Activation of a polyvalent cation-sensing receptor decreases magnesium transport via claudin-16

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Abstract

Renal magnesium is mainly reabsorbed by a paracellular pathway in the thick ascending limb of Henle. The expression of claudin-16 increased magnesium transport in Madin–Darby canine kidney (MDCK) cells. Little is known about the regulatory mechanism of magnesium transport via claudin-16. Here we examined the effect of a polyvalent cation-sensing receptor (CaSR) on the intracellular distribution of and transport of magnesium by claudin-16. FLAG-tagged claudin-16 was stably expressed in MDCK cells using a Tet-OFF system. The activation of CaSR by magnesium, calcium, neomycin, and gadolinium did not affect the expression of FLAG-tagged claudin-16, CaSR, or ZO-1, a tight junctional scaffolding protein. These activators decreased the phosphoserine level of FLAG-tagged claudin-16 and the association of FLAG-tagged claudin-16 with ZO-1. The activation of CaSR induced a decrease in PKA activity. Immunofluorescence microscopy revealed that FLAG-tagged claudin-16 is distributed at the cell–cell border under unstimulated conditions, whereas it translocates to the intracellular compartment, mainly lysosome, with the activation of CaSR. In contrast, the distribution of ZO-1 was unaffected by the activation. The expression of FLAG-tagged claudin-16 increased transepithelial electrical resistance (TER) and transepithelial magnesium transport without affecting FITC-dextran (MW 4000) flux. The activation of CaSR decreased TER and magnesium transport, which were recovered by co-treatment with dibutyryl cAMP, a membrane-permeable cAMP analogue. Taken together, CaSR activation may decrease PKA activity, resulting in a decrease in phosphorylated claudin-16, the translocation of claudin-16 to lysosome and a decrease in magnesium reabsorption.

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1. Introduction

Serum magnesium levels are kept within a narrow range. Magnesium homeostasis is controlled by the balance between intestinal absorption and renal excretion. Under physiological conditions, a decrease in magnesium intake is supplemented by enhanced magnesium absorption in the intestine and reduced excretion in the kidney. Renal magnesium filtered in the glomeruli is mainly reabsorbed in the thick ascending limb (TAL) of Henle [1]. In this segment, magnesium is passively transported from the lumen to the interstitial space through the paracellular pathway, driven by the lumen-positive transepithelial voltage [2,3].

A primary barrier to the passage of solutes through the paracellular pathway is formed by the tight junction (TJ) complex. The permeability to ions via the paracellular pathway may be controlled by tight junctional proteins [4]. The TJ complex is composed of various membrane integral proteins, scaffolding proteins, and cytoskeletal proteins. The membrane integral proteins include occludin, a 65 kDa protein bearing four transmembrane domains [5], and claudins, 20–28 kDa proteins that share a common topology with occludin [6]. Claudins
consist of a family of at least 24 homologous isoforms sharing several essential features [7,8]. The carboxyl terminus of most claudins has a PDZ-binding motif that can interact with the PDZ domains of scaffolding proteins ZO-1, ZO-2, and ZO-3 [9,10]. Different claudins can exist within the same junction where they interact in a homophilic or heterophilic manner [11–13]. By changing the combination of claudins in each tissue, one may show the variation in paracellular permeability.

Claudin-16 (CLDN-16) is exclusively expressed in the TAL where most of the magnesium is reabsorbed. Using a positional cloning approach, Simon et al. [14] found mutations in the CLDN-16 gene in cases of familial hypomagnesemia with hypercalciumia and nephrocalcinosis. Therefore, CLDN-16 seems to function as a paracellular magnesium transport pathway. At present, little is known about the regulatory mechanism of the expression and intracellular distribution of CLDN-16. Numerous mutants of CLDN-16, which are identified in FHHNC, induce a mistargeting of CLDN-16 to the TJ [15,16]. We recently reported that phosphorylation by a cAMP/PKA-dependent pathway is necessary for CLDN-16 to distribute to the

Fig. 1. Inducible expression of FLAG-tagged CLDN-16 in the MDCK Tet-OFF cells. Cells were cultured in the presence (+) or absence (−) of 100 ng/ml doxycycline. The cell lysates were separated by SDS-PAGE, followed by immunoblotting with anti-FLAG, anti-ZO-1, anti-CaSR or anti-actin antibody.

