Regulated mucin secretion is essential for the formation of the mucus layer that protects the underlying epithelial cells from foreign particles. Alterations in the quantity or quality of secreted mucins are therefore detrimental to airway and colon physiology. Based on various biochemical assays in several human cell lines, we report here that Na+/Ca2+ exchanger 2 (NCX2) works in conjunction with transient receptor potential cation channel subfamily M member 4 (TRPM4), and perhaps TRPM5, Na+ channels to control Ca2+-mediated secretion of both mucin 2 (MUC2) and MUC5AC from HT29-18N2 colon cancer cells. Differentiated normal bronchial epithelial (NHBE) cells and tracheal cells from patients with cystic fibrosis (CFT1-LC3) expressed only TRPM4 and all three isoforms of NCXs. Blocking the activity of TRPM4 or NCX proteins abrogated MUC5AC secretion from NHBE and CFT1-LC3 cells. Altogether, our findings reveal that NCX and TRPM4/TRPM5 are together, our findings reveal that NCX and TRPM4/TRPM5 are both required for mucin secretion. We therefore propose that these two proteins could be potential pharmacological targets to control mucus-related pathologies such as cystic fibrosis.

Human genome encodes 21 different mucin proteins that are secreted to compose the mucous layer, which protects the underlying tissue from pathogens, allergens, and chemical agents (1). Of these mucins, Mucin 2 (MUC2),2 Mucin 5AC (MUC5AC), and Mucin 5B (MUC5B) are the best characterized gel-forming mucins (2). MUC2 is the major constituent of colon mucus (3), whereas MUC5AC and MUC5B are the main mucins expressed in the airways (4).

Mucins of about 500 kDa are synthesized in the endoplasmic reticulum (ER) and transported to the Golgi complex where, after extensive glycosylation, these proteins can reach sizes of up to 2.5 million Da (5, 6). After sorting at the Golgi, the heavily glycosylated mucins are packed into micrometer-sized granules that mature to contain highly condensed mucins (2). The mechanism of mucin sorting and export at the Golgi are not known. Mucin containing granules undergo a process of maturation, which is accompanied by condensation of mucins. A pool of mature granules fuses to plasma membrane by two different pathways: 1) baseline or exogenous agonist-independent reaction and 2) exogenous agonist dependent or the stimulated pathway. The key issue in the late stages of mucin release pathways is to tightly regulate the number of granules that fuse to plasma membrane by SNARE-dependent reaction. We have recently shown that baseline mucin secretion in colon cancer cells, and in mouse colon in vivo, depends on KChIP3, a calcium sensor, and intracellular calcium oscillations. KChIP3, however, does not appear to have a role in the extracellular agonist-dependent stimulated mucin secretion (7).

High levels of an extracellular stimulus (for example, ATP released in paracrine fashion and its metabolite adenosine, and possibly by inflammatory mediators) is well known to trigger mucin secretion by the involvement of a G protein–coupled

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2 The abbreviations used are: MUC2, mucin-2; CF, cystic fibrosis; MUC5AC, mucin-5AC; NCX, sodium-calcium exchanger; NHBE, normal human bronchial epithelial cells; PMA, phorbol 12-myristate 13-acetate; TRPM4, transient receptor potential cation channel subfamily M member 4; TRPM5, transient receptor potential cation channel subfamily M member 5; MARCKS, myristoylated alanine-rich protein kinase C substrate; PM, plasma membrane; SOCE, store-operated calcium entry; TG, thapsigargin; ALL, anti-liquid interface; IL-13, interleukin-13; ER, endoplasmic reticulum; DAPI, 4′,6-diamidino-2-phenylindole; AUC, area under the curve; PKC, protein kinase C, SNARE, soluble NSF attachment protein receptor(s); IP3, inositol 1,4,5-trisphosphate; shRNA, short hairpin RNA; qPCR, quantitative PCR.
receptor (like P2Y2) that activates phospholipase C to generate diacylglycerol, which activates PKC, and IP₃ that releases Ca²⁺ from apical ER (5). Activated PKC phosphorylates a protein called myristoylated alanine-rich protein kinase C substrate (MARCKS) on the cytoplasmic face of the plasma membrane (PM). This phosphorylation, or binding to Ca²⁺-calmodulin, dissociates MARCKS from the PM, which is then recruited to mucin granules by Hsp70 and cytoeine string protein (8). MARCKS-bound granules then dock to the apical surface by an actin-dependent process and fuse to plasma membrane by a MARCKS-bound granules then dock to the apical surface by an actin-dependent process and fuse to plasma membrane by a MARCKS-bound granules then dock to the apical surface by an actin-dependent process and fuse to plasma membrane by a SNARE-dependent fusion process, which involves VAMP8, SNAP23, and Munc18b (5, 9, 10). The syntxin family member involved in this fusion reaction is not known.

TRPM5, a Ca²⁺-activated monovalent cation-selective channel, which is expressed in the HT29-18N2 colonic cancer cell line, is required for stimulated MUC5AC secretion (11). Although differentiated HT29-18N2 colonic cancer cells secrete MUC5AC, normal colonic cells secrete MUC2. Is TRPM5 required for MUC2 secretion by colon cells? Cells of the airways, on the other hand, secrete MUC5AC, but instead of TRPM5 express its close homolog TRPM4 (12, 13). Knockdown efficiency estimated by qPCR revealed greater than 65% reduction of TRPM4 mRNA levels in TRPM4 KD cells (Fig. S1A) and 85 and 75% reduction in TRPM4 and TRPM5 mRNA levels, respectively, in TRPM4 + TRPM5 KD cells (Fig. S1B), and 60% reduction of NCX2 mRNA levels in NCX2 KD cells (Fig. S1C). We also tested the effect of TRPM4 and TRPM5 shRNA on the expression of other transgenes. Our results show that TRPM4 shRNA did not affect TRPM5 levels (Fig. S1A), however, TRPM5 shRNA produced a 60% decrease in TRPM4 levels (and 80% reduction of TRPM5 levels, as published in Ref. 11 (Fig. S1D)).

