The Epac1 signaling pathway regulates Cl⁻ secretion via modulation of apical KCNN4c channels in diarrhea

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Running title: Regulation of apical KCNN4c channel by Epac1 signaling in epithelial Cl⁻ secretion

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Background: Apical KCNN4c channel provides driving force for cAMP induced Cl⁻ secretion. Results: Epac1-Rap1A-RhoA-ROCK signaling affect Cl⁻ secretion via effects on the apical expression of KCNN4c channels. Conclusion: This mechanism couples the surface expression of KCNN4c channels and Cl⁻ secretion in diarrhea. Significance: Apical KCNN4c channels are a new target for adjunct therapy in diarrhea.

SUMMARY

The apical membrane of intestinal epithelia expresses intermediate conductance K⁺ channel (KCNN4) which provides driving force for Cl⁻ secretion. However, its role in diarrhea and regulation by Epac1 is unknown. Previously we have established that Epac1 upon binding of cAMP activates PKA-independent mechanism of Cl⁻ secretion via stimulation of Rap2-PLCε-[Ca²⁺]i signaling. Here we report Epac1 regulates surface expression of KCNN4c channel through its downstream Rap1A-RhoA-ROCK signaling pathway for sustained Cl⁻ secretion. Depletion of Epac1 protein and apical addition of TRAM-34, a specific KCNN4 inhibitor significantly abolished cAMP stimulated Cl⁻ secretion and apical K⁺ conductance [I(Kap)] in T84WT cells. Current-Voltage relationship of basolaterally permeabilized monolayers treated with Epac1 agonist, 8-pCPT-2'-O-Me-cAMP, showed the presence of an inwardly rectifying and TRAM-34 sensitive K⁺ channel in T84WT cells which was absent in Epac1KDT84 cells. Reconstructed confocal images in Epac1KDT84 cells revealed redistribution of KCNN4c proteins into sub-apical intracellular compartment while biotinylation assay shows ~83% lower surface expression of KCNN4c proteins compared to T84WT cells. Further investigation revealed an Epac1 agonist activates Rap1 to facilitates [I(Kap)]. Both RhoA inhibitor (GGTI298) and ROCK inhibitor (H1152) significantly reduced cAMP agonist stimulated I(Kap) while later additionally show reduced co-localization of KCNN4c with apical membrane marker WGA in T84WT cells. In vivo mice ileal loop experiments show reduced fluid accumulation by TRAM-34, GGTI298 or H1152 when injected together with cholera toxin into the loop. We conclude that Rap1A dependent signaling of Epac1 involving RhoA-ROCK is an important regulator of intestinal fluid transport via modulation of apical KCNN4c channels, a finding with potential therapeutic value in diarrheal diseases.

INTRODUCTION

Epithelial ion transport is a dynamic process that refers to both secretion and absorption of fluid and electrolytes across the gastro-intestinal cells (1-4). Disturbances of this normal vectorial transport process under pathophysiologic conditions like pathogenic infection or genetic mutation, often manifest diarrhea where either active secretion is enhanced or absorption process is inhibited or in some cases both are affected (5). In the case of secretory diarrhea, there is an
enhanced Cl⁻ secretion along with Na⁺ and water drawn osmotically into the intestinal lumen, leading to a massive loss of important electrolytes and fluid from the body and potentially death (6-10). For the sustained loss of Cl⁻ through the apical Cl⁻ channels (CFTR, CaCC) not only must Cl⁻ be replenished through the basolateral NKCC1 co-transporter but various K⁺ channels must also maintain a favorable hyperpolarized condition to counteract Cl⁻ efflux mediated depolarization of the cells. Thus, in epithelial function, second messenger activated K⁺ channels are essential players in the process of transepithelial Cl⁻ secretion (11). In gastrointestinal epithelial cells, they recycle K⁺ across the membrane via multiple types of K⁺ channels to maintain the driving force for Cl⁻ exit. Ca²⁺-activated K⁺ channel(s) have been suggested to participate in the generation of the driving force for Cl⁻ secretion. Indeed, it is usually assumed that only a basolateral K⁺ conductance produced from Ca²⁺ stimulation would actually facilitate the efficiency of the secretion mechanism (12-13). However, there is considerable experimental evidence to suggest that Ca²⁺-activated K⁺ channel of the intestinal apical membrane has a significant role in Cl⁻ secretion (14-15). Though the Ca²⁺-activated KCNN4 channel is expressed both at apical and basolateral membrane in colonic epithelia, contribution of only basolateral KCNN4 channel is credited for maintaining the favorable electrochemical gradient to sustain Cl⁻ secretion thus neglecting the contribution of an apical KCNN4 channel(16). The significance of an apical K⁺ channel was first highlighted by Young et al in 1989 when he mathematically proved apical K⁺ channels actually increase Cl⁻ secretion in polarized secretory epithelia (17). Recently, Rajendran et al demonstrated apical KCNN4c(apical version of KCNN4 channel) provides the driving force for Cl⁻ secretion and also contributes to stool K⁺ loss during secretory diarrhea in rat distal colon(18).