Fig. 2. Decrease of phosphorylated CLDN-16 by CaSR agonists. The cells expressing the FLAG-tagged CLDN-16 were incubated with CaSR agonists including MgCl2 (5 and 10 mM), CaCl2 (5 and 10 mM), neomycin (0.1 and 0.5 mM), and GdCl3 (0.1 and 0.5 mM) for 1.5 h. (A) The total cell lysate was separated by SDS-PAGE, followed by immunoblotting with anti-FLAG, anti-ZO-1, anti-CaSR or anti-actin antibody. (B) The total cell lysates were immunoprecipitated with polyclonal anti-FLAG antibody. Then, the immune pellets were immunoblotted with anti-phosphoserine (P-Ser), anti-ZO-1, or monoclonal anti-FLAG antibody.
TJ [17]. To control permeability to magnesium, the incorporation of CLDN-16 into the TJ would be a key regulatory step. A calcium-sensing receptor (CaSR) is expressed in the renal tubules and senses changes in extracellular calcium and magnesium levels [18]. In cases of familial hypocalciuric hypercalcemia (FHH), an inherited disorder with mutations in the CaSR gene, individuals exhibit low urinary calcium excretion even if their plasma calcium concentration is high [19]. Hebert et al. [20] proposed that the elevation of plasma calcium and magnesium concentrations induces the activation of CaSR followed by a reduction in the reabsorption of NaCl in the TAL. This in turn decreases the lumen-positive potential that is the driving force for calcium and magnesium transport. However, it is unknown whether the activation of CaSR directly affects the expression and function of CLDN-16.

In the present study, we investigated the effect of CaSR agonists on the intracellular distribution of and transport of magnesium by CLDN-16 using Madin–Darby canine kidney (MDCK) Tet-OFF cells. We found that the agonists decrease the level of phosphorylated CLDN-16 and induce the translocation of CLDN-16 from the TJ to intracellular compartments, in particular to lysosome. The transepithelial transport of magnesium from the apical-to-basal compartments was decreased by the agonists. We suggest that the activation of CaSR decreases the distribution of CLDN-16 in the TJ and is partially involved in the down-regulation of magnesium reabsorption in the TAL.

2. Materials and methods

2.1. Materials

Monoclonal mouse anti-FLAG and monoclonal anti-phosphoserine antibodies were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Polyclonal rabbit anti-FLAG was from Medical & Biological Laboratories (Aichi, Japan). Polyclonal goat anti-actin and polyclonal rabbit anti-ZO-1 antibodies, and ExactaCruz C were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other reagents were of the highest grade of purity available.

2.2. Cell culture and transfection

The MDCK (type II) Tet-OFF cell line was obtained from BD Biosciences Clontech (Franklin Lakes, NJ, USA). FLAG-tagged CLDN-16 was stably expressed in the MDCK Tet-OFF cells [21]. Cells were grown in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich) supplemented with 5% fetal calf serum (HyClone, Logan, UT, USA), 0.07 mg/ml penicillin-G potassium, 0.14 mg/ml streptomycin sulfate, 0.1 mg/ml G418, and 0.1 mg/ml hygromycin B in a 5% CO2 atmosphere at 37 °C. The expression of FLAG-tagged CLDN-16 was induced by adding 0.1 μg/ml doxycycline.

2.3. Preparation of membrane fraction and immunoprecipitation

Confluent MDCK cells were scraped into cold PBS and precipitated by centrifugation. Then, the cells were lysed in a RIPA buffer containing 150 mM NaCl, 0.5 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 50 mM Tris–HCl (pH 8.0), a protease inhibitor cocktail (Sigma-Aldrich), 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate, and sonicated for 20 s. After centrifugation at 6000 g for 5 min, the supernatant was collected (total cell lysate). The total cell lysate (500 μg) was incubated with polyclonal anti-FLAG antibody and ExactaCruz B for 12–16 h at 4 °C with gentle rocking. After centrifugation at 5000 g for 1 h, the pellet was washed three times with the RIPA buffer. The total cell lysate and immunoprecipitate were solubilized in a sample buffer for SDS-polyacrylamide gel electrophoresis. Protein concentrations were measured using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin as the standard.

2.4. SDS-polyacrylamide gel electrophoresis and immunoblotting

SDS-polyacrylamide gel electrophoresis was carried out as described previously [22]. In brief, the total cell lysate (30 μg) or immunoprecipitate was applied to the SDS-polyacrylamide gel. Proteins were blotted onto a PVDF membrane and incubated with each primary antibody for 16 h at 4 °C followed by a peroxidase-conjugated secondary antibody for 1 h at room temperature. Finally, the blots were stained with an ECL Western blotting kit from GE Healthcare UK Ltd. (Amersham Place, England).