MUC2 and MUC5AC secretion by HT29-18N2 goblet cells requires TRPM4/TRPM5 and NCX2

We first measured MUC5AC secretion in TRPM4 KD, TRPM5 KD (described previously (11)), TRPM4 + TRPM5 KD, and NCX2 KD cells, which were differentiated by serum starvation, and treated with 100 μM ATP for 30 min at 37 °C. Cells expressing TRPM4 or TRPM5 shRNA displayed a similar reduction of ATP-dependent MUC5AC secretion compared with control cells (36 and 33%, respectively). Interestingly, TRPM4 + TRPM5 KD and NCX2 KD cells also presented comparable reduction in MUC5AC secretion compared with control cells (43 and 48% reduction) (Fig. 1D). Next, we studied their role in ATP-mediated MUC2 secretion. Control and KD cells were differentiated and treated with 100 μM ATP for 30

Results

Expression of TRPM4, TRPM5, and NCX2 in HT29-18N2 colonic goblet cells

We used HT29-18N2 cells that differentiate into mucin producing cells after 6 days of incubation in protein-free medium as a means to unravel the requirements for MUC2 and MUC5AC secretion (11). These HT29-18N2 cells are a good model system to study MUC5AC and MUC2 secretion. We acknowledge the caveats that MUC5AC is not ordinarily expressed in gastrointestinal cells. It is also important to note that the expression levels of MUC2 might be different in HT29-18N2 compared with the physiologically relevant cells in the colon. Total mRNA was extracted from differentiated HT29-18N2 cells and expression of NCXs, TRPM4, and TRPM5 was monitored by reverse transcription of the mRNA and PCR with specific primers for each gene (Table 1). MUC5AC and MUC2 were used as positive controls for differentiation of HT29-18N2 cells, and respective control cDNA (MUC2 cDNA, Clontech Laboratories) was used to test each set of primers (control cDNA for: NCX1, NCX2, and TRPM4; lungs cDNA for: NCX3, TRPM5, and MUC5AC; colon cDNA for: MUC2). Our results show that TRPM4, TRPM5 (as shown before in Ref. 11), and NCX2 (SLC8A2 gene) are expressed in differentiated HT29-18N2, whereas NCX1 and NCX3 (SLC8A1 and SLC8A3 genes, respectively) are not expressed in these cells (Fig. 1A).

We generated shRNA-dependent HT29-18N2 cell lines stably depleted of TRPM4, TRPM4 + TRPM5, or NCX2 as described under “Experimental procedures.” The HT29-18N2 cell line stably depleted of TRPM5 has been described previously (11). Knockdown efficiency estimated by qPCR revealed greater than 65% reduction of TRPM4 mRNA levels in TRPM4 KD cells (Fig. S1A); 85 and 75% reduction in TRPM4 and TRPM5 mRNA levels, respectively, in TRPM4 + TRPM5 KD cells (Fig. S1B), and 60% reduction of NCX2 mRNA levels in NCX2 KD cells (Fig. S1C). We also tested the effect of TRPM4 and TRPM5 shRNA on the expression of other transgenes. Our results show that TRPM4 shRNA did not affect TRPM5 levels (Fig. S1A), however, TRPM5 shRNA produced a 60% decrease in TRPM4 levels and 80% reduction of TRPM5 levels, as published in Ref. 11 (Fig. S1D)).

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Table 1

| Genes (genes) | Forward primers (5’-3’) | Reverse primers (3’-5’) |
|--------------|------------------------|------------------------|
| SLC8A1 | CTGTGGAGAGCTGAAATCCAG | CTCTCCTTTGTCGTGCTAA |
| SLC8A2 | GGGATTCTAGCTGCTACTCA | CCGCAGGCGTCTGCTAA |
| SLC8A3 | TACATCCCTCTTTAGAGAGG | CTCTCCTTTGTCGTGCTAA |
| MUC5AC | CACCTTCAAGTGGTACGGG | CCTTTCCTACCCAGCCTCT |
| MUC2 | TGAGGCGCATGCTCTTTC | GCACCACTTCCTCCACCC |
| TRPM4 | CAAAGAGGGACAGAACAA | CCTTTCGCGAAGAGTCTAC |
| TRPM5 | CCAAGAGGAGAACCTTCTGA | GCTGTGTTCCACGCTTAAAC |
| HPR1T | CCTGCTTCTCCTCAGCTCAG | AAACCTTCTCAAATCTCCAGC |
| GAPDH | GAAGCTTGAAGTTGCGGAGTC | CATCGGCCAAGTCTTGGGA |
TRPM4 and NCXs mediated mucin secretion