KCNN4 is an intermediate conductance calcium activated K⁺ channel of the KCNN family and functions primarily in a variety of non-excitable cells including intestinal epithelia (19-21). Currently, three splice variants are known - KCNN4a, b & c - expressed in smooth muscle cells, basolateral and apical membrane of colonic enterocytes, respectively (22). Though Ca²⁺ is the main modulator of the KCNN family of ion channels, cAMP has also been suggested to modulate these ion channels. This coupling or coordination between Ca²⁺ and cAMP has been most widely appreciated in the BK channel where cAMP and PKA dependent phosphorylation shifts the affinity of the BK channels for Ca²⁺, making it more active at physiological [Ca²⁺]. (23). However, it has been demonstrated that BK channels play no essential role in the generation of the driving force for colonic electrogenic Cl⁻ secretion (24). An earlier study reported a dual mode of activation for the KCNN4 channel by these second messengers during physiological responses of the cells (25-26). In line with these observations, we have demonstrated previously a link between two second messengers: cAMP and Ca²⁺ via Epac1 (Exchange protein directly activated by cAMP) -Rap2 signaling, which is involved in cholera toxin (CT) stimulated Cl⁻ secretion. However, activation of Rap1 by cAMP is also achieved by the binding of cAMP to Epac proteins. The role of Rap1 in intestinal epithelial ion transport remains relatively unexplored. Epac activates Rap1 by catalyzing the conversion of GDP-Rap1 to GTP-Rap1, which is independent of classical cAMP/PKA signaling. Active GTP-Rap1 may act via its downstream signaling RhoA-ROCK in the pathogenesis of secretory diarrhea.

Hence, the present study meant to explore the hypothesis that Epac1 and its associated signaling may influence apical KCNN4c channel function via Rap1-RhoA-ROCK signaling pathway in cAMP-stimulated Cl⁻ secretion. We employed electrophysiology techniques that allows measurement of agonist induced short circuit current (Isc) and apical K⁺ conductance [I[K(ap)] in a polarized epithelium. The results indicate that activation of apical KCNN4c channels by Epac1 signaling is required to support Cl⁻ secretion induced by cAMP. Furthermore, our results strongly suggest that Epac1 and its downstream signaling might regulate the surface amount of functional KCNN4c protein. More importantly, the second key observation arise from our study that potentially targets the apical KCNN4c
channel could provide a novel option to combat secretory diarrhea with ORS therapy.

