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Cholesterol Stimulates the Transient Receptor Potential Melastatin 4 Channel in mpkCCDc14 Cells

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We have shown that cholesterol regulates the activity of ion channels in mouse cortical collecting duct (CCD) mpkCCDc14 cells and that the transient receptor potential melastatin 4 (TRPM4) channel is expressed in these cells. However, whether TRPM4 channel is regulated by cholesterol remains unclear. Here, we performed inside-out patch-clamp experiments and found that inhibition of cholesterol biosynthesis by lovastatin significantly decreased, whereas enrichment of cholesterol with exogenous cholesterol significantly increased, TRPM4 channel open probability (Po) by regulating its sensitivity to Ca2+ in mpkCCDc14 cells. In addition, inside-out patch-clamp data show that acute depletion of cholesterol in the membrane inner leaflet by methyl-β-cyclodextrin (MβCD) significantly reduced TRPM4 Po, which was reversed by exogenous cholesterol. Moreover, immunofluorescence microscopy, Western blot, cell-surface biotinylation, and patch clamp analysis show that neither inhibition of intracellular cholesterol biosynthesis with lovastatin nor application of exogenous cholesterol had effect on TRPM4 channel protein abundance in the plasma membrane of mpkCCDc14 cells. Sucrose density gradient centrifugation studies demonstrate that TRPM4 was mainly located in cholesterol-rich lipid rafts. Lipid-protein overlay experiments show that TRPM4 directly interacted with several anionic phospholipids, including PI(4,5)P2. Depletion of PI(4,5)P2 with either wortmannin or PGE2 abrogated the stimulatory effects of exogenous cholesterol on TRPM4 activity, whereas exogenous PI(4,5)P2 (diC8-PI(4,5)P2, a water-soluble analog) increased the effects. These results suggest that cholesterol stimulates TRPM4 via a PI(4,5)P2-dependent mechanism.

Keywords: TRPM4, Cholesterol, PI(4,5)P2, Lipid rafts, Lovastatin

Abbreviations: TRPM4, transient receptor potential melastatin 4; CCD, cortical collecting duct; MβCD, methyl-β-cyclodextrin; ENaC, epithelial sodium channel; NSCCa, Ca2+-activated nonslective cation current; ROMK, renal outer medullary K+ channel; CsA, cyclosporine A.
INTRODUCTION

The transient receptor potential melastatin 4 (TRPM4) channel is activated by an increase in intracellular Ca\(^{2+}\), which is permeable equally to Na\(^+\) and K\(^+\) (Wu et al., 2016; Ding et al., 2017). The channel has a relatively broad tissue expression pattern and its dysregulation is implicated in numerous diseases (Abriel et al., 2012). Recent studies have shown that TRPM4 mutations are associated with isolated cardiac conduction disease, right bundle-branch block, tachycardia, and Brugada syndrome (Kruse et al., 2009; Liu et al., 2010; Liu H. et al., 2013). Our previous study has shown that high salt diet-induced TRPM4 expression contributes to early stage endothelial damage in Dahl salt-sensitive hypertensive rat (Ding et al., 2017). We have also shown that TRPM4 channel is responsible for a Ca\(^{2+}\)-activated nonselective cation current (NSCCa) in CCD principal cells (Wu et al., 2016). Therefore, investigation of the regulation of TRPM4 would provide important information for many cellular functions which mediated by intracellular calcium.

Cholesterol, a major sterol in the mammalian plasma membrane, modulates the function of various ion channels (Levitan et al., 2010). Statins are potent inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase, the rate-limiting enzyme in the synthesis of cholesterol. We have shown that inhibition of cholesterol synthesis with lovastatin reduces the activity of epithelial sodium channel (ENaC) and that enrichment of cholesterol enhances activity of ENaC (Wei et al., 2007; Wang et al., 2009; Zhai et al., 2018). In contrast, inhibition of cholesterol synthesis with lovastatin stimulates the renal outer medullary K+ channel (ROMK) in mpkCCDc14 cells (Liu et al., 2015) and inwardly rectifying K+ channels in CHO cells (Romanenko et al., 2009). In addition, we have shown that lovastatin even antagonizes cyclosporine A (CsA)-induced cell apoptosis by reducing cholesterol synthesis in renal epithelial cells (Liu B. C. et al., 2013). However, it remains unclear how cholesterol regulates TRPM4 channels.