A. Ctr1 HT29 Neg

B. MUC5AC

C. MUC5AC

D. Scr. shRNA TRPM4-shRNA TRPM5-shRNA TRPM4/5-shRNA NCX2-shRNA

E. Scr. shRNA TRPM4-shRNA TRPM5-shRNA TRPM4/5-shRNA NCX2-shRNA

F. MUC5AC MUC2 Merg

Figure 1. TRPM4/TRPM5 and NCX2 are required for mucin secretion by HT29-18N2 colonic cancer cells. A, RNA levels of NCX1, NCX2, NCX3, TRPM4, TRPM5, MUC2, and MUC5AC in positive control cDNA (control cDNA for NCX1, NCX2, TRPM4; lungs cDNA for NCX3, TRPM5, and MUC5AC; and colon cDNA for MUC2; MTC cDNA Panels, Clontech Laboratories) and differentiated HT29-18N2 cDNA were analyzed by agarose gel. B, Intracellular MUC5AC levels of control, TRPM4, TRPM5, TRPM4 + TRPM5, and NCX2 KD cells. Differentiated cells were lysed and analyzed by dot blot with anti-MUC5AC and anti-actin antibodies. Intensities of MUC5AC spots were normalized to the actin levels. Results are mean ± S.E. (n = 3). C, intracellular MUC2 levels of differentiated control, TRPM4 KD, TRPM5 KD, TRPM4 + TRPM5 KD, and NCX2 KD cells. Cells were lysed and analyzed by dot blot with an anti-MUC2 and anti-actin antibody. Intensities of MUC2 spots were quantified using ImageJ and normalized to the actin levels. Results are mean ± S.E. (n = 3). D, control, TRPM4, TRPM5, TRPM4 + TRPM5, and NCX2 stable knockdown cells were starved and incubated for 30 min at 37 °C with 100 μM ATP or vehicle. Secreted MUC5AC was collected, processed for dot blot analysis with an anti-MUC5AC antibody, and quantified using ImageJ. The y axis represents relative values with respect to the values of untreated cells of each condition. Average values ± S.E. are plotted as bar graphs (n = 3). Statistics are shown for 100 μM ATP condition. E, differentiated control, TRPM4 KD, TRPM5 KD, TRPM4 + TRPM5 KD, and NCX2 KD cells were incubated for 30 min at 37 °C with 100 μM ATP or vehicle. Secreted MUC2 was collected and analyzed by dot blot with an anti-MUC2 antibody and analyzed with ImageJ. The y axis of the plot represents relative values with respect to the values of untreated cells for each condition. Average values ± S.E. are plotted as bar graphs (n = 3). Statistical analyses are only shown for 100 μM ATP stimulation. F, control, TRPM4 KD, TRPM5 KD, TRPM4 + TRPM5 KD, and NCX2 KD cells were differentiated by starvation. After starvation, cells were processed for cytotoxic washout, fixed, and permeabilized for analysis by immunofluorescence microscopy with anti-MUC5AC antibody (red), anti-MUC2 antibody (green), and DAPI (blue). Scale bar = 5 μm. Ctrl, control; Neg, reverse transcription without reverse transcriptase; Scr., scrambled shRNA; TRPM4/5 shRNA, TRPM4 + TRPM5 KD cells. *, p < 0.05; **, p < 0.01.

NCX2 and TRPM4 control ATP-mediated Ca²⁺ entry into HT29-18N2 goblet cells

Mucin secretion is a Ca²⁺-dependent process. However, whether the source of Ca²⁺ is intracellular, extracellular, or both is unclear (11, 15, 16). In the airways, PMA (phorbol 12-myristate 13-acetate) can trigger mucin secretion without any dependence on extracellular Ca²⁺ (15–17). We have reported previously that TRPM5 regulates extracellular Ca²⁺ entry, independent of voltage-gated calcium channels, and this event is essential for the physiological ATP-mediated mucin secretion from HT29-18N2 cells (11). Moreover, there is growing evidence on the requirement of extracellular Ca²⁺ for mucin secretion in different cell types under physiological conditions (18–20). It is likely that PMA, a diacylglycerol mimic,
which strongly activates PKC (8), Munc-13 (21), and many other proteins (22, 23), can override the dependence on extracellular Ca\(^{2+}\) in contrast to physiological stimuli such as ATP, menthol, and cold. We therefore retested the role of external Ca\(^{2+}\) in ATP-dependent mucin secretion to understand the involvement of TRPM4/5 and NCX2.

First, we monitored ATP-dependent MUC5AC and MUC2 secretion in the presence (1.2 mM CaCl\(_2\)) or absence (0.5 mM EGTA) of extracellular Ca\(^{2+}\) (30 min at 37 °C). Extracellular medium was collected and analyzed by dot blotting with anti-MUC5AC and anti-MUC2 antibody, respectively. Quantification of the dot blot showed that both MUC5AC (Fig. 2A) and MUC2 secretion (Fig. 2B) require extracellular Ca\(^{2+}\). This confirms our previous findings that MUC5AC secretion by the ATP-mediated pathway depends on extracellular Ca\(^{2+}\). ATP-mediated calcium entry is a combination of ROCE (receptor-operated calcium entry, likely involving TRP channels) and SOCE (store-operated calcium entry, dependent on Orai channels) (24). We used thapsigargin (TG) to inhibit sarco/endo-plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) (25), which ordinarily promotes Ca\(^{2+}\) release from ER and triggers SOCE. Our results show that TG triggered similar levels of calcium release from the ER and higher levels of calcium entry as observed after addition of 1.2 mM CaCl\(_2\) to the extracellular medium compared with ATP stimulation (Fig. 2C, quantification in Fig. 2D), but it did not promote MUC5AC secretion (Fig. 2E). These data reveal that ATP-mediated Ca\(^{2+}\) release from intracellular stores (in the absence of extracellular Ca\(^{2+}\)) or SOCE are insufficient to promote stimulated mucin secretion from HT29-18N2 cells. Extracellular Ca\(^{2+}\) entry (involving TRPM5 as shown previously (11)), is, however, necessary for both MUC5AC and MUC2 release.