MATERIAL & METHODS

Reagents- Unless otherwise stated, all chemicals used in this study were obtained from Sigma-Aldrich. Cell culture media and Fetal Bovine Serum (FBS), were purchased from Cell Clone (catalog no. cc3021) and Himedia (catalog no. RM9970), respectively. Puromycin (catalog no. ant pr-1) was purchased from Invivogen (USA). cDNA synthesis reagents were purchased from Invitrogen (catalog no. 11904-018) and Real Time PCR master mix from Applied Biosystem (catalog no.4309155). Penicillin-streptomycin was obtained from Gibco. Wheat Germ Agglutinin (WGA) was purchased from Molecular Probes. TRIZOL (catalog no. 15596-026), FITC (catalog no. A11036) and alexa 568 conjugated secondary antibody from Invitrogen. C3 toxin (catalog no. CT04) was purchased from Cytoskeleton Inc., USA. 8-pCPT-2’-O-Me-cAMP (catalog no. C051-01) was purchased from Biolog Life Science Institute, Germany. KCNN4abc antibodies was a gift from Dr. V. M. Rajendran’s laboratory at University of West Virginia, USA and GAPDH was obtained from cell signaling.

Cell Culture- Wild type human colonic T84 intestinal epithelial cells (T84WT) and Epac1 Knockdown in T84WT cells (Epac1KDT84) cells obtained from Gastroenterology Division of the John Hopkins University. T84WT cells were routinely maintained in 1:1 ratio of Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F-12 medium supplemented with 10% fetal bovine serum, 100U/ml penicillin and 100 µg/ml streptomycin. For Epac1KDT84 cells, additional puromycin (10 µg/ml) was added in the media. Briefly, T84WT cells between passages 8 and 20 were seeded onto polycarbonate membrane, 12-mm Snapwell permeable support cell culture inserts (0.4 µm pore size; Costar, USA, catalog no. 3407) and grown for 10 to 14 days, during which time the media were changed every 48 hours. Monolayer resistance was determined using an EVOM ohmmeter with STX2 electrodes (World Precision Instruments, Inc. USA). Monolayers were considered polarized and mounted in Ussing’s chamber when resistance was equal to or greater than 1,500 Ω/cm².

RNA Interference:

Rap1A and Rap1B Knockdown by Lentiviral shRNA

The RNA interference Consortium (TRC) Lentivirus-based short hairpin RNA (shRNA) were used to knockdown Rap1A and Rap1B in T84WT cells according to our previously described method (27). In brief, gene sequence-specific shRNA clones constructed within the lentivirus plasmid vector pLKO.1-puromycin were obtained from Sigma-Aldrich. Four lentivirus-mediated shRNAs for each gene were designed to trigger the gene silencing which are listed below (Table 1). Stable cell lines of T84WT with expression of Rap1A and Rap1B knockdown were generated by infecting cells with respective gene-specific Lentiviral particles, and positively transduced cells were selected with 10µg/ml of puromycin-containing medium. mRNA expression in transduced cells was evaluated by qPCR as well as by measuring Isc. The Lentiviral shRNA construct specific for green fluorescent protein (GFP) were transduced in T84WT cells and served as negative control. The efficiency of lentivirus-mediated siRNA infection in T84WT cells was determined using fluorescence microscopy to observe lentivirus mediated GFP expression (data not shown).

Real-time PCR (qPCR)- Analysis of mRNA expression was performed compared with GAPDH using the difference of PCR cycles to reach a threshold amplification (ΔCT), and the relative amount of the target mRNA is given as 2^(-ΔCT). Real-time PCR was performed on an Applied Biosystems AB7900 real-time PCR detection system programmed as follows: Step 1, 1 cycle, 50°C for 2 min; step 2, 1 cycle, 95°C for 10 min; step 3, 45 cycles, 95°C for 15 s, 60°C for 30 s, 72°C for 30 s; then step 4(dissociation, ramp rate 2%), 95°C for 15 s, 60°C for 15 s, and 95°C for 15 s. Amplification reactions were performed in a final volume of 25μl containing cDNA from 30 ng of reverse-transcribed total RNA, 300 nm each of forward and reverse primers for Rap1A (F-GACCTGAGGGAACAGATTTTAC; R- CCTGCTCTTTTGCCAACACTAC; accession number NM_001010935) and Rap1B (F-TTCCATCACGACAGATTTTAC; R- CCCTACAACCTTTTCTCTT; accession number NM_001010942.1) and SYBR Green
PCR Master. The forward and reverse primers for GAPDH were GTCTCTCTGTACCTCAACA and CAGGAAATGAGCTTGACAAAA, respectively. To control for specific PCR products, a dissociation curve was generated after the end of the last cycle.

