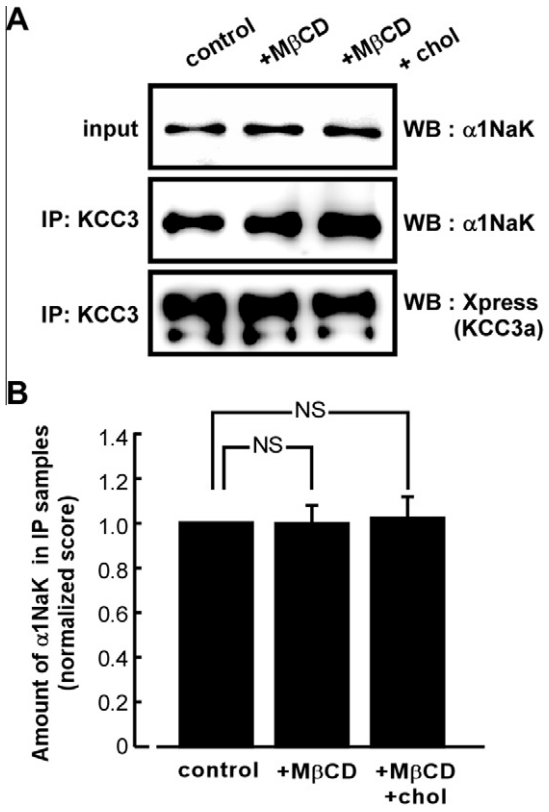

Fig. 4. Effects of MβCD and cholesterol on association of KCC3a and α1NaK. (A) The tet-on cells were treated with and without 10 mM MβCD at 37 °C for 30 min (+MβCD and control, respectively). After washing out of MβCD, the cells were treated with cholesterol (15 μg/ml) at 37 °C for 60 min (+MβCD + chol). Immunoprecipitation (IP) was performed with the detergent extracts of the cells by using anti-KCC3 antibody and protein A-agarose. The detergent extracts (1/750 of total protein; top panels) and immunoprecipitation samples (1/3 (middle panels) and 1/10 (bottom panels) of IP samples) were detected by Western blotting (WB) using anti-α1NaK antibody (top and middle panels) and anti-Xpress antibody (detecting for KCC3a; bottom panel). Typical example from eight similar experiments is shown. (B) Amount of α1NaK in the IP samples was quantified. In each case, the score was calculated using the following equation: Arbitrary score = (amount of α1NaK in the IP sample)/(amount of KCC3a in the IP sample). The score for control is normalized as 1. $n = 8$; NS, not significant ($P > 0.05$).

3.3. Effects of MβCD and cholesterol on association of KCC3a with α1NaK

To study whether association of KCC3a with α1NaK in the tet-on cells was affected by MβCD or MβCD plus cholesterol, immunoprecipitation was performed by using an anti-KCC3 antibody. The subsequent Western blotting of these immune pellets with an anti-α1NaK antibody gave a single band for α1NaK (100 kDa) in three cases (Fig. 4A). Amount of α1NaK in the immune pellets was not significantly decreased by the MβCD treatment (Fig. 4B), suggesting that cholesterol depletion did not destruct the KCC3a-α1NaK association. In control experiment, the anti-α1NaK antibody gave no band in the immune pellets obtained with the rabbit IgG used (not shown).

4. Discussion

Recently, we found that association of KCC3a with α1NaK in lipid rafts of LLC-PK1 cells increases Na^+,K^+-ATPase activity, the increment of which was inhibited by a KCC inhibitor (DIOA) [5,6].

In the present study, we focused on cholesterol, which is one of key components in lipid rafts. In the KCC3a-expressing cells, depletion of cholesterol by MβCD significantly inhibited the KCC3a-increased Na^+,K^+-ATPase activity in parallel with a shift of distribution of both KCC3a and α1NaK from the DRM to non-DRM fractions. Repletion of cholesterol after the MβCD treatment significantly rescued the MβCD-inhibited Na^+,K^+-ATPase activity accompanied by a redistribution of KCC3a and α1NaK in the DRM fraction, suggesting that the destructed lipid rafts generated by depletion of cholesterol can be functionally reconstructed by repletion of exogenous cholesterol.

Importantly, KCC3a was co-immunoprecipitated with α1NaK even after destruction of lipid rafts by MβCD, indicating that molecular association of KCC3a with α1NaK still retains in the non-raft environment. Our results suggest that cholesterol is essential for eliciting up-regulation of Na^+,K^+-ATPase activity by KCC3a in the KCC3a–α1NaK complex.

In LLC-PK1 cells, Liang et al. [10] reported that non-pumping Na^+,K^+-ATPase and pumping Na^+,K^+-ATPase are present in caveolae and non-caveolae, respectively, and that depletion of cholesterol moves some of the non-pumping Na^+,K^+-ATPase into the pumping pool and increases total Na^+,K^+-ATPase activity. Consisting with their results, we found that depletion of cholesterol by MβCD slightly but significantly increased Na^+,K^+-ATPase activity of the LLC-PK1 cells that did not express KCC3a (tet-off cells). (Supplementary Fig. 1). Exogenous expression of KCC3a recruited endogenous Na^+,K^+-ATPase (α1NaK) in lipid rafts of LLC-PK1 cells [5]. Therefore, cholesterol depletion may cause different effects on Na^+,K^+-ATPase alone and KCC3a–Na^+,K^+-ATPase complex. Furthermore, Chen et al. [11] reported that cholesterol depletion decreased expression level of α1NaK in LLC-PK1 cells that do not express KCC3a, whereas our present study showed that no change of α1NaK expression was induced by cholesterol depletion in the KCC3a-expressing LLC-PK1 cells.

Recently, it has been reported that α1NaK is associated with BK_{Ca} channel in lipid rafts of human melanoma IGR39 cells, and that treatment of the cells with MβCD eliminates this association [12]. In contrast, we found that KCC3a was associated with α1NaK even after treatment of the cells with MβCD, suggesting that cholesterol may be essential to maintain up-regulation of Na^+,K^+-ATPase activity by KCC3a, and that the KCC3a–α1NaK complex itself may be insufficient to generate the synergistic effect.

In gastric gland in stomach, younger parietal cells in the luminal region of the glands secretes more actively than do older parietal cells in the basal region [13,14]. Functional complex of KCC3a and α1NaK is present in younger parietal cells, suggesting it is involved in the mechanism of gastric acid secretion [5]. It would be an interesting subject to clarify difference of regulatory mechanism of the cholesterol content between younger and older parietal cells in a future study.

In conclusion, we have found that the cholesterol depletion does not affect molecular association between α1NaK and KCC3a, but destructs the functional association between them.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.06.089.

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