Second, we measured intracellular Ca\(^{2+}\) levels with the calcium dye Fura-2/AM in differentiated HT29-18N2 cells upon stimulation with 100 μM ATP. Our data confirm that in the absence of extracellular Ca\(^{2+}\) (medium containing 0.5 mM EGTA), ATP triggered Ca\(^{2+}\) release from the intracellular stores, and, importantly, it promoted Ca\(^{2+}\) entry into the cells after addition of 1.2 mM CaCl\(_2\) to the extracellular medium (Fig. 2C, black symbols). We then tested whether TRPM4, TRPM5,
and NCX2 had a role in ATP-mediated Ca\(^{2+}\) entry into the cells. We measured intracellular Ca\(^{2+}\) levels in differentiated NCX2 KD, TRPM4 KD, TRPM5 KD, TRPM4 + TRPM5 KD, and control HT29-18N2 cells with the calcium dye Fura-2/AM and 100 \(\mu M\) ATP as the stimulus (in the presence or absence of extracellular Ca\(^{2+}\)).

Our data show that Ca\(^{2+}\) release from internal stores was not affected in any of the knockdown cells compared with control cells (Fig. 2F), however, there was a clear reduction in Ca\(^{2+}\) entry after addition of extracellular Ca\(^{2+}\). Quantification of the data shown in Fig. 2F reveal a 20% reduction in Ca\(^{2+}\) entry for TRPM4 and TRPM5 KD cells, a 37.5% reduction for the TRPM4 + TRPM5 KD cells, and a 40% reduction in NCX2 KD cells compared with control cells (Fig. 2G). Considering that both TRPM4 and TRPM5 are Na\(^+\) channels activated by similar stimuli (i.e., intracellular Ca\(^{2+}\) and PKC) (13), and NCX2 can promote Ca\(^{2+}\) entry by operating in the reverse mode (26), we suggest that functional coupling of TRPM4/TRPM5 and NCX2 facilitates reverse mode NCX activity. We do not claim a direct interaction of TRPM4/TRPM5 and NCX2, but we propose that TRPM4/TRPM5 and NCX2 are in the same pathway of ATP-mediated calcium entry to promote mucin secretion from HT29-18N2 cells.

**MUC5AC is secreted by differentiated airway cells**

We tested whether TRPM4/TRPM5 and NCX have a role in mucin secretion in two different airway cell lines: 1) NHBE, normal human bronchial epithelial cells, and 2) CFT1-LC3 cells, a human tracheal epithelial cell line derived from a patient with cystic fibrosis (CF) presenting homozygous mutation F508 in cystic fibrosis transmembrane conductance regulator (CFTR) and transformed with human papilloma virus E6/E7 genes and a retroviral vector alone (27). CFT1-LC3 cells are used extensively to study defective hydroelectrolyte transport characteristic of CF (28). Patients with CF exhibit accumulation of sticky mucus in the lungs, pancreas, intestine, and reproductive organs. Because secreted mucins are a major component that control the rheological properties of mucus (2), understanding the pathway of mucin secretion could potentially provide new targets to potentially alleviate symptoms associated with CF (29).

To study MUC5AC secretion by airway cells, we used the air-liquid interface (ALI) procedure, which effectively promotes normal human tracheal and bronchial epithelial cells differentiation into mucin-secreting cells (30). Briefly, airway epithelial cells are plated on a porous membrane to form a monolayer. The basal plasma membrane of these cells is exposed to the medium, whereas the apical surface faces the air. We used this standard protocol that includes incubation with interleukin-13 (IL-13) to differentiate NHBE and CFT1-LC3 cells into secretory goblet cells (see Fig. 3A). To assess if both cell lines are differentiated into mucin-producing cells by this protocol, we first tested the mRNA and protein levels of MUC5AC. Total RNA was extracted from undifferentiated and differentiated NHBE and CFT1-LC3 cells, and expression of MUC5AC was tested by RT-PCR and qPCR using specific primers for MUC5AC (and GAPDH as reference gene). As shown in Fig. 3B, MUC5AC levels in differentiated compared with undifferentiated NHBE and CFT1-LC3 cells increased by 3.5- and 3-fold, respectively (Fig. 3B). MUC5AC protein levels were determined in cell lysates of undifferentiated and differentiated NHBE and CF cells, respectively, with specific anti-MUC5AC antibody by the dot blot procedure. Our data reveal that both NHBE and CFT1-LC3 cells up-regulate MUC5AC protein levels upon differentiation compared with undifferentiated cells (Fig. 3C). Finally, we used a combination of three different mucin secretagogues (100 \(\mu M\) ATP, 10 ng/ml of IL-13, and 300 nm PMA) (8, 11, 31) to trigger MUC5AC secretion. Although ATP is a potent stimulus for mucin secretion (16), we supplemented it with IL-13 (which stimulates MUC5AC expression and also increases its secretion) (31) and limiting quantities of PMA (strong activator of PKC and known to cause MUC5AC secretion) (8, 11) to increase the amount of MUC5AC secreted by both NHBE– and CFT1-LC3– differentiated cells, thereby making detection easier in the extracellular space. Importantly, only differentiated cells secrete MUC5AC after stimulation with ATP, IL-13, and PMA (Fig. 3D).

**TRPM4 and NCX1/2/3 are expressed in differentiated airway cells**

After differentiation, RNA from NHBE and CFT1-LC3 cells was extracted, retro-transcribed into cDNA, and analyzed by PCR (primers in Table 1). Our results show that TRPM4, but not TRPM5, and all 3 members of NCX family (NCX1, NCX2, and NCX3) are expressed in differentiated NHBE and CFT1-LC3 cells. MUC5AC was detected as a positive control for differentiation of cells and HPRT1 was used as a loading control (Fig. 3E). Taken together, our findings reveal that both differentiated NHBE and CF-derived cells express specific members of the TRPM4/TRPM5 and NCX family of proteins. We therefore tested whether NCX’s reverse mode pathway is required for mucin secretion by these airway cells.