2.5. Confocal microscopy

MDCK cells expressing the FLAG-tagged CLDN-16 were plated at a confluent density on cover glasses and, if necessary, were preincubated with lysisotracker for 30 min at 37 °C. Immunofluorescence microscopy was performed as described previously [23]. Immunolabeled cells were visualized on an LSM 510 confocal microscope (Carl Zeiss, Germany) set with a filter appropriate for Texas Red (543 nm excitation, 585–615 nm emission), and FITC (488 nm excitation, 530 nm emission). Images were further processed using Adobe Photoshop (Adobe System, San Jose, CA, USA).

2.6. Measurement of PKA activity

PKA activity was assessed using a Kinase Activity Assay Kit for PKA (BioSource, Nivelles, Belgium). MDCK cells expressing the FLAG-tagged CLDN-16 were lysed in Omnia Cell Extraction Buffer. After centrifugation at 16,000 g for 30 min, the supernatant was used for the assay. PKA activity was determined following the manufacturer’s instructions. The fluorescence intensity was measured with excitation at 360 nm and emission at 485 nm using a Multilabel Counter 1420 ARVOxs (Perkin Elmer, Wellesley, MA, USA). Data were represented as relative fluorescence intensity.

2.7. Measurement of paracellular permeability

MDCK cells expressing the FLAG-tagged CLDN-16 were plated at a confluent density on Transwells with polyester membrane inserts (6.5 or 12 mm...
diameter, 0.4 μm-pore size, Corning Incorporated-Life Sciences, Acton, MA, USA). TER was measured using a Millicell-ERS epithelial volt-ohmmeter (Millipore, Billerica, MA, USA). TER values (ohms×cm²) were normalized based on the area of the monolayer and were calculated by subtracting the blank values from the filter and the bathing medium. The paracellular diffusion of FITC-dextran (4000 Da) for 1 h from the apical-to-basal compartments was measured with a Multilabel Counter 1420 ARVOsx. Transepithelial transport of Mg²⁺ from the apical-to-basal compartments was measured using xylidyl blue-I (XB-I) as described previously[17].

2.8. Statistics

Results are presented as means±S.E.M. Differences between groups were analyzed with a one-way analysis of variance, and corrections for multiple comparison were made using Tukey’s multiple comparison test. Significant differences were assumed at P<0.05.

3. Results

3.1. Endogenous expression of CaSR in MDCK Tet-OFF cells

We have generated stable cell lines expressing FLAG-tagged CLDN-16 by using a tetracycline-inducible system[21]. The clones show strong transgene expression in the absence of doxycycline. The induction of CLDN-16 expression had no obvious effect on the endogenous expression of ZO-1, CaSR, or actin (Fig. 1).

3.2. Effects of CaSR agonists on the phosphorylation of CLDN-16

The activation of CaSR induces a modulation of cellular signaling thereby affecting hormone secretion and renal
Electrolyte handling [20]. CaSR is responsive not only to calcium and magnesium but also to neomycin and gadolinium. In MDCK cells expressing FLAG-tagged CLDN-16, CaSR agonists affected neither the endogenous expression of CaSR and ZO-1 nor the exogenous expression of FLAG-tagged CLDN-16 (Fig. 2A). We recently reported that CLDN-16 is phosphorylated at serine residue via a cAMP/PKA-dependent pathway in the presence of serum [21]. CaSR agonists decreased the level of phosphorylated CLDN-16 (Fig. 2B). Furthermore, these agonists decreased the association of CLDN-16 with ZO-1. These findings are similar to the results obtained with a PKA inhibitor [17]. ZO-1 binds directly to the PDZ-binding motif of claudins and connects them to the underlying actin cytoskeleton. By the activation of CaSR, CLDN-16 appears to dissociate from the TJ complex. The activation reduced production of intracellular cAMP mediated by the inhibition of adenylate cyclase in the TAL dissected from the outer medulla of the mouse kidney [24]. We suggest that CaSR agonists decreased PKA activity, resulting in the decrease in level of phosphorylated CLDN-16.

3.3. Translocation of CLDN-16 from TJ to lysosome by CaSR agonists

In unstimulated cells, FLAG-tagged CLDN-16 was distributed in the TJ concomitant with ZO-1 (Fig. 4). CaSR agonists partially induced the translocation of CLDN-16 from the TJ to intracellular compartments. In contrast, the distribution of ZO-1 was not changed by CaSR agonists. Next, we performed a double immunofluorescence analysis of CLDN-16 and a lysosome marker. In unstimulated cells, FLAG-tagged CLDN-16 did not overlap with lysotracker (Fig. 4). Conversely, FLAG-tagged CLDN-16 was colocalized with lysotracker in the agonist-treated cells (Fig. 5).

