Intestinal TMEM16A control luminal chloride secretion in a NHERF1 dependent manner

Tultul Saha\textsuperscript{a}, Joydeep Aoun\textsuperscript{a,b}, Mikio Hayashi\textsuperscript{c}, Sheikh Irshad Ali\textsuperscript{a}, Paramita Sarkar\textsuperscript{a}, Prasanta Kumar Bag\textsuperscript{d}, Normand Leblanc\textsuperscript{b}, Nadia Ameen\textsuperscript{f}, Owen M. Woodward\textsuperscript{f}, Kazi Mirajul Hoque\textsuperscript{a,*,\textdagger}

\textsuperscript{a}Pathophysiology Division, National Institute of Cholera & Enteric Diseases, Kolkata, India
\textsuperscript{b}Department of Pharmacology, The Center for Cardiovascular Research, Center of Biomedical Research Excellence for Molecular and Cellular Signal Transduction in the Cardiovascular System, University of Nevada, Reno School of Medicine, Reno, NV, United States
\textsuperscript{c}Dept. of Cell Physiology, Institute of Biomedical Science, Kansai Medical University, Hirakata, Japan
\textsuperscript{d}Dept. of Biochemistry, University of Calcutta, 35 Ballygunge Circular Road, Kolkata, India
\textsuperscript{e}Department of Pediatrics/Gastroenterology and Hepatology, Cellular and Molecular Physiology, Yale School of Medicine, New Haven, CT, United States
\textsuperscript{f}Dept. of Physiology, University of Maryland School of Medicine, Baltimore, MD, United States

\textbf{A B S T R A C T}

TMEM16A (Transmembrane protein 16A or Anoctamin1) is a calcium-activated chloride channel (CaCC), that exerts critical roles in epithelial secretion. However, its localization, function, and regulation in intestinal chloride (Cl\textsuperscript{−}) secretion remain obscure. Here, we show that TMEM16A protein abundance correlates with Cl\textsuperscript{−} secretion in different regions of native intestine activated by the Ca\textsuperscript{2+}-elevating muscarinic agonist carbachol (CCH). Basal, as well as both cAMP- and CCH-stimulated Isc, was largely reduced in Ano1± mouse intestine. We found CCH was not able to increase Isc in the presence of apical to serosal Cl\textsuperscript{−} gradient, strongly supporting TMEM16A as primarily a luminal Cl\textsuperscript{−} channel. Immunostaining demonstrated apical localization of TMEM16A, whereas it colocalized with NHERF1 in mouse colonic tissue. Cell death in NHERF1 in human colonic T84 cells caused a significant reduction of both cAMP- and CCH-stimulated Isc. Immunoprecipitation experiments revealed that NHERF1 forms a complex with TMEM16A through a PDZ-based interaction. We conclude that TMEM16A is a luminal Cl\textsuperscript{−} channel in the intestine that functionally interacts with CFTR via PDZ-based interaction of NHERF1 for efficient and specific cholinergic stimulation of intestinal Cl\textsuperscript{−} secretion.