**Inhibition of NCX’s reverse mode blocks MUC5AC secretion in both NHBE and CF cells**

To test the involvement of NCX in MUC5AC secretion, we applied a mixture containing 100 \(\mu M\) ATP, 10 ng/ml of IL-13, and 300 nm PMA or a vehicle to CFT1-LC3 and NHBE cells (1 h at 37 °C). These exogenous stimuli promoted MUC5AC secretion from both NHBE– and CFT1-LC3– differentiated cells (Fig. 4, A and B, control bars, respectively) compared with cells treated with the vehicle alone. We then tested whether inhibition of NCX’s reverse mode affected mucin secretion from these cells by using the benzoxoxyphenyl analog KB-R7943, which preferentially inhibits NCX’s reverse mode (32, 34–36). Although KB-R7943 affects all 3 isoforms of human NCXs, it is 3-fold more effective in inhibiting NCX3 compared with its effects on NCX1 and NCX2 (33). Our results demonstrate that 50 \(\mu M\) KB-R7943, a concentration previously described to inhibit NCX in HT29-18N2 cells (11), completely abolished MUC5AC secretion in NHBE and CFT1-LC3– differentiated cells (Fig. 4, A and B, respectively). We cannot rule out the possibility that KB-R7943 affects mucin secretion by its effects on other membrane channels and transporters, but it is certainly not via SOCE (34), the \(N\)-methyl-D-aspartate receptor (35), neuronal nicotinic acetylcholine receptors (36), and the...
norepinephrine transporter (37), as none of these components are directly involved in MUC5AC secretion.

To further assess the involvement of NCX’s reverse mode in MUC5AC secretion by CF cells, we tested the effect of two other inhibitors of NCX: SN-6 and benzamil. SN-6, a benzyloxyphenyl analog, is developed from KB-R7943 and known to inhibit NCXs’ reverse mode more effectively than the forward mode. SN-6 is a more selective inhibitor of NCX than KB-R7943 and at the optimal concentration of 5 μM does not affect other receptors, transporters, or ion channels (38, 39). Moreover, SN-6 inhibits NCX1 more potently than NCX2 and NCX3 (38). Benzamil is an amiloride analog, which inhibits both the forward and reverse mode of all three NCX isoforms (40). As shown in Fig. 4B, these two inhibitors reduced MUC5AC secretion compared with cells treated with vehicle alone. Importantly, the effect of SN-6 (93% reduction) was very similar to the effect of KB-R7943 (95% reduction) and much stronger than benzamil (65% reduction), which is in accordance with our hypothesis that NCX’s reverse mode, and not the other membrane currents, regulates MUC5AC secretion (Fig. 4B).

**Extracellular Ca²⁺ and TRPM4 are required for MUC5AC secretion in CF cells**

Our results indicate that the NCX family of exchangers is required for MUC5AC secretion from colonic (HT29-18N2), bronchial (NHBE), and CF derived (CFT1-LC3) cells. Knockdown of TRPM4 affected MUC5AC secretion from HT29-18N2 cells and we asked whether it also had a role in MUC5AC secretion by CFT1-LC3 cells. To test this, we used 9-phenanthrol, which is known to specifically inhibit TRPM4 (41). We tested its specificity in TRPM4 KD HT29-18N2 cells, and our
TRPM4 and NCXs mediated mucin secretion

Discussion

Secreted mucins are the main components of mucus that protects the epithelium from pathogens, allergens, and toxic compounds. The overall mechanism of how these heavily glycosylated mucins are released from the specialized epithelial goblet cells is not well understood (42). The 21 different mucins expressed in humans (endorsed by the HUGO gene nomenclature committee) can be classified into two kinds: the plasma membrane bound (transmembrane) and the gel-forming (secreted) mucins (43). The membrane-tethered mucins (like MUC1, MUC4, or MUC16) are involved in hydration and lubrication of epithelia, and protection against pathogens, whereas the secreted mucins (such as MUC5AC, MUC2, or MUC5B) are important for the formation, function, and rheological properties of the mucus layer (2, 43). A screen for proteins involved in the release of MUC5AC, a secreted gel-forming mucin, by colonic goblet cancer cells revealed new candidates of which TRPM5, a sodium channel, was described previously (11).

Our new findings reveal that the sodium importer TRPM4 and possibly its ortholog TRPM5 function via a sodium/calcium exchanger (NCX) to control mucin secretion in cells of the airways and the colon. This scheme reveals a program that regulates ATP-dependent mucin secretion by controlling calcium entry through TRPM4/TRPM5 and NCXs. It is important to note that both TRPM4 and TRPM5 sodium channels, and only NCX2, are expressed in colon cells; whereas the airway cells express TRPM4 and all 3 sodium/calcium exchangers. What is the significance of these differential combinations of TRPMs and NCXs in these tissues? We entertain the possibility that the combination of TRPM4 + TRPM5 and NCX is adapted based on the stimuli to control the quantity of mucin secretion from the respective cells. In a tissue with a need for sustained mucin release to generate a large mucus layer (i.e. allergic response, inflammation), it is sufficient to utilize only one NCX, but two different sodium channels that can be activated after stimuli (to maintain the signal). In the airways, on the other hand, where a sudden increase in mucin secretion is needed to protect the tissue (i.e. after exposure to allergens or pathogens), it is important to exploit all NCX members to provoke a strong