Electrophysiology:
Measurement of transepithelial short-circuit current (Isc)- T84WT and Epac1KDT84 cells grown on snapwell inserts were mounted in an Ussing chamber and transepithelial potential differences were clamped to zero mV using VCC MC6 multi-channel voltage–current clamp amplifier (Physiologic Instruments) as previously described (11). The short circuit current (Isc) was continuously recorded using Ag–AgCl electrodes in 3M KCl agar bridges. Apical and basolateral solutions were maintained at 37°C by heated water jackets and were separately perfused and oxygenated with 100% O2. The bath Ringer solution contain (in mM): 140 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 10 Glucose and 10 HEPES adjusted to pH 7.4. The change in Isc induced by the treatment was expressed as the difference from the baseline to the steady state.

Measurement of Apical Potassium and Chloride Current- Apical K+ conductance [I(Kap)] was assessed applying a high basolateral to low-apical [K+] gradient across the monolayers followed by permeabilization with the monovalent ionophore nystatin used previously (27, 28). After mounting snapwell inserts on Ussing chamber normal Ringer solution in apical hemi-chamber was replaced with low K+ buffer solution containing (in mM) 137 NMDG-Glutamate, 5 K-glucenate, 0.4 MgSO4, 1.25 CaCl2, 0.43 KH2PO4, 0.35 K2HPO4, 10 Glucose and 10 HEPES while basolateral chamber was replaced with high K+ buffer solution of following composition (in mM): 143 K-glucenate, 0.4 MgSO4, 1.25 CaCl2, 0.43 KH2PO4, 0.35 K2HPO4, 10 Glucose and 10 HEPES.

Apical Cl– conductance [I(Clap)] were assessed after permeabilization of the basolateral membrane with 50µg/ml nystatin and the establishment of a basolateral to apical Cl– concentration gradient described previously (27). For the measurement of I(Clap) current, we did not use the customery Ussing convention for Iscs, where the voltage is V2-V1, in which V1 represents basolateral and V2 apical (apical-basolateral). Instead, we used the physiological convention of outside-inside, which, when the basolateral side has been permeabilized to Cl–, can also be thought of as basolateral-apical. In the measurement of I(Kap), we kept the “Ussing convention” of apical-basolateral. The switching of conventions results in a switching polarity but nothing else, and allows for a comparison of the properties of the channels described here with those of others (27).

Current-voltage (I-V) relationship study: To study the K+ conductance of apical plasma membrane for the generation of a I-V relationship, the voltage across the monolayer was sequentially stepped from a holding voltage of 0mV to values between -100mV to +100mV in 20mV increments with a pulse duration of 5s and corresponding currents were recorded. A 50s interval between each pulse was sufficient for recovery from activation. The protocol was performed after sustained stimulation of forskolin or 8-pCPT-2’-O-Me-cAMP. I-V plot of current recordings of nystatin-permeabilized T84WT cells were performed under symmetrical (basal/apical) K+ gradient (143 mM).

Western Blot- T84WT and Epac1KDT84 cells grown to confluency on transwell permeable support(75 mm diameter, catalog no. 3419, costar) were washed and scraped in PBS solution and then homogenized by sonication in RIPA buffer with protease inhibitor cocktail(1:100; Sigma) to obtain cell lysate. Total cell lysate from different region of mice intestine were prepared as reported previously (29). These cell lysate were separated by 10% SDS-PAGE and blotted to nitrocellulose membrane. These membranes were blocked with 5% nonfat dried milk for 1 h at room temperature and incubated over night with primary antibody at 4°C. The primary antibody used were anti-KCNN4b antibody (dilution 1:3000) and anti-GAPDH antibody (dilution 1:5000). The KCNN4b antibody we used also detects both KCNN4b and KCNN4c as documented by a previous study (22). IRDye 800- or 680-conjugated anti-mouse or anti-rabbit IgG (1:15000; Rockland immunochemicala, Gilbertsville,
PA) were used as secondary antibodies for 1h followed by three washes. The fluorescence signal was analyzed by using the Odyssey infrared system at 700 and 800 nm wavelength (LI-COR Bioscience) previously described (27).