The cell membrane contains specialized microdomains referred to as lipid rafts which are enriched in cholesterol and sphingolipids. In lipid rafts, PI(4,5)P2 is thought to be localized in lipid rafts in mpkCCDc14 cells. We also show that enrichment of membrane cholesterol increases, whereas depletion of cholesterol by lovastatin decreases, TRPM4 activity by regulating its sensitivity to Ca\(^{2+}\) in mpkCCDc14 cells. Our results suggest that plasma membrane cholesterol stimulates TRPM4 via a PI(4,5)P2 dependent mechanism.

METHODS

Cell Culture

The mpkCCDc14 line is an immortalized mouse collecting duct principal cell line, which was cultured as described previously (Bens et al., 1999). These cells were cultured in a 1:1 mixture of DMEM and Ham’s F-12 medium (GIBCO) supplemented with 20 mM HEPES, 2 mM L-glutamine, 50 nM dexamethasone, 1 nm triiodothyronine, 2% heat-inactivated FBS, and 0.1% penicillin-streptomycin. The mpkCCDc14 cells were plated at a density of 75,000 cells·cm\(^{-2}\) and grown on permeable supports to maintain cell polarization (Costar Transwells; 0.4 µm pore, 24 mm diameter) and cultured for at least 7 days prior to the experiments.

Cell-Surface Biotinylation and Western Blot Assay

Biotinylation of the plasma membrane from mpkCCDc14 cells was performed as described previously (Wu et al., 2016). Briefly, after each treatment, the cells were incubated with a freshly prepared solution of 1.0 mg/ml EZ-Link sulfo-N-hydroxysuccinimide disulfide-biotin (Pierce, 21331) in borate buffer for 30 min at 4°C. The biotin reaction was quenched for 5 min with 0.1 mM lysine. An equal amount of lysate protein (1 mg) from each sample was respectively incubated with 50 µl of 1.0 mg/ml EZ-Link sulfo-N-hydroxysuccinimide disulfide-biotin and washed four times with RIPA buffer. Equal amounts of samples from either whole-cell or biotinylated plasma membranes were loaded and separated by a 10% SDS-polyacrylamide gel and transferred to polyvinylidene fluoride membranes. The membranes were then blocked in 5% non-fat dry milk for 1 h, followed by incubation with rabbit polyclonal anti-TRPM4 antibody (1:200 dilution; Alomone Labs; ACC-044) at 4°C for overnight with gentle shaking. The beads were washed four times with RIPA buffer. Equal amounts of samples from either whole-cell or biotinylated plasma membranes were loaded and separated by a 10% SDS-polyacrylamide gel and transferred to polyvinylidene fluoride membranes. The membranes were then blocked in 5% non-fat dry milk for 1 h, followed by incubation with rabbit polyclonal anti-TRPM4 antibody (1:200 dilution; Alomone Labs; ACC-044) at 4°C for overnight. Rabbit polyclonal anti-GAPDH (1:1000 dilution; Santa cruz; sc-25778) was used as internal controls. Bands were visualized with enhanced chemiluminescence (Bio-Rad, Cat. No., 170-5061) and quantified via densitometry using the ImageJ software (NIH ImageJ software).
**Sucrose Gradient Assay**