Figure 4. Involvement of NCXs in MUC5AC secretion by airway cells. A, differentiated NHBE cells were pretreated for 15 min at 37 °C with DMSO (Control) or 50 μM KB-R7943 (NCX inha.). After 15 min, a mixture containing 100 μM ATP + 10 ng/ml of IL-13 + 300 nM PMA, and the respective drug (DMSO or KB-R7943) was added to the cells and incubated for 1 h at 37 °C. Secreted MUC5AC was collected and processed for dot blot with an anti-MUC5AC antibody. The y axis represents relative values with respect to the values of the basal of each condition. Results are average values ± S.E. (n = 3). B, differentiated CFT1-LC3 cells were pretreated for 15 min at 37 °C with DMSO (Control), 50 μM KB-R7943 (KB-R), 5 μM benzamil (Benz.), or 5 μM SN-6. After 15 min, a mixture containing 100 μM ATP + 10 ng/ml of IL-13 + 300 nM PMA, and the respective drug (DMSO, KB-R7943, benzamil, or SN-6) was added to the cells and incubated for 1 h at 37 °C. Secreted MUC5AC was collected and processed for dot blot with an anti-MUC5AC antibody. The y axis represents relative values with respect to the values of the basal of each condition. Results are average values ± S.E. (n = 3). C, differentiated CFT1-LC3 cells were incubated for 1 h at 37 °C with DMSO (Control) or 10 μM 9-phenanthrol (TRPM4 inh) in the presence or absence of stimuli (100 μM ATP + 10 ng/ml of IL-13 + 300 nM PMA). Secreted MUC5AC was collected and processed for dot blot with an anti-MUC5AC antibody. The y axis represents relative values with respect to the values of the basal of each condition. Results are average values ± S.E. (n = 3). D, CFT1-LC3 differentiated cells were incubated for 1 h at 37 °C with a mixture containing 100 μM ATP + 10 ng/ml of IL-13 + 300 nM PMA or vehicle in the presence (w/Ca2+) or absence of Ca2+ (w/o Ca2+). Secreted MUC5AC was collected and processed for dot blot analysis with an anti-MUC5AC antibody. The dot blots were quantified using ImageJ. The y axis represents relative values with respect to the values of vehicle-treated cells for each condition. Average values ± S.E. are plotted as bar graphs (n = 3), NCX inh, NCX inhibitor KB-R7943; TRPM4 inh, TRPM4 inhibitor 9-phenanthrol. *, p < 0.05; **, p < 0.01.

data reveal that 9-phenanthrol inhibited MUC5AC secretion in control cells only. The effect of 9-phenanthrol treatment was not enhanced in cells depleted of TRPM4 (Fig. S2B). Briefly, CFT1-LC3–differentiated cells were stimulated with a mixture containing 100 μM ATP, 10 ng/ml of IL-13, and 300 nM PMA (1 h at 37 °C), resulting in a significant increase in extracellular MUC5AC (2-fold increase) that was completely abolished by 10 μM 9-phenanthrol treatment (Fig. 4C). To test the requirement of extracellular calcium in MUC5AC secretion by differentiated CFT1-LC3 cells, the cells were treated with stimuli (100 μM ATP, 10 ng/ml of IL-13, and 300 nM PMA) in the presence (1.2 mM CaCl2) or absence (0.5 mM EGTA) of extracellular Ca2+ (1 h at 37 °C). As shown in Fig. 4D, removal of extracellular Ca2+ strongly reduced stimulated mucin secretion (Fig. 4D).

In sum, our results show that NCX’s reverse mode, TRPM4, and extracellular calcium are required for MUC5AC secretion in CFT1-LC3 cells.
increase in calcium uptake to trigger rapid mucin secretion. Another possibility we entertain, based on our data, is that TRPM4 is the main regulator for both MUC2 and MUC5AC release from the colonic and airway cells, whereas TRPM5 likely acts as a modulator of mucin secretion and has an important role in mucous-related pathologies. Importantly, our data reveal that knockdown of TRPM4 + TRPM5 and NCX has a greater effect on the physiologically secreted MUC2 by colonic cells (HT29-18N2) and MUC5AC release from the airway cells (NHBE and CFT1-LC3), respectively. MUC2 and MUC5AC are the physiologically relevant secreted mucins of the colon and airway goblet cells, respectively, so knocking down the specific isoform of TRPM and NCX expressed in these cells has a greater impact on the secretion of corresponding mucins. MUC5AC is expressed and secreted by colonic cancer cells and not the normal colon cells, which might explain a partial effect on its secretion by TRPM4/TRPM5 and NCX knockdown. Meaning, there is an optimal pairing of the calcium import machinery and the mucins for efficient mucin secretion in a cell type-specific manner.

The isoforms of these two classes of proteins involved vary in a tissue-specific manner, however, the general principle is the same as described below (Fig. 5). We present two albeit speculative distinct modes of mucin secretion: baseline mucin secretion (Fig. 5A) and stimulated mucin secretion (Fig. 5B).

**Baseline mucin secretion**

In the absence of an external stimulus, TRPM4/TRPM5 are not active. Under these conditions, the cells control mucin secretion by coupling the function of intracellular calcium oscillations to KChIP3 (7). An increase in intracellular calcium threshold by ryanodine receptor-mediated release of calcium from the ER, loads calcium onto KChIP3 causing its release from the mature granule, which subsequently fuse to plasma membrane. This procedure is independent of low affinity calcium sensor synaptotagmin 2 (17) and used by cells to control mucin secretion in the absence of extracellular calcium (Fig. 5A).