**Surface Biotinylation**—Cell monolayers were grown on six well (top three inserts for T84WT and bottom three for Epac1KDT84 cells) transwell inserts (cat. No. 3450, Corning, MA) for detection of total and surface amount of KCNN4c and KCNN4b by surface biotinylation as described earlier (30,31). Briefly, cells grown onto transwell insert were serum starved for 4h, followed by rinsing three times with ice-cold phosphate-buffered saline (150 mM NaCl, 20 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4) and once in borate buffer (154 mM NaCl, 1.0mM boric acid, 7.2 mM KCl, and 1.8 mM CaCl<sub>2</sub>, pH 9.0). Cells (both surface) were then incubated for 20 min with 0.5mg/ml NHS-SS-biotin (biotinylation solution; Pierce Chemicals) and repeated once. Cells were then washed three times with the quenching buffer (20 mM Tris and 120 mM NaCl, pH 7.4) to scavenge the unbound NHS-SS-biotin. Monolayers were washed with ice-cold PBS and scraped off their filter support in N<sup>+</sup> buffer (60 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM KCl, 5 mM Na3EDTA, 3mM EGTA, and 1% Triton X-100). The lysate was agitated for 30 min and spun at 14000 xg for 15 min to remove the insoluble cell debris. An aliquot was retained as the total cellular KCNN4 protein. Protein concentration was determined and 1 mg of lysate was then incubated with streptavidin-agarose beads for overnight. The streptavidin-agarose beads were washed five times in N<sup>+</sup> buffer to remove nonspecifically bound proteins. All the above procedures were performed at 4°C or in ice. Biotinylated surface proteins were then solubilized in equivalent volume of sample buffer (5 mM Tris-HCl pH 6.8, 1% SDS, 10% glycerol, and 1% 2-mercaptoethanol) boiled for 5 min. Dilution of the total and surface KCNN4c and KCNN4b were resolved by 10% SDS-PAGE and immunoblotted with anti-KCNN4abc antibody. Western analysis and quantification of the surface fraction were performed using the Odyssey system and Odyssey software (LI-COR) described in the method section. Multiple volumes for each total and surface sample were used with linear regression with intensity of signal to obtain a single value for each sample. Percentage of surface KCNN4c was calculated from the ratio [(surface KCNN4c signal/total KCNN4c signal)] x dilution factor of surface and total KCNN4c samples and expressed as percentage of total KCNN4c.