1. Introduction

Transepithelial secretion of ions and fluid across the intestinal epithelia is important to retain proper conditions (pH, salt concentration, osmolarity, etc.) for effective digestion, and to rinse the epithelial surface. Transport of chloride ions (Cl\textsuperscript{−}) across secretory epithelial cells provides an essential driving force for fluid movement and ions out into the lumen to maintain luminal hydration \cite{1,2}. Its rate and direction control the extent of secretion by classical intracellular second messengers such as adenosine 3′,5′-cyclic monophosphate (cAMP) and calcium (Ca\textsuperscript{2+}). These pathways exist in native intestinal epithelia and colonic tumor cell lines where several ion channels are regulated either by Ca\textsuperscript{2+} - or cAMP-mediated agonists to induce Cl\textsuperscript{−} secretion \cite{3,4}. In intestinal epithelial cells, many studies have been concentrated on the cAMP-dependent Cystic Fibrosis Transmembrane Regulator (CFTR) Cl\textsuperscript{−} channels and resulting defects in its activation by cAMP agonists observed in Cystic Fibrosis (CF). An increase in intracellular [Ca\textsuperscript{2+}], in enterocytes of the intestine elicited by enterotoxins and hormones, stimulates intestinal ions and fluid secretion by stimulating Cl\textsuperscript{−} channels called Calcium-Activated Cl\textsuperscript{−} Channels (CaCCs). In the early 1980s, studies have reported in \textit{Xenopus laevis} oocytes that CaCCs can activate fertilization by promoting a fast-electrical inhibition to restrain sperm entry \cite{5,6}. Although, the distinct molecular identification of these channels was stay unattainable before transmembrane protein 16A (TMEM16A; subsequently renamed as ANO1) was determined as a CaCC in 2008 \cite{7-9}. Subsequently, TMEM16A has received immense attention, and considerable research has depicted the physiological function and properties of this protein \cite{10}. The function of TMEM16A has

\textsuperscript{\dagger} Corresponding author. Dept. of Physiology, University of Maryland, School of Medicine, 655 West Baltimore Street Baltimore, MD, United States. E-mail address: mkazi@som.umaryland.edu (K.M. Hoque).

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remained controversial in the intestinal epithelium. There has been an ambiguity about whether TMEM16A channels are expressed on the apical membranes of enterocytes and contribute to luminal Cl- secretion. It is also unclear whether TMEM16A channels can be regulated by the PDZ (PSD-95, Dlg, ZO-1) containing proteins present in the mammalian intestine. The multi-PDZ domain-containing protein NHERF1 (Na+/H+ exchanger regulatory factor 1) is a scaffolding protein that interacts with ezrin through its ezrin/radixin/moesin (ERM) domain, which associates with actin. It has been shown to bind to a list of ion channels, transporters, and receptors [11]. Previously we have demonstrated that the presence of NHERF1 is essential for basal as well as maximal stimulation of TMEM16A by an increase in intracellular calcium in the murine intestine [12]. In this study, we focused on determining the expression and function of TMEM16A in mouse intestine and its possible association with NHERF1. Our data suggest that NHERF1 may mediate the coupling of TMEM16A to CFTR at the apical membrane for efficient and specific cholinergic stimulation of intestinal Cl- secretion.

2. Materials and methods

2.1. Animal and tissue preparation

Six to eight weeks old C57BL/6 male mice were used. Experiments were approved by the Institutional Animal Ethics Committee of NICED, Kolkata, India [no.NICED/CPCSEA/AW/(225)/IAECMH/3]. Ano1 ± heterozygous offspring, developed by Jason R.

Rock were made available to us as a gift by Dr. Sean Ward, University of Nevada, Reno School of Medicine [13]. Mouse intestinal tissue was removed by sharp dissection after euthanasia and sera-musculature stripping done as described previously [14,15].

2.2. Measurement of transepithelial short circuit current (Isc)

Sections of intestinal tissues; wild type T84 cells (T84WT) and NHERF1 knock down (NHERF1KD) T84 cells grown as confluent monolayers on 12-mm Snapwell inserts were mounted in Ussing chamber to measure short circuit current (Isc) [3,16]. Both mucosal (apical) and serosal (basolateral) sides were bathed with Ringer solution, which contained (in mM): 140 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 10 Glucose and 10 HEPES adjusted to pH 7.4 [16,17]. To examine basolateral Isc, a Cl- gradient in the apical to basolateral direction was imposed. The solution contained (mM): Basolateral – 140 Na+, 5 K+, 2 Ca2+, 1 Mg2+, 6 Cl–, 145 gluconate and apical – 140 Na+, 5 K+, 1 Ca2+, 1 Mg2+, 149 Cl–, 0 gluconate. Carbachol (CCH) and forskolin(FSK) were purchased from SIGMA, and MONNA inhibitor was purchased from Tocris Bioscience.