3.4. Decrease of TER and Mg$^{2+}$ transport by CaSR agonists

As shown in Figs. 4 and 5, CaSR agonists caused the translocation of CLDN-16 from the TJ to lysosome. We examined the effect of the agonists on TER, the flux of FITC-dextran, and the transepithelial transport of Mg$^{2+}$ from the apical-to-basal compartments. Removal of doxycycline induced an increase in the TER and transepithelial transport (Fig. 6A and C). In contrast, the flux of FITC-dextran from the apical-to-basal compartments was unchanged by doxycycline (Fig. 6B). CaSR agonists significantly decreased the TER and transepithelial Mg$^{2+}$ transport. In contrast, the flux of FITC-dextran was unchanged by the agonists. These results suggest that CaSR agonists induced the translocation of CLDN-16 from the TJ to lysosome, resulting in the loss of function.

3.5. Loss of CaSR-induced inhibitory effect by dibutyryl cAMP

We reported that CLDN-16 is phosphorylated by a cAMP/PKA-dependent pathway [17]. As shown in Fig. 3, CaSR agonists decreased PKA activity. Therefore, it is suggested that CaSR agonists reduced intracellular cAMP content. The reduction in the phosphorylation of CLDN-16 by neomycin and gadolinium was recovered by dibutyryl cAMP (DBcAMP), a membrane-permeable cAMP analogue (Fig. 7A). Immunofluorescence microscopy showed that CLDN-16 was distributed in the TJ concomitant with ZO-1 in the cells treated with DBcAMP and CaSR agonists (Fig. 7B). Furthermore, the decrease in TER and transepithelial Mg$^{2+}$ transport from the apical-to-basal compartments caused by CaSR agonists was completely reversed by DBcAMP (Fig. 7C and D). These results suggested that CaSR agonists negatively regulate the cAMP/PKA-dependent pathway.

4. Discussion

Different claudins can exist within the same junction and make ion-selective pores. The exogenous expression of a single claudin influences paracellular permeability. Claudin-4 or -14 expression reduces permeability to sodium in MDCK II cells [25]. Claudin-8 expression reduces permeability to not only monovalent alkali metal cations but also H$, \text{NH}_4^+$, and HCO$_3^-$ [26,27]. By contrast, claudin-2 or -15 expression increases the permeability to sodium [25]. These differences of ionic charge selectivity may be due to the charges on the first
extracellular loop. In the first extracellular loop of CLDN-16, there are ten negatively charged amino acids [14]. The abundant negative charges may recognize divalent cations and CLDN-16 enhances the transport of calcium and magnesium. Some mutants with negatively charged amino acids in the first extracellular loop, which are observed in FHHNC, lost the tight junctional localization and the function to transport magnesium [15,16]. To control permeability to magnesium, the incorporation of CLDN-16 into the TJ may be a key regulatory step.

Little is known about how the intracellular distribution of claudins is regulated. Several reports suggest that the function and/or localization of claudins is regulated by its phosphorylation. Mutant WNK4 phosphorylates the cytosolic carboxyl terminus of claudin-4 and increases permeability to chloride [28]. The localization and the solubility in Triton X-100 of claudin-4 were not affected by mutant WNK4, suggesting that the increase in permeability to chloride with mutant WNK4 expression was not caused by the change in the translocation of claudin-4. Similarly, claudin-7 is a substrate of WNK4 and phosphorylation of claudin-7 increases its permeability to chloride [29].

The carboxyl terminus of claudin-3 is phosphorylated by PKA in ovarian cells [30]. WT and T192A mutant claudin-3 are localized in the intercellular junction, whereas T192D mutant claudin-3, mimicking the phosphorylated state, is diffused from the TJ. In contrast, the phosphorylation of claudin-5 by PKA increases its localization to the TJ in endothelial cells [31]. Similarly, PKA phosphorylates CLDN-16 and increases its localization to the TJ in MDCK cells [17]. WT CLDN-16 treated with a PKA inhibitor and S217A mutant CLDN-16, a non-phosphorylated form, is dissociated from the TJ and translocated to lysosome. The effect of phosphorylation on the intracellular distribution of claudins may be different in each claudin.