**Immunofluorescence study**—Transwell insert grown T84WT cell monolayers were fixed on ice in 3% paraformaldehyde solution (Electron Microscopy Sciences Cat # 15710, Hartfield, Pa) in PBS, pH 7.4, at 4°C for 20 min. Monolayers were then washed in ice-cold PBS and quenched with 50 mM NH₄Cl in PBS for 15 min on ice and excised as circles from the inserts. The fixed cell monolayers were then permeabilized for 30 min in 0.1% saponin/PBS before being blocked for 30 min in PBS + 1% BSA supplemented with 10% FBS. Cell monolayers were incubated with primary antibody in PBS + 1% BSA for 1h at room temperature in a moist environment. After three 10-min washes in PBS + 0.1% saponin, monolayers were incubated with alexa 568-conjugated goat secondary antibody diluted 1:100 in PBS + 1% BSA for 1 h at room temperature in a moist and dark environment. Inserts were then rinsed four times for 10 min in PBS + 0.1% saponin, cut into rectangles, placed on glass slides, and mounted in mounting medium (Sigma), and examined on a Zeiss LSM 510 laser scanning confocal fluorescence microscope (X63 objective, oil immersion). To label the apical surface of T84WT cells, confluent cell monolayers were labeled with 1 µg/ml FITC-conjugated Wheat Germ Agglutinin(WGA) prior to permeabilization and staining for KCNN4c channel. For staining of mouse intestinal tissue section, the intestine was dissected and the colon was rinsed with ice-cold saline and fixed in 3% paraformaldehyde prior to standard paraffin embedding as described (32,33). Briefly, individual sections were heat fixed, deparaffinized, followed by microwave treatment in 0.01M sodium citrate solution for antigen recovery. Endogenous peroxidase activity was blocked by a 30 min preincubation in H₂O₂ (Sigma). Sections were then washed in PBS and blocked with 5% normal goat serum (NGS, Sigma) in PBS for 30 min at room temperature. Subsequently, sections were incubated with primary antibody diluted in 5% NGS-PBS for 1h.
at room temperature followed by FITC-conjugated goat secondary antibody and images were obtained using a Zeiss LSM 510/META confocal microscopy.

**In Vitro Activation of Rap1**- Activation of Rap1 was determined by the EZ-Detect Rap activation kit (Pierce # 89872, Rockford, IL, USA) as previously described with slight modification (27). Serum-deprived cells were stimulated with or without FSK and 8-pCPT-2'-O-Me-cAMP for 20 min at 37°C and then lysed in lysis buffer (provided in the kit) in the presence of protease inhibitor cocktail. After lysis samples were centrifuged at 14,000xg at 4°C for 10 min, and the protein concentration of the lysates were measured. 500 µg of cell lysates were immediately affinity-precipitated at 4°C for 1 h with 20µg of glutathione-S-transferase-tagged RalGDS-RBD (Ras-binding domain of the Ral guanine nucleotide dissociation stimulator) following the manufacturer’s protocol. Activated Rap1 (Rap1-GTP) was eluted in 50 µl Laemmli sample buffer and detected by western blotting using an antibody specific to Rap1.

**Mouse Ileal Loop Experiment**- The ileal loop experiment was performed in mice of 6-8 weeks old by a modified Rabbit Ileal Loop (RIL) assay originally described by De and Chatterje (34). Prior approval for the animal experiment was obtained from the Institutional Animal Ethics Committee (Approval no. Apro/75/24/11/2010 of 24th Nov 2010). Following gut sterilization, the animals were kept fasting for 24 h prior to surgery and fed only water ad libitum. Anaesthesia was induced by a cocktail of ketamine (35 mg per kg of body weight) and xylazine (5 mg per kg of body weight). A laparotomy was performed and an ileal loop of 5 cm length was constricted at terminal ileum by tying with non-absorbable silk. The following fluids were instilled in each loop by means of a tuberculin syringe fitted with disposable needle through ligated end of the loop as the ligature is tightened. Pure CT (1 µg; positive control), saline (negative controls), CT (1µg) + TRAM 34 (different conc. in µM as indicated in Fig 7), CT (1µg) + H1152 (1 µM), and CT (1µg) + different concentration of GGTI 298 in µM (a specific inhibitor of Rap1A). The intestine was returned to the peritoneum, and the mice were sutured and returned to their cages. After 6 h, these animals were sacrificed by cervical dislocation, and the loops were excised. The fluid from each loop was collected and the ratio of amount of fluid contained in the loop with respect to the length of the loop (fluid accumulation ratio, g/cm) was calculated as a reflection of efficacy of various inhibitors.

**Statistical Analysis**- Results are expressed as means ± SEM. Paired and unpaired t-test was used to compare mean values within one experimental series. P<0.05 was accepted to indicate statistical significance.