2.3. Immunoblot analysis

Total lysates were prepared and samples were separated in 8% SDS-PAGE and transferred to Nitrocellulose membrane (0.45 μm, Bio Rad) [16]. After incubation with primary antibodies(rabbit anti-TMEM16A, Novus biological #NBP2-29662; rabbit anti-NHERF1, Abcam #ab5199; rabbit anti-beta actin, Cell signaling Technology #4967).membranes were washed and incubated with alkaline phosphatase conjugated secondary antibody (goat anti-rabbit IgG, BioRad).The membranes were incubated with BCIP-NBT for the detection of target protein.

2.4. Immunofluorescence labeling

Mouse intestinal tissue sections were fixed in 3% paraformaldehyde prior to paraffin embedding [16,17].Thereafter, sections were incubated with rabbit anti-TMEM16A (Novus biological, #NBP2-29662) and mouse anti-NHERF1(Santa Cruz Biotechnology, #sc-271552) antibody overnight at 4 ℃ followed by exposure to goat anti-rabbit- IgG Alexa Fluor 488 or goat anti-mouse-IgG Alexa Fluor 680 secondary antibody (Invitrogen; 1:500) for 1h at room temperature. Mouse tissue images were obtained using an Olympus Fluoview FV1000 laser scanning confocal microscope.

2.5. Cell culture, plasmid constructs and transfection

T84WT cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F-12 (Cell Clone) medium supplemented with 10% fetal bovine serum(FBS, Cell Clone), 100 units/ml penicillin, and 100 μg/ml streptomycin.NHERF1 shRNA (TRCN0000043736) was purchased from Sigma-Aldrich. Lentivirus generation was performed as described previously [3]. HEK293T, Caco2, HT29 cells were maintained in DMEM (HiMedia) supplemented with 10% FBS. Full length human NHERF1 in pcmV-2-Flag vector (plasmid # 28291) was obtained from Addgene [18,19]. Full length mouse Ano1 tagged with mCherry (monomeric cherry) and Ano1 with deletion of C-terminal 4aa (ΔC4) were obtained from Dr. Mikio Hayashi. HEK293T cells were transfected with plasmid DNAs using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

2.6. Co-immunoprecipitation

Protein was isolated from transfected HEK293T cells and pre-cleaned with protein G-agarose beads (Pierce) and incubated overnight with Anti-Flag antibody (SIGMA) at 4 ℃. The protein antibody complex was immobilized by protein G agarose beads for 3 h at 4 ℃. The beads were washed and mixed in 2X Laemmli sample buffer. To check the interaction between endogenously expressed TMEM16A and NHERF1 in T84 cell, T84 cell lysates were first immunoprecipitated with anti-NHERF1 and then western blotting was done.

2.7. RNA extraction and real time PCR

Total RNA was extracted using Trizol (SIGMA) reagent as per manufacturer’s instructions and cDNA preparation was done using superscript cDNA preparation kit. Real time PCR was performed on an Applied Biosystems AB7900 real time PCR detection system [14]. Amplification reactions were performed containing cDNA, 300 nM each of forward and reverse primers and SYBR Green PCR Master Mix. The primer sequences are listed in Table S1. The normalized mRNA expression was calculated according to the (2ΔΔCt) method [20].

2.8. Statistical analysis

Statistical data and analysis were performed using Origin 6.0(Ori-ginLab, Northampton, MA, USA) software. Data are expressed as mean ± SE. Statistical significance was determined by using paired or unpaired t-test as appropriate. One-way ANOVA with Bonferroni test was done when required. P value < 0.05 was considered statistically significant as indicated.

3. Results

3.1. Ano1 expression and function are detectable along the mouse intestine

Expression of Ano1 mRNA in mouse intestine was analyzed by qPCR. Ano1 transcripts are differentially expressed across the intestinal epithelial tissues with highest expression in the colon (Fig 1A). To establish a comprehensive understanding of the protein involved in calcium stimulated Cl- secretion, region-specific changes in Cl- secretion were assessed in intestinal mucosa by stimulation with 100 μM CCH. This concentration is consistent with previous literature showing that EC50 values for a carbachol-induced increase in Isc in T84 cells remain high.