In the isolated perfused cortical TAL, the transport of magnesium is passive and dependent on the transepithelial voltage [2,3]. Secretion of magnesium and calcium into the lumen increases under conditions of a negative transepithelial dilution potential, whereas reabsorption increases with a positive transepithelial dilution potential. We recently reported that the transport of calcium is also regulated by the transepithelial voltage in the MDCK cells [23]. Interestingly, reabsorption of calcium was enhanced by the expression of CLDN-16, whereas secretion was not affected by the expression. We hypothesized that the high concentration of calcium in the bath solution inhibited the excretion of calcium via CLDN-16. In the present study, we found that the activation of CaSR decreases the level of phosphorylated CLDN-16. Furthermore, the activation decreased the association of CLDN-16 with ZO-1 and induced the translocation of CLDN-16 from the TJ to lysosome, thereby reducing the transepithelial transport of magnesium. So far, Müller et al. [32] reported that mutations in CLDN-16 that loss association with ZO-1 lead to lysosomal mistargeting. Therefore, it must be necessary that CLDN-16 associates with ZO-1 to localize at the TJ. At present, we do not know how phosphorylation of CLDN-16 may affect its association with ZO-1.

We reported that dephosphorylated CLDN-16 is ubiquitinated [17]. Ubiquitinated proteins in plasma membrane are internalized from the cell surface and targeted to lysosome [33,34]. The

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**Fig. 7.** Loss of CaSR-induced inhibitory effect by dibutyryl cAMP. The cells expressing the FLAG-tagged CLDN-16 were incubated with CaSR agonists including 0.5 mM neomycin and 0.5 mM GdCl₃ for 1.5 h in the presence and absence of 0.5 mM DBcAMP. (A) The total cell lysates were immunoprecipitated with polyclonal anti-FLAG antibody. Then, the immune pellets were immunoblotted with anti-phosphoserine (P-Ser) or monoclonal anti-FLAG antibody. (B) Cells were double stained with anti-FLAG (green) and anti-ZO-1 antibodies (red). The co-localization of FLAG-tagged CLDN-16 and ZO-1 appears yellow in the merged images. The scale bar represents 10 μm. TER (C) and the transport of Mg²⁺ from the apical-to-basal compartments (D) were measured after treatment with 0.5 mM neomycin or 0.5 mM GdCl₃ for 1.5 h in the presence of 0.5 mM DBcAMP. NS, not significantly different (P>0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
ubiquitinated CLDN-16 might be pulled out of the membrane, thereby CDLN-16 loses its association with ZO-1.

CaSR is distributed along the nephron from the proximal tubule to the collecting duct, being particularly abundant in the basolateral membrane of the TAL. It has been proposed that CaSR could function as the physiological receptor for calcium and magnesium. CaSR is a G protein-coupled receptor, and its activation leads to two cell signaling pathways [20]. Coupling to a Gq/11 protein activates phospholipase C, resulting in an elevation of the intracellular free Ca\(^{2+}\) concentration and the activation of protein kinase C. The activation of CaSR leads to a reduction in the intracellular cAMP content. This might occur via coupling to a pertussis toxin-sensitive Gi protein, which inhibits adenylate cyclase, or via an indirect mechanism involving arachidonic acid metabolites. At present, we do not know what molecule is involved in the activation of adenylate cyclase, but our recent findings showed that serum factors influence the phosphorylation of CLDN-16. Furthermore, 2′,5′-dideoxy adenosine, an adenylate inhibitor, decreased the phosphorylation of CLDN-16, indicating that CLDN-16 is phosphorylated by serum factors via the activation of adenylate cyclase [21]. Interestingly, the activation of CaSR induces a reduction in PKA activity and phosphorylated CLDN-16. DBcAMP abolished the translocation of CLDN-16 into lysosome and the reduction in the transepithelial transport of magnesium caused by CaSR agonists. We suggest that the activation of CaSR decreases renal magnesium reabsorption in two ways: (1) via a decrease in the lumen’s positive potential [35] and (2) via the internalization of CLDN-16 from the TJ as shown in the present studies. The CLDN-16 expression affects permeability to sodium in MDCK cells [17] and LLC-PK\(_1\) cells [15]. Therefore, CLDN-16 might be also involved in the regulation of the transepithelial voltage.

In conclusion, we found that the phosphorylation and distribution of CLDN-16 in the TJ are negatively regulated by CaSR agonists. Elevated concentrations of divalent cations in the TAL caused the translocation of CLDN-16 from the TJ to lysosome, resulting in a reduction in the amount of magnesium reabsorption. This regulatory mechanism might prevent excess reabsorption of divalent cations and back-flow from the peritubular space to tubule lumen.

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