**RESULTS**

**KCNN4 channel blocker inhibits cAMP stimulated Cl− secretion in intestinal epithelial cells**- To directly test the hypothesis that apical K+ channels play a role in providing the driving force for the cAMP stimulated Cl− secretion, we first investigated the pharmacological profile of various K+ channel blockers added on the apical side after the adenylate cyclase activator, forskolin (FSK), stimulated Isc, a measure of Cl− secretion. FSK elicits Cl− secretion in T84WT cells via elevation of cAMP and activation of protein kinase A and Epac1 (27,35). Figure 1A shows inhibitory effect in Isc responses of FSK to cAMP stimulation by various K+ channel blockers. We found that (CLT) clotrimazole (91 ± 4% of control) which is often used as a probe of Ca2+-activated K+ channels (36,37), a specific Ca2+-activated KCNN4 channel blocker almost completely inhibited Cl− secretion stimulated by FSK. However, addition of apamin, a specific SK channel blocker, IBTX (Iberiotoxin), a specific BK channel blocker, TEA (Tetraethylammonium), a non-specific K+ channel blocker and ZnCl2, a KCNQ1/KCNE3 channel blocker, had no effect on FSK stimulated Isc. BaCl2, a nonspecific K+ channel blocker, only required a 5 mM concentration to significantly (21 ± 4 µA/cm2 vs. 32 ± 2 µA/cm2, (34%) P<0.05) reduce FSK stimulated Isc. The complete inhibition by lipophilic CLT and TRAM-34 represents both apical and basolateral KCNN4 channels, while inhibitory effect of impermeable BaCl2 in intact cells or apical TRAM-34 in basolaterally permeabilized T84 cells represents only inhibition
of apical KCNN4 channels. Zn had a biphasic effect on KCNQ1/KCNE3 in T84WT cells (38) and was used to establish that KCNQ1/KCNE3 is not on the apical membrane. This is critical since basolateral membrane K+ channels composed of KCNQ1 and the KCNE3 ancillary subunit play a critical role in epithelial tissue Cl− secretion by establishing electrical driving force. The complete inhibition of FSK-stimulated Isc by CLT and TRAM-34 compared to the lack of effect of apamin, IBTX or Zn suggests a possible role for apical KCNN4 channels but not BK, SK or KCNQ1/KCNE3 channels in cAMP-stimulated Cl− secretion.

To further show that TRAM-34 inhibits FSK stimulated Cl− secretion via blockage of the apical KCNN4 channel, we examined $I_{K(ap)}$ by applying a basolateral to apical K+ gradient with K+ as the sole permeant ion as described in the method section (39). Nystatin permeabilization of the basolateral membrane and in the presence of this K+ gradient, FSK elicited a rise in $I_{K(ap)}$ which was significantly (32 ± 5 µA/cm² vs. 8±2µA/cm², P<0.01) inhibited by 10µM TRAM-34 (Fig. 1B) and TRAM-34 did not have any effect on $I_{Cl(ap)}$ measured after permeabilization of the basolateral membrane and the establishment of a basolateral to apical Cl− conductance gradient previously described (27). In contrast, the addition of a CFTR inhibitor CFTRinh-172 to the apical membrane led to a marked inhibition of $I_{Cl(ap)}$ (Fig. 1C). All these data when taken together suggests that apical KCNN4 channels may be required for cAMP stimulated transepithelial Cl− secretion.