T. Saha et al. Biochemistry and Biophysics Reports 25 (2021) 100912
ranged between 10 and 30 μM while the maximum response was achieved by serosal addition of 100 μM carbachol as published elsewhere [21–23]. The greatest CCH-dependent ΔIsc in mouse intestine was evident in jejunum, ileum and colon compared to duodenum (Fig. 1B). This data is consistent with a study that demonstrated stimulation of Isc by CCH in all intestinal segments but differs from that of others [24,25].

We next examined the effect of MONNA, a potent and selective blocker of TMEM16A on basal and CCH-stimulated Isc in colonic tissue. Both basal (17.52 ± 3.5 vs. 10.5 ± 3.20 μA/cm², P < 0.05) and CCH-stimulated (38.45 ± 2.9 vs. 3.25 ± 0.75 μA/cm², P < 0.001) Isc were significantly inhibited by apical incubation of MONNA (Fig. 1C) [26]. However, no discernible inhibition (45.60 ± 4.25 vs 36.23 ± 3.23 μA/cm²) was monitored in CCH-enhanced Isc upon serosal application of MONNA (Fig. 1D). We further detected serosal Cl⁻ conductance in the presence of an apical to serosal Cl⁻ gradient. Under this condition CCH was not able to increase the Isc; whereas in the presence of serosal to

**Fig. 1.** Ano1 mRNA expressed differentially and carbachol (CCH) stimulates Isc both in mouse small and large intestine. (A) Represents the quantity of Ano1 gene expression. Data are means ± S.E (n = 3). (B) CCH stimulated Isc in different parts of the mouse intestine, as indicated. *P < 0.01 (ANOVA with Bonferroni’s test). (C) Effects of MONNA on basal and CCH-stimulated Isc and (D) representative tracing of apical and serosal incubation of MONNA in response to CCH stimulation in mouse colonic tissue. Data are means ± S.E (n = 4–7). (E) Showing representative Isc response to CCH in the presence of apical to serosal and serosal to apical Cl⁻ gradient. Ap, apical and Bl, serosal side of the mouse colon (n = 3).

**Fig. 2.** Ano1 ± mouse caused reduction of basal and CCH-stimulated Isc. (A) Basal and CCH-stimulated Isc in the small intestine (jejunal) and colonic tissue of WT and Ano1 ± mice. Data are means ± S.E (n = 6). *P < 0.05. (B) Showing FSK-stimulated Isc response in WT and Ano1 ± mouse colonic mucosa. Data are means ± S.E (n = 4–6). (C) Representative western blot image of TMEM16A protein in different parts of wild type and Ano1 ± enterocytes. (D) Quantitative analysis of the immunoblots was determined by ImageJ analysis. Data are means ± S.E of three independent experiments. (* denotes significant difference at p value < 0.05, NS, non significant at p < 0.05).
apical Cl\(^{-}\) gradient, intestinal tissue displayed an immediate rise in Isc (Fig. 1E). We have observed the negative Isc when there is a mucosal to serosal Cl\(^{-}\) gradient. Apart from actively secreting Cl\(^{-}\), in addition to Na\(^{+}\) the colon also actively absorbs Cl\(^{-}\). It is therefore possible that the negative Isc may be due to the luminal to serosal Cl\(^{-}\) gradient yielding a Cl\(^{-}\) absorptive current flowing in the opposite direction to the known Cl\(^{-}\) secretory Isc. Another possibility might be the presence of an unknown/unusual electrogenic transport mechanism in the colonic tissue.