**Stimulated mucin secretion**

When cells are stimulated (for example, by ATP or IL-13), there is a rapid burst of Ca^{2+} released from the ER, which activates TRPM4/TRPM5. Once activated, TRPM4/TRPM5 permeate sodium into the cytoplasm. The local increase in Na^{+} concentration in the proximity of NCX triggers them to act in the reverse mode and pump calcium into the cells. This increases local Ca^{2+} concentration and engages the low affinity calcium sensor synaptotagmin 2 to promote fusion of mucin granules to plasma membrane (17). This procedure accounts for a rapid burst in mucin secretion under conditions such as exposure of cells to exogenous stimuli (Fig. 5B).

In conclusion, our data are beginning to reveal the mechanism by which cells use intracellular versus extracellular calcium to control baseline and stimulated mucin secretion. We suggest that KChIP3 functions in baseline secretion (7), and TRPM4/TRPM5/NCXs in stimulated mucin secretion are potential pharmacological targets to help control dysregulated mucin secretion observed in patients with diseases of the airways and the colon.

**Experimental procedures**

**Reagents and antibodies**

All chemicals were obtained from Sigma, except IL-13 (R&D Systems, Minneapolis, MN), anti-MUC2 antibody clone 996/1 (RRID:AB_297837) (Abcam, Cambridge, UK), and anti-MUC5AC antibody clone 45M1 (RRID: AB_934745) (Neomarkers, Waltham, MA). Secondary antibodies for immunofluorescence microscopy and dot blotting were from Life Technologies (ThermoFisher Scientific, Waltham, MA).

**Cell lines**

HT29-18N2 cells (from ATCC, RRID: CVCL_5942), CFT1-LC3 cells (a kind gift of Dr. J. Yankaskas, University of North Carolina at Chapel Hill) (RRID:CVCL_G214), and NHBE cells were purchased from Lonza (Basel, Switzerland) (RRID:CV-
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CL_S124). All cell lines were authenticated by the suppliers, and their phenotype previously demonstrated (44, 45). HT29-18N2, NHBE, and CFT1-LC3 cell lines tested negative for mycoplasma contamination by the Lookout® mycoplasma PCR detection kit (Sigma).

**Differentiation of HT29-18N2 cells**

HT29-18N2 cells were differentiated to goblet cells as described previously (11). Briefly, cells were seeded in complete growth medium (Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, and 1% penicillin/streptomycin), and the following day (D0) medium was changed to PFH-MII protein-free medium (GIBCO, ThermoFisher Scientific). After 3 days (D3), medium was replaced with fresh PFHMII medium and cells were grown an 3 additional days. At day 6 (D6) cells were seeded in complete growth medium for different experiments following replacement of complete growth medium for PFHMII medium at day 7 (D7). All experimental procedures were performed at day 9 (D9).

**Differentiation of CF and NHBE cells**

CFT1-LC3 or NHBE cells were plated in 24-mm Transwell plates with 0.4-μm pore polyester membrane insert (Costar, Corning number 3470, Sigma) pre-coated with collagen type I (0.03 mg/ml) (BD Biosciences, San Jose, CA). B-ALI growth medium supplemented with SingleQuots™ (according to the manufacturer’s instructions) (Lonza) was then added to both apical compartment was changed to B-ALI Differentiation Medium (D0) until the day of experiment (D21), the medium was changed to basolateral sides) and lysed in 1% Triton X-100, 1 mM DTT, 1× PBS for 1 h at 4 °C and centrifuged at 10,000 × g for 30 min (cell lysate).

**Dot blot analysis**

Supernatants and cell lysates were spotted on nitrocellulose membranes (0.45 μm) using a Bio-Dot Microfiltration Apparatus (Bio-Rad) (manufacturer’s protocol) and membranes were incubated in blocking solution (4% BSA, 0.1% Tween/PBS) for 1 h at room temperature. The blocking solution was removed and the membranes incubated with anti-MUC5AC antibody diluted 1:1000 or anti-MUC2 antibody diluted 1:1000 in blocking solution, overnight at 4 °C. Membranes were then washed in 0.1% Tween/PBS and incubated with a donkey anti-mouse or anti-rabbit Alexa 647-coupled antibody (Life Technologies) for 30 min at room temperature. For the detection of β-actin, cell lysates were separated on SDS-PAGE, transferred to nitrocellulose membranes, and processed as described for the dot blot analysis using anti-β-actin antibody (RIRD:AB_476692) at a dilution of 1:5000. Membranes were washed and imaged with LI-COR Odyssey® scanner (resolution = 84 μm) (LI-COR, Nebraska). Quantification of the dot blots was performed with ImageJ (FIJI, version 2.0.0-rc-43/1.51g) (46). Linearity and specificity of MUC2 antibody in our assay are provided in Fig. S3. Fig. S3A shows the detection of MUC2 antibody to compare basal and stimulated secretion (Fig. S3A). Fig. S3B shows serial dilutions of cell lysate to determine the linearity and detection limit of our assay (Fig. S3B).