Lipid raft fractionation was isolated as described previously (Liu et al., 2015). Briefly, mpkCCD14 cells suspension were homogenized in 0.5% Brij 96V (Sigma)/TNEV buffer (10 mM Tris-HCl, pH 7.5; 150 mM NaCl; 5 mM EDTA; 2 mM Na vanadate; and protease inhibitor cocktail) on ice for 30 min. The supernatant (500 μl) was mixed with an equal volume of 80% sucrose in TNEV and transferred into a centrifuge tube (13 × 51 mm; Beckman Coulter, Palatine, IL, United States). Three milliliters of 35% sucrose in TNEV was carefully layered on top of the mixture, followed by another 1 ml layer of 5% sucrose. The sucrose gradient was then centrifuged in a SW 50.1 rotor (Beckman Coulter) at 34,000 rpm (~ 110,000 g) for 20 h at 4°C. After centrifugation, fractions were collected starting from the top to bottom of the tube. Thirteen fractions (~ 400 μl) were collected, and equal volumes of each fraction were analyzed by 10% gradient SDS-PAGE and immunoblotted with TRPM4 antibodies and caveolin-1 (1:1000 dilution; Cell Signaling Tech cat# 32675).

**Patch-Clamp Recording**

Single-channel currents were recorded from mpkCCD14 cells under the voltage-clamp mode with an Axopatch-200B amplifier (Molecular Devices, Sunnyvale, CA), using inside-out patch-clamp configurations, as described previously (Wu et al., 2016). Data were acquired and sampled with a low-pass, 1 kHz, eight-pole Bessel filter using a Digidata 1440A analog-digital interface (Axon Instruments, Inc.). The mpkCCD14 cells were thoroughly washed with NaCl solution containing (in mM) 145 NaCl, 1 MgCl2, 1 CaCl2, 10 glucose, and 10 HEPES, adjusted pH to 7.4 with NaOH. This NaCl solution was used for filling the bath in the patch chamber and filling the patch pipette. The patch pipette was pulled with Borosilicate glass, giving a tip resistance of ~8–8 MΩ when filled with NaCl solution. Single-channel currents were obtained at a holding potential of 80 mV for inside-out recordings, and only the patches with the seal resistance >2 GΩ were used. Experiments were conducted at room temperature (22–25°C). Prior to analysis, the single-channel traces were further filtered at 100 Hz. The single-channel amplitude was constructed by all-point amplitude histogram and the histograms were fit using multiple Gaussian and optimized using a simplex algorithm. \( P_{0} \) was calculated as \( P_{0} = \frac{N \cdot P_{0}}{N} \), where N (N = estimated by the current amplitude histogram during at least 5 min recording period when the channel was maximally activated by Ca\(^{2+}\)) is the number of active channels in the patch. According to a total of 20 inside-out patches, we found that the channel activity can be maximally activated after we excised the patch membrane and exposed the inner leaflet of the membrane to 1 mM Ca\(^{2+}\) in the bath. Under the condition, we recorded more than 5 min and the channel activity remained very high. This maximal activation of the channel after we excised the patch membrane into a bath solution containing 1 mM Ca\(^{2+}\) was also used for the estimation of N. We also switched to a solution containing 10 mM EGTA without any calcium to mark the zero current levels. More importantly, since lovastatin significantly inhibited the TRPM4 open probability by reducing its sensitivity to Ca\(^{2+}\). In order to obtain the number of active channels in the patches, single channel current was recorded from inside-out patches exposed the patch membrane to the bath containing 5 mM CaCl\(_2\), followed by a bath solution with 10 mM EGTA without any calcium. The free Ca\(^{2+}\) concentration after chelating CaCl\(_2\) with EGTA was determined using free Web software Winamac (Stanford University, Stanford, CA, United States), as previously described (Wu et al., 2014).