Fig. 3. TMEM16A localizes to the apical membrane of mouse enterocytes and immunoprecipitates with NHERF1 in co-transfected HEK293T cells. (A) Representative western blot showing NHERF1 expression in mouse intestinal tissue as indicated. The histogram showing the densitometric quantification of NHERF1 protein expression, right to the immunoblot. This experiment was performed in triplicate. (B) Immunofluorescence demonstrated TMEM16A (green) and NHERF1 (red) in mouse colon, the yellow signal indicated co-localization of TMEM16A with NHERF1 (merged). Representative of two independent experiments. (C) TMEM16A co-immunoprecipitates with Flag-NHERF1 in co-transfected HEK293T cells. TMEM16A was detected only in the Co-IP complex from cell co-transfected with plasmids expressing both mouse Ano1 and Flag-Nherf1. (D) Depicted a reduced TMEM16A abundance in Co-IP complex from cell lysates with truncated (delC4) Ano1 compared to the result obtained with the control (full length). Representative blot of three independent experiments for (C) and (D). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
3.2. *Ano1* ± mice exhibited reduced CCH- and cAMP-stimulated Isc

To further clarify that TMEM16A contributes to calcium-stimulated Cl\(^{-}\) secretion, we have used *Ano1* ± heterozygous mice to measure CCH-stimulated Isc. Heterozygous mice was used because TMEM16A homozygous knock-out mice die soon after birth caused by tracheomalacia [13] and no intestinal specific knock out mouse was available. A discernible reduction of both residual and CCH-stimulated Cl\(^{-}\) secretion was found in *Ano1* ± mouse jejunal and colon (Fig. 2A). *Ano1* ± colon exhibited ~84% (39.45 ± 10.29 vs. 6.38 ± 3.4 μA/cm\(^2\), \(P < 0.01\)) reduction of CCH stimulated Isc, while residual Isc was reduced by ~47% (17.52 ± 3.02 vs. 9.24 ± 1.75 μA/cm\(^2\), \(P < 0.05\)). This decrease in CCH-stimulated Isc mirrored the Isc response to CCH in the presence of MONNA (Fig. 1C). We next investigated the involvement of TMEM16A in controlling CFTR-mediated Cl\(^{-}\) secretion by measuring FSK-stimulated (adenylate cyclase activator) Isc in *Ano1* ± colon. As shown in Fig. 2B, colonic tissue from *Ano1* ± mice showed significantly reduced Isc in response to FSK. The TMEM16A protein abundance was determined by using intestinal epithelial cell lysates prepared from the luminal surface of both WT and *Ano1* ± mice (Fig. 2C). The densitometry of TMEM16A expression in the western blot relative to beta-actin is presented to the right of the immunoblot (Fig. 2D). A decreased TMEM16A expression was noticed in *Ano1* ± intestine compared to WT tissue.

3.3. **TMEM16A binds with NHERF1 by its C-terminal PDZ binding motif**

Previous studies have shown the tight regulation of apical Cl\(^{-}\) channels by inclusion in a macromolecular complex [27]. We investigated whether intestinal TMEM16A also complex with these scaffolds to regulate CCH-stimulated Cl\(^{-}\) secretion. We had previously documented that basal and CCH-stimulatedIscc was reduced by 60% and 92% in *Nherf1*+/− but not in *Nherf2*+/− mouse colonic tissue [12]. Western blotting reconfirms NHERF1 protein expression in mouse intestine (Fig. 3A). The densitometric quantification of NHERF1 protein is depicted in the right panel of the immunoblot. Sequence analysis of TMEM16A revealed a C-terminal Type 1 PDZ binding domain. The C-terminal sequence of TMEM16A in mouse (NP_848757.5 for isoform 1 and NP_001229278.2 for isoform-2) and human (NP_060513.5) is G-D-A-L and G-G-V-L, respectively. To test for a possible TMEM16A/NHERF1 interaction, immunostaining was done in mouse colonic tissue that showed apically expressed TMEM16A co-localized with NHERF1 as illustrated by the yellow immunofluorescence when images were merged (Fig. 3B).