**Immunofluorescence analysis**

Differentiated HT29-18N2 (Control, TRPM4 KD, TRPM5 KD, TRPM4 + TRPM5 KD, or NCX2 KD) cells were grown on coverslips. To visualize intracellular MUC5AC and MUC2 granules, differentiated HT29-18N2 cells were washed twice at room temperature with PBS for 5 min. Next, cells were permeabilized by incubation in a buffer composed of 20 mM HEPES, pH 7.4, 110 mM KOAc, 2 mM MgOAc, and 0.5 mM EGTA (adapted from Ref. 47) with 0.001 mM digitonin for 5 min on ice, followed by washing for 7 min on ice with the same buffer without detergent. Then, cells were fixed in 4% paraformaldehyde for 15 min, further permeabilized for 5 min with 0.001% digitonin in the same buffer without detergent and blocked with 4% BSA in 1× PBS for 15 min. The anti-MUC5AC antibody was added to cells at 1:5000 in 4% BSA/PBS overnight at 4 °C. The anti-MUC2 antibody was added to cells at 1:500 in 4% BSA/PBS overnight at 4 °C. Next day, the cells were washed with PBS and incubated for 1 h at room temperature with a donkey anti-rabbit Alexa 555 (for MUC2), anti-mouse Alexa 647 (for MUC5AC) (Life Technologies), diluted at 1:1,000 in 4% BSA/PBS, and DAPI (1:20,000). Finally, cells were washed in PBS and mounted in Mucin secretion assay for HT29-18N2 cells

HT29-18N2 cells were differentiated for 6 days and then split into 6-well plates. One day later (D7), the medium was exchanged with fresh PFHMII medium and cells were grown for 2 more days. On D9, cells were washed with isotonic solution containing: 140 mM NaCl, 2.5 mM KCl, 1.2 mM CaCl₂, 0.5 mM MgCl₂, 5 mM glucose, and 10 mM HEPES (305 mosmol/liter, pH 7.4, adjusted with Tris). A mix containing 100 μM ATP, 10 ng/ml of IL-13, and 300 nM PMA or vehicle was then added to both compartments, and cells were incubated for 1 h at 37 °C. Medium from the apical compartment was collected and centrifuged for 5 min at 800 × g at 4 °C (supernatant). The cells were then washed 2 times in PBS (apical and basolateral sides) and lysed in 1% Triton X-100, 1 mM DTT, 1× PBS for 1 h at 4 °C and centrifuged at 10,000 × g for 30 min (cell lysate).

**Mucin secretion assay for CFT1-LC3 and NHBE cells**

Mucin secretion assays for CFT1-LC3 and NHBE cells were performed on day 21 (D21), 3 days after removing IL-13 from the medium to allow the accumulation of mucins. Both apical and basolateral compartments of Transwells were washed with isotonic solution containing: 140 mM NaCl, 2.5 mM KCl, 1.2 mM CaCl₂, 0.5 mM MgCl₂, 5 mM glucose, and 10 mM HEPES (305 mosmol/liter, pH 7.4, adjusted with Tris). A mix containing 100 μM ATP, 10 ng/ml of IL-13, and 300 nM PMA or vehicle was then added to both compartments, and cells were incubated for 1 h at 37 °C. Medium from the apical compartment was collected and centrifuged for 5 min at 800 × g at 4 °C (supernatant). The cells were then washed 2 times in PBS (apical and basolateral sides) and lysed in 1% Triton X-100, 1 mM DTT, 1× PBS for 1 h at 4 °C and centrifuged at 10,000 × g for 30 min (cell lysate).
Flucin resistance (10 were collected, filtered, and directly added to HT29-18N2 cells.

**Expression profile and qPCR**

Undifferentiated and differentiated cells (HT29-18N2, NHBE, and CFT1-LC3) were lysed and total RNA extracted with the RNeasy extraction kit (Qiagen, Netherlands). Total RNA was treated with DNase I (New England Labs, Ipswich, MA) for 1 h at 37 °C and purified by phenol extraction. cDNA was synthesized with Superscript III (Invitrogen). Primers for each gene (sequence shown below, Table 1) were designed using Primer-BLAST (NCBI) (48), limiting the target size to 300 bp and the annealing temperature to 60 °C. To determine expression levels of MUC5AC, NCX2, TRPM5, and TRPM4, quantitative real-time PCR was performed with Light Cycler 480 SYBR Green I Master (Roche Applied Science, Switzerland) according to the manufacturer’s instructions.

**Generation of stable shRNA knockdown cell lines**

HEK293 cells (ATCC, negative for mycoplasma) were co-transfected with the plasmid, vesicular stomatitis virus-G, pPRE (packaging), and REV by calcium phosphate to produce lentiviruses. 48 h post-transfection, the secreted lentiviruses were collected, filtered, and directly added to HT29-18N2 cells. Stably infected HT29-18N2 cells were selected either by purocytin resistance (10 μM) or sorted for GFP positive signal by FACs.

**Measurement of intracellular [Ca2+]**

HT29-18N2 cells were plated on glass coverslips, loaded with 5 μM Fura-2/AM for 30 min at room temperature, washed, and bathed in an isotonic solution containing: 140 mM NaCl, 2.5 mM KCl, 1.2 mM CaCl2, 0.5 mM MgCl2, 5 mM glucose, 10 mM HEPES (305 mosmol/liter, pH 7.4, adjusted with Tris). Ca2+ free solutions were obtained by replacing CaCl2 with equal amounts of MgCl2 plus 0.5 mM EGTA. Different stimuli were added to the bath solution as indicated in the figure legend. All experiments were carried out at room temperature as previously described (49). AquaCosmos software (Hamamatsu Photonics) was used for capturing the fluorescence ratio at 505 nm obtained post- excitation at 340 and 380 nm. Images were computed every 5 ms. Area under the curve (AUC) was calculated using SigmaPlot 10 software.

**Statistical analysis**

All data shown are mean ± S.E. Statistical analysis was performed using Student’s t test (continuous data, two groups) or one-way analysis of variance for the comparison of more than 2 groups (GraphPad Prism 6 (RRID:SCR_002798) or SigmaPlot 10 (RRID:SCR_003210) software). Bonferroni’s test was used for post hoc comparison of the mean values. Criteria for a significant statistically significant difference were: *p < 0.05; **p < 0.01.

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