**Confocal Microscopy**

Confocal microscopy experiments were performed as previously reported (Wu et al., 2016). Briefly, after fixation with 4% paraformaldehyde at room temperature for 10 min, the cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min and blocked with 5% BSA/PBS-T for 30 min. Rabbit polyclonal anti-TRPM4 antibody (alomone ACC-044; 1:100 dilution) was added in 1% BSA/TBS-T for overnight at 4°C. The sections were washed in TBS-T and incubated with Alexa Fluor 488 conjugated donkey anti-rabbit IgG (Invitrogen A21206, 1:100 dilution) and Alexa-594-conjugated cholera toxin B (CTB) (Invitrogen C34777) for 1 h. All slides were imaged using a confocal microscope (Olympus, Fluoview1000, Japan). To detect cholesterol levels in the plasma membrane of mpkCCD14 cells, the cells were incubated with 5 μg/ml filipin (Sigma, Cat#: F9765) for 30 min. Filipin staining was viewed by confocal microscope using DAPI filter. The control fluorescent intensity is used as a calibrator, and relative fluorescent intensity is calculated against this calibrator. All slides were imaged using a confocal microscope (Olympus, Fluoview1000, Japan) and analyzed using Olympus Fluoview FV1000 version 3.1 software. Identical acquisition settings were used for all images. To quantify colocalizations, the image analysis program ImageJ was used. Both Pearson and Manders coefficients were calculated.

**Lipid-Protein Overlay**

To test the lipid-binding properties of TRPM4, Protein lipid overlay assays were performed using Pip Strips from Invitrogen (Chicago, IL, United States) as previously described (Zhang et al., 2010). The strip is a piece of nitrocellulose membrane on which 15 phospholipids at 100 pM and a blank sample were loaded by the manufacturer. Briefly, strips were blocked in TBS-Tween (0.1%, TBS-T) and 3% BSA for 1 h. The mpkCCD14 cells lysate was then diluted in the blocking buffer to 0.5 μg/ml and incubated overnight at 4°C. To detect the possible binding of TRPM4 to spotted phospholipids, the Strips were incubated with rabbit polyclonal antibodies directed against TRPM4 (1:200; ACC-044; Alomone Labs, Jerusalem, Israel) similar to the western blot method.

**Chemicals**

All chemicals for electrophysiological recordings were purchased from Sigma-Aldrich (St Louis, MO, United States) except when specified. DiC8-PI(4,5)P\(_2\) was purchased from Echelon Biosciences. Both wortmannin and prostaglandin E2 (PGE2)
were used to pre-treat mpkCCDc14 cells for 30 min for patch-clamp experiments.

**Data Analysis.**
Data are reported as mean values ±SEM. Statistical analysis was performed with GraphPad Prism 5 software (GraphPad; La Jolla, CA) was used for all statistical calculations. Student t test was used between two groups. Analysis of variance was used for multiple comparisons. Results were considered significant if \( P < 0.05 \).

**RESULTS**

**Inhibition of Cholesterol Biosynthesis Decreases, Whereas Enrichment of Cholesterol Increases, Transient Receptor Potential Melastatin4 Channel Activity by Regulating its Sensitivity to Ca\(^{2+}\) in mpkCCDc14 Cells.**

To manipulate the plasma membrane cholesterol content, lovastatin and exogenous cholesterol were used as described previously (Song et al., 2014). The mpkCCDc14 cells were treated with 5 \( \mu \)M lovastatin, 30 \( \mu \)g/ml exogenous cholesterol, or 5 \( \mu \)M lovastatin plus 30 \( \mu \)g/ml exogenous cholesterol for 48 hrs. Then, plasma membrane cholesterol levels in mpkCCDc14 cells were evaluated by filipin staining. The data show that exogenous cholesterol significantly increased, whereas lovastatin significantly decreased, the cholesterol levels in the plasma membrane of mpkCCDc14 cells, and that co-treatment of the cells with both lovastatin and exogenous cholesterol did not alter cholesterol levels (Figures 1A,B). To further determine whether these treatments affect TRPM4 channel activity by regulating its sensitivity to Ca\(^{2+}\), the channel activity was recorded by exposing the patch membrane to the bath containing different concentrations of free Ca\(^{2+}\) using the inside-out patch-clamp technique. We have previously demonstrated that the concentration of Ca\(^{2+}\) required for 50% of maximal activation of TRPM4 (EC\(_{50}\)) was \( \sim 32.6 \mu \)M under basal conditions without manipulation of membrane cholesterol levels (Wu et al., 2016). Here, we found that the EC\(_{50}\) after reducing membrane cholesterol with lovastatin was only \( \sim 5.76 \mu \)M and that this effect was significantly reversed to \( \sim 10.2 \mu \)M by enrichment of membrane cholesterol with exogenous cholesterol (Figure 2). These results indicate that elevation of membrane cholesterol increases the TRPM4 channel activity by enhancing its sensitivity to Ca\(^{2+}\) in mpkCCDc14 cells.