We further examined the TMEM16A/NHERF1 interaction by co-immunoprecipitation experiment. HEK293T cells were co-transfected with mouse *Ano1* and Flag-Nherf1. NHERF1 and TMEM16A were only detected in the FLAG immunoprecipitated fraction where co-transfected cell lysate was used (Fig. 3C). To further check the role of PDZ binding motif of TMEM16A, cells were co-transfected with *Ano1* (full-length or C-terminal four amino acid truncated) and Flag-Nherf1. In contrast with full length *Ano1*, a little interaction was observed in case of *Ano1*delC4 with Flag-Nherf1 (Fig. 3D). This confirms the association between TMEM16A and NHERF1, and also suggests the role of the C-terminal PDZ binding motif of TMEM16A for this interaction.

3.4. **TMEM16A interacts with NHERF1 to potentiate Cl\(^{-}\) secretion in human colonic epithelia**

The expression of TMEM16A and NHERF1 was also detected in human intestinal cell lines (T84, Caco2, HT29) (Fig. S1). Co-immunoprecipitation experiment in T84 cells determined the interaction between endogenous TMEM16A and NHERF1 (Fig. 4A). To verify the regulatory role of NHERF1 in the context of Cl\(^{-}\) secretion, NHERF1 expression was silenced using lentivirus-containing shRNA in T84 cells and the reduction of NHERF1 expression was determined by real time PCR (Fig. S2) and western blot (Fig. 4B and C). As shown in Fig. 4D, the CCH-stimulated Cl\(^{-}\) secretion was reduced drastically in NHERF1 KD cells compared to WT and vector-transduced cells. To further investigate whether NHERF1 might be involved in determining TMEM16A and CFTR-mediated luminal Cl\(^{-}\) secretion, WT and NHERF1KD T84 monolayers were stimulated by cAMP and Ca\(^{2+}\) mediating secretagogues. When monolayers were stimulated with FSK followed by CCH, a synergistic effect of FSK and CCH-stimulated Cl\(^{-}\) secretion was evident in the intestinal epithelia. However, a significant reduction of Cl\(^{-}\) secretion was apparent in NHERF1KD T84 cells upon FSK and CCH stimulation (Fig. 4E).

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**Fig. 4. Functional involvement of NHERF1 in TMEM16A mediated Cl\(^{-}\) secretion.** (A) NHERF1 was co-immunoprecipitated with TMEM16A endogenously from T84 cell lysates. No signal was detected using protein-G-conjugated beads alone (IP control). (B) Western blot showing NHERF1 expression in wild type T84 (T84WT), vector control (T84VC) and NHERF1 knocked down cells (T84NF1KD736). (C) Histogram showing densitometric quantification of NHERF1 expression, right to the immunoblot. Result represents means ± S.E. (\(n = 3\)). (D) Representative traces showing the response of T84NF1KD736 cells to CCH-stimulated Isc. Inset showing summarized data from six independent experiments. Data are means ± S.E. (E) Effect of FSK pre-stimulation on CCH-stimulated Isc in WT and NHERF1KD T84 cell monolayers. Monolayers grown on filters were exposed to FSK (10 μM) and then stimulated with serosal CCH (100 μM). Data are mean ± SEM from 4 to 6 monolayers of each condition.
of serosal localization and function of TMEM16A. The secretory response is that FSK modifies Ca$^{2+}$ and chloride channels, to further enhance the extent of the secretory process. The current findings not only expand knowledge of TMEM16A in intestinal epithelia. The present findings depicted apical localization of TMEM16A and potential interaction with NHERF1, that has shown to exert a vital role in both cAMP- and CCH-stimulated luminal Ca$^{2+}$ secretion. The current findings not only expand knowledge of the intestinal TMEM16A function but provides a rationale for studying the formation and regulation of the TMEM16A-NHERF1-CFTR macromolecular complex, which has clinical implications for therapy of CF transport defect-related disease including CF.

**Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

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**4. Discussion**

CaCCs have been proposed to be involved in transepithelial transport, particularly in Cl$^{-}$ and fluid secretion elicited by CCH in secretory epithelia. Despite abundant evidence of a role for CaCC-mediated transepithelial Ca$^{2+}$ secretion, TMEM16A’s importance remains controversial. Earlier studies showed segmental heterogeneity regarding Ano1 mRNA detection in mouse intestine [1]. Here, we observed a gradual retrograde decrease of Ano1 mRNA expression from the distal colon toward the small intestine, as shown in Fig. 1A. Since TMEM16A is a bonafide CaCC, lsc measurements were performed across intestinal tissues to determine if protein abundance measured by western blot correlated with lsc under basal and CCH stimulation in WT and Ano1++/− heterozygous mice. We found that basal Cl$^{-}$ secretion was higher in jejunum and ileum compared with duodenum and colon (data not shown). In contrast, CCH had similar stimulatory effects in jejunum, ileum, and colon compared to the duodenum, which displayed a lower response to CCH. Both the basal and CCH-stimulated Cl$^{-}$ secretion in Ano1++/− mice were significantly reduced in the small intestine (jejunum), and colon (Fig. 2A). In agreement with functional data (ΔIsc), we observed a reduced expression of TMEM16A in Ano1++/− mice as compared with WT tissues. Together, these observations demonstrate a correlation between the functional response elicited in both small and large intestine to CCH and protein abundance but only a partial correlation with Ano1 mRNA abundance. The interdependence of CFTR and TMEM16A functions is controversial. Recently, it has been reported that the forskolin-induced lsc in isolated colonocytes of colon-specific KO of Ano1 (Cdx2-Ano1fl/fl) was significantly reduced than WT (Ano1fl/fl) colonocytes. However, there were no differences in CFTR protein abundance between WT and Cdx2-Ano1fl/fl colonocytes [28]. They also demonstrated that the cholera toxin-induced fluid accumulation was significantly reduced in the jejunum in Ano1-deficient mice, supporting a role for Ano1 in cAMP-induced intestinal secretion. Unfortunately, they did not provide any evidence of any differences in CFTR protein abundances in the jejunum. However, our finding in the current study is consistent with their findings, at least at the functional level, that there is a significant reduction of cAMP-stimulated Cl$^{-}$ secretion in Ano1++/− colon compared to WT colon [12,28]. These data strongly suggest that TMEM16A is essential not only for Ca$^{2+}$-dependent but also for cAMP-dependent Cl$^{-}$ secretion and their interdependence in the intestinal tissue [29]. The present study demonstrated crosstalk between cAMP and calcium signaling with a consequent synergistic enhancement of secretory responses when T84 cells were exposed to FSK first, followed by CCH (Fig. 4E). One possible explanation for the cAMP’s ability to enhance CCH-stimulated secretory response is that FSK modifies Ca$^{2+}$ signaling via stimulation of Epac (exchange protein directly activated by cAMP) because it has been shown to transduce cAMP into [Ca$^{2+}$], signaling [3]. These findings suggest that the transduction of CAMP into [Ca$^{2+}$] signaling may act in addition to the previously described cooperative opening of potassium and chloride channels, to further enhance the extent of the secretory response to CCH stimulation [30,31].

Our present study demonstrated clear apical membrane expression of TMEM16A in mouse intestinal tissue. We were unable to detect TMEM16A expression on the basolateral side of the epithelial cells, a finding consistent with the demonstration of TMEM16A expressed in the luminal membrane of salivary gland cells and airway epithelium [10, 32]. Functionally, this study documented that the TMEM16A inhibitor Monna inhibited CCH-stimulated Cl$^{-}$ secretion when applied from the luminal side (Fig. 1D) but was insensitive to serosal Monna, supporting a lack of serosal involvement of TMEM16A in the mouse intestine. Furthermore, in the presence of an apical to serosal Cl$^{-}$ gradient, CCH was unable to increase lsc, indicating the functional absence of a serosal CaCC (Fig. 1E). Together, these data evidenced lack of serosal localization and function of TMEM16A.