**Exogenous Cholesterol Restores the Inhibition of Transient Receptor Potential Melastatin4 by Deletion of Membrane Cholesterol With Methyl-\( \beta \)-Cyclodextrin in mpkCCDc14 Cells**
To further determine whether acute depletion of cholesterol with M\( \beta \)CD affects TRPM4 channel activity, we performed excised inside-out patch-clamp experiments in mpkCCDc14 cells. Here, we found that acute extraction of cholesterol out of the inner leaflet of the patch membrane with M\( \beta \)CD significantly reduced TRPM4 Po and that application of exogenous cholesterol
reversed the reduction of TRPM4 channel activity induced by MβCD (Figures 3A,B). These data together suggest that cholesterol in the inner leaflet of the plasma membrane is required for maintaining TRPM4 activity.

**Enrichment of Plasma Membrane Cholesterol Stimulates Transient Receptor Potential Melastatin4 via a PI(4,5)P2-Dependent Mechanism**

We have previously shown that cholesterol regulates ROMK channels by altering PI(4,5)P2 localization (Liu et al., 2015). It is known that PI(4,5)P2 stimulates TRPM4 channels (Nilius et al., 2006). Therefore, we would ask whether PI(4,5)P2 is required for cholesterol to stimulate TRPM4. Wortmannin, at high concentrations, is a PI4K inhibitor, therefore preventing the synthesis of PI(4,5)P2 and depleting membrane PI(4,5)P2 (Saleh et al., 2009). Our data show that depletion of PI(4,5)P2 by wortmannin (20 μM) abrogated exogenous cholesterol-induced TRPM4 channel activity (Figures 4A,B). In contrast, application of 20 nM wortmannin, which is unable to alter PI(4,5)P2 levels, had no effects on cholesterol-induced TRPM4 channel activity (Figures 4C,D). Since PGE2 depletes PI(4,5)P2 via activation of Gq-coupled EP1 receptors (Harraz et al., 2018), PGE2 (2 μM) was also used to examine whether cholesterol can stimulate TRPM4 without PI(4,5)P2. Our data showed that PGE2 abolished the stimulatory effects of exogenous cholesterol on TRPM4 activity, whereas exogenous PI(4,5)P2 (diC8-PI(4,5)P2, a water-soluble analog) increased the effects (Figures 4E,F). These data suggest that elevation of plasma membrane cholesterol stimulates TRPM4 via a PI(4,5)P2-dependent mechanism.
PI(4,5)P₂ Binds to Transient Receptor Potential Melastatin4 Channels in mpkCCD\textsubscript{c14} Cells

Previous studies have demonstrated that PI(4,5)P₂ is a strong positive modulator of TRPM4 (Nilius et al., 2006). Consistently, our inside-out data show that diC8-PI(4,5)P₂ (20 μM) significantly increased the TRPM4 activity (Figures 5A,B). To further determine whether TRPM4 can bind to PI(4,5)P₂, lipid-protein overlay experiments were performed by using PIP Strips. Our data show that TRPM4 physically binds to almost all phosphatidylinositols (PI) including PI(4,5)P₂ (Figure 5). These data suggest that PI(4,5)P₂ stimulates TRPM4 channels probably via a physical interaction.

Transient Receptor Potential Melastatin4 is Mainly Located in Cholesterol-Rich Lipid Rafts in mpkCCD\textsubscript{c14}

Our previous studies have shown that cholesterol in lipid rafts maintains PI(4,5)P₂ in lipid rafts (Liu et al., 2015). To determine whether TRPM4 is also located in lipid rafts to physically interact with PI(4,5)P₂, we labeled lipid rafts with fluorescence-tagged cholera toxin (CTX) and TRPM4 with its specific antibody. Quantitative analysis with ImageJ showed that TRPM4 channel was colocalized with lipid rafts (Pearson coefficient was 0.726 ± 0.049 and Manders coefficients were 0.902 ± 0.042 [M1], red color and 0.932 ± 0.037 [M2], green color). Confocal microscopy data showed that TRPM4 channel was co-localized with lipid rafts (Figure 6A). Consistently, the
data from sucrose density gradient assays also show that fractions 2–5 are denoted as the lipid raft fractions as indicated by caveolin-1, a marker of membrane lipid rafts and TRPM4 was mainly enriched in lipid raft membranes in mpkCCDc14 cells (Figure 6B). These data indicate that plasma membrane cholesterol stimulates TRPM4 by holding PI(4,5)P₂ in lipid rafts.

**Changes in Plasma Membrane Cholesterol Have No Effect on Transient Receptor Potential Melastatin4 Expression.**

Since plasma membrane cholesterol could regulate TRPM4 activity, we examined whether manipulation of cholesterol levels will affect the expression of TRPM4 in mpkCCDc14 cells.
Confocal microscopy experiments were performed using control cells and cells treated for 48 hrs with 30 μg/ml exogenous cholesterol, 5 μM lovastatin, or 5 μM lovastatin plus 30 μg/ml exogenous cholesterol. These data showed that enrichment of cholesterol or inhibition of cholesterol biosynthesis has no effect on TRPM4 expression in mpkCCDc14 cells (Figures 7A,B). To confirm the results from confocal microscopy experiments, Western blot and cell-surface biotinylation assay data also
showed that the total and membrane levels of TRPM4 in mpkCCDc14 cells were unaltered by cholesterol enrichment or inhibition of cholesterol biosynthesis (Figures 7C,D). To further examine whether manipulating the membrane cholesterol of mpkCCDc14 cells can affect TRPM4 cell membrane abundance, the number of active channels was recorded by exposing the patch membrane to the bath containing 5 mM CaCl2, followed by a bath solution with 10 mM EGTA and no calcium. Our data showed that neither cholesterol enrichment nor inhibition of cholesterol biosynthesis affects the number of active channels in the patches from mpkCCDc14 cells (Figures 7E,F). Thus, the potentiating effect of cholesterol on TRPM4 activity cannot be attributed to an enhanced its surface expression.

**DISCUSSION**

The present study shows that pharmacological approaches to manipulate the plasma membrane cholesterol content regulate TRPM4 channel activity in mpkCCDc14 cells. Inhibition of cholesterol biosynthesis decreases, whereas enrichment of...
cholesterol in cell membrane increases, TRPM4 channel activity by regulating its sensitivity to Ca\(^{2+}\) in mpkCCD\(_{14}\) cells. Our results suggest that elevation of plasma membrane cholesterol stimulates TRPM4 via a PI(4,5)P\(_2\) dependent mechanism.

Different mechanisms have been proposed to account for cholesterol regulation of ion channels. Several lines of evidence suggest that cholesterol may directly regulate ion channels by binding to specific sites of the channels or indirectly regulate ion channels by promoting the interaction with intracellular signal cascades including PI(4,5)P\(_2\). Here we show that TRPM4 is located in lipid rafts where cholesterol is located. However, we also show that cholesterol no longer stimulates TRPM4 channels when PI(4,5)P\(_2\) is depleted by inhibition of phosphatidylinositol 5-kinase with a high concentration of wortmannin, indicating that cholesterol does not directly stimulate TRPM4 channels. Our previous studies have demonstrated that PI(4,5)P\(_2\) is co-localized with cholesterol in the microvilli where a majority of lipid rafts is located (Zhai et al., 2018; Zhai et al., 2019) and that inhibition of cholesterol synthesis reduces PI(4,5)P\(_2\) in the microvilli by causing PI(4,5)P\(_2\) diffusion into planar regions (Liu et al., 2015). Therefore, decreases in membrane cholesterol would decrease the activity of TRPM4 channels which is located in lipid rafts by reducing PI(4,5)P\(_2\) which is also located in lipid rafts. Conversely, increases in membrane cholesterol would increase the activity of TRPM4 channels by elevating PI(4,5)P\(_2\). We also favor the nation that TRPM4 is located in the lipid rafts and that exogenous cholesterol acts as a shuttle to collect free PI(4,5)P\(_2\) in non-lipids to translocate PI(4,5)P\(_2\) into lipid rafts to stimulate TRPM4.