NHERF1 is expressed in enterocytes and has an established history of regulating key transport proteins within the apical membrane of a variety of epithelial cell types, including enterocytes [33]. In this study, we attempted to find whether NHERF1 interacts with TMEM16A and controls CCH-stimulated Cl$^{-}$ secretion. Mouse TMEM16A revealed a PDZ binding motif (GDAL) at the C terminal tail that carries a Leucine at ‘0’ position and Aspartate at ‘-2’ position being the affinity determining position, which may serve to link TMEM16A to the actin cytoskeleton through intermediary scaffolding proteins [34]. Here, we evidenced that TMEM16A forms a complex with NHERF1 in endogenously as well as in heterologous expression system (Figs. 4A and 3C), and this complex formation was diminished when the four-terminal amino acids (GDAL) on TMEM16A were truncated (Fig. 3D). This association was further confirmed functionally by knocking down NHERF1 in T84 cells, where a significant decrease of CCH-stimulated lsc was observed compared to T84WT and vector-transduced cells. Dual-immunofluorescence imaging of TMEM16A and NHERF1 also demonstrated that the two proteins were expressed in proximity within the apical microvilli domain (Fig. 3B). We examined the effect of NHERF1 deletion in the interaction between cAMP and CCH-stimulated Cl$^{-}$ secretion in human colonic epithelial cells. We observed a significant reduction of both cAMP and CCH-stimulated Cl$^{-}$ secretion, suggesting that CFTR and TMEM16A proteins might functionally interact via NHERF1 at the apical membrane, a conclusion that supports a previous study that showed a direct interaction between CFTR and TMEM16A [35]. These findings provide the first evidence of the cellular mechanism by which TMEM16A modulates luminal Cl$^{-}$ secretion impacting CFTR function through NHERF1 interaction. Lee and co-workers have shown that the reduction in cAMP-induced Cl$^{-}$ currents in colon specific Ano1 KO was not a result of the downregulation of CFTR because its protein level was not changed by the deletion of Ano1 [28]. We found a significant reduction of both cAMP and CCH-stimulated Cl$^{-}$ secretion in NHERF1 depleted cells, confirming the functional association between CFTR and ANO1 depends on NHERF1 interaction and expression, perhaps not due to the change of protein abundance. Together, based on these observations, we speculate that NHERF1 clusters TMEM16A-CFTR close to the microvillar plasma membrane via a PDZ based interaction, which is critical for muscular regulation of Cl$^{-}$ secretion in the intestinal epithelium. Further studies will be needed to characterize the precise role of NHERF1 in the regulation of TMEM16A, exceptionally whether NHERF1 might be important for TMEM16A to bind ERM proteins since a direct interaction between moesin and the channel has been found [36].

In conclusion, our study addressed some current controversies regarding the expression, localization, and functional regulation of TMEM16A in intestinal epithelia. The present findings depicted apical localization of TMEM16A and potential interaction with NHERF1, that has shown to exert a vital role in both cAMP- and CCH-stimulated luminal Cl$^{-}$ secretion. The current findings not only expand knowledge of the intestinal TMEM16A function but provides a rationale for studying the formation and regulation of the TMEM16A-NHERF1-CFTR macromolecular complex, which has clinical implications for therapy of Cl$^{-}$ transport defect-related disease including CF.

**Author statement**

TS, JA, IA, KMH designed experiments, contributed to the experimental analysis and interpretation of data. KMH and TS conceived the work, and drafted manuscript. TS, JA, IA, PS performed experiments and take responsibility for the integrity of the data and accuracy of the data analysis. BW contributed reagents. NL, NA, PB and OMW provided intellectual input for writing the manuscript later edited and revised the manuscript critically. MH conducted experiments to engineer Ano1 construct and Ano1C4 deletion.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence
the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2021.100912.

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