Since it is known that PI(4,5)P\(_2\) sensitizes TRPM4 to Ca\(^{2+}\) (Zhang et al., 2005), in the experiments we used wortmannin and PGE\(_2\) to reduce PI(4,5)P\(_2\), however, in order to achieve a basal activity we increased the concentration of Ca\(^{2+}\) (1 mM) before we applied cholesterol. As shown in Figures 4C,D, under the condition that PI(4,5)P\(_2\) was reduced and Ca\(^{2+}\) was elevated, cholesterol failed to increase TRPM4 activity. The failure should not be due to a saturated activation of the channel by 1 mM Ca\(^{2+}\), because additional PI(4,5)P\(_2\) still elevated the channel activity (Figure 4E). Although our data suggest that the effect of cholesterol on TRPM4 channel activity is PI(4,5)P\(_2\)-dependent, we cannot rule out the possibility that TRPM4 can directly interact with cholesterol, because lipids may well be coordinated in the channel complex and interact by allosteric linkage. Indeed, previous studies have suggested that TRPM4 channel contains putative cholesterol binding sites (Autzen et al., 2018). However, our data suggest that it is unlikely that enrichment of cholesterol stimulates TRPM4 activity by direct interaction with the channel via the cholesterol-binding sites, because cholesterol no longer stimulates TRPM4 channels when PI(4,5)P\(_2\) is depleted. We argue that the direct interaction between cholesterol and TRPM4 may only play a role in maintaining TRPM4 localization in lipid rafts.

Our previous report suggests that TRPM4 accounts for the nonselective cation channel activity found in the CCD principal cells (Wu et al., 2016). However, it still remains unclear whether TRPM4 channel activity is responsible for K\(^+\) secretion and Na\(^+\) reabsorption under physiological conditions, because it requires a high concentration of intracellular Ca\(^{2+}\) to activate TRPM4 channels. Based on our findings that endogenous TRPP2 and TRPV4 assemble to form a non-selective calcium-permeable channel complex in the CCD principal cells (Zhang et al., 2013). Activation of TRPP2/TRPV4 would allow Ca\(^{2+}\) influx to generate sufficient magnitude (μM) in the subapical membrane to activate TRPM4 channels. Therefore, the present study indicates that TRPM4 channel activity is responsible for K\(^+\) secretion and Na\(^+\) reabsorption under physiological conditions. Since cholesterol accumulation causes kidney dysfunction and contributes to hypertension, the stimulation of TRPM4 by elevated cholesterol would have pathophysiological significance.

**CONCLUSION**

Our data show that TRPM4 channel is localized in lipid rafts in mpkCCD\(_{14}\) cells. In addition, enrichment of membrane cholesterol increases, whereas deletion of cholesterol by lovastatin decreases, TRPM4 activity by regulating its sensitivity to Ca\(^{2+}\) in mpkCCD\(_{14}\) cells. Our data also suggest that plasma membrane cholesterol stimulates TRPM4 via a PI(4,5)P\(_2\) dependent mechanism.

**DATA AVAILABILITY STATEMENT**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**AUTHOR CONTRIBUTIONS**

Z-RZ and H-PM: designed the study; M-MW, Y-XC, B-LZ, MY, Y-CY, DZ, XA, Q-SW, JL, CL, and L-LT: carried out experiments; M-MW and B-LZ analyzed data and prepared figures; M-MW, Z-RZ, and H-PM: drafted and revised the paper; all authors approved the final version of the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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