Detection of apical expression of KCNN4 channels by western blot and confocal microscopy in T84WT cells and mouse intestinal epithelia- To examine KCNN4 protein expression in different part of mouse intestine and different intestinal epithelial cell lines, Western blot analysis was performed using a KCNN4abc antibody. Previous studies (22,40) have shown that two isoforms, KCNN4c and KCNN4b, localize to the apical and basolateral membranes, respectively, in rat colon. We hoped to determine whether they are also present in human intestinal carcinoma cell lines and in mouse intestine. Figure 2A shows strong KCNN4c immunoreactive signal in different region of mouse intestine as well as in T84WT, and HT29.19a cells. KCNN4 protein migrates as two major bands in all cases with apparent molecular weights of 37 kDa and 40 kDa. This finding confirms the expression of apical (37 kDa) and basolateral (40 kDa) version of KCNN4 channel termed KCNN4c and KCNN4b, respectively, in epithelial cell homogenates as previously reported (40). To confirm apical expression of KCNN4 on mouse colon and on T84WT cells grown in transwell inserts, a confocal study was employed using a KCNN4abc antibody. The xy and xz images of T84WT cells shown in Fig. 2B confirmed apical expression of KCNN4 channel by its co-localization with apical surface marker wheat germ agglutinin (WGA) which binds specifically to sugar residues expressed on the cell’s apical membrane. We further confirmed apical expression of KCNN4 in the mouse colon by immunostaining as shown in Fig. 2C.

Epac1 influenced apical K+ conductance, $[I_{K(ap)}]$ by regulating apical KCNN4 channel in T84WT cells- Our previous study identified an additional cAMP signaling pathway that activates PKA independent, Epac1-mediated intestinal Cl− secretion through a non-CFTR Cl− channel (27). Epac1 increases [Ca2+]i through activating Epac1-Rap2-PLCε-Ca2+ signaling cascades (27). Because KCNN4 channels are regulated by [Ca2+]i, we next investigated the role of Epac1, if any, in $I_{K(ap)}$ using nystatin permeabilized T84WT cells. In the presence of an apically directed K+ gradient, addition of FSK failed to raise $I_{K(ap)}$ in Epac1KDT84 cells, which was significantly different from T84WT cells (5±0.8 µA/cm² vs. 32.3±2.7 µA/cm², p<0.005 Fig. 3A). Unexpectedly, serosal application of [Ca2+]i elevating agent, carbachol (CCH) in Epac1KDT84 cells also resulted in significant reduction of $I_{K(ap)}$ when compared to T84WT cells (20±5 vs. 69±4 µA/cm², p<0.01, Fig. 3B). These data of $I_{K(ap)}$ in Epac1KDT84 cells compared to T84WT cells in response to FSK and CCH indicate that Epac1 is required for the agonist stimulated Cl− secretion, influencing the $I_{K(ap)}$. Next, we sought to ascertain whether apical KCNN4c contributes to the $I_{K(ap)}$ observed in the previous experiments. We studied the current-voltage (I-V) relationships in basolaterally permeabilized monolayers of T84WT and Epac1KDT84 cells treated with
Epac1 agonist, 8-pCPT-2'-O-Me-cAMP or in the presence of TRAM-34 in a symmetric K⁺ ion concentration. Figure 3C demonstrated the presence of a moderately inwardly rectified, K⁺ channel in T84WT cells that was activated by 8-pCPT-2'-O-Me-cAMP and blocked by TRAM-34. Interestingly, 8-pCPT-2'-O-Me-cAMP was not able to activate this inwardly rectifying current in Epac1KDT84 cells. Furthermore, with the symmetric K⁺ ion concentration, the I-V curve became more or less linear when a voltage ramp (-60 to +60mV) was applied. We also determined the calcium dependency of 8-pCPT-2'-O-Me-cAMP stimulated I_{K(ap)} current in T84WT cells in the presence or absence of [Ca²⁺]ᵢ chelator, BAPTA-AM. As shown in the inset of figure 3C, treatment of 8-pCPT-2'-O-Me-cAMP increased I_{K(ap)}, which was significantly inhibited by BAPTA-AM. Furthermore, addition of the KCNN4 channel specific activator, DC-EBIO at the apical side of intact T84WT cells resulted in a 44±5 µA/cm² rise in Isc. In contrast DC-EBIO enhanced Isc only 5±1 µA/cm² and 4±3 µA/cm² in Epac1KDT84 cells and T84WT + TRAM34 (10 µM), respectively (Fig. 3D). Together these data indicate Epac1 may influence an inwardly rectifying Ca²⁺-activated I_{K(ap)} by regulating apical KCNN4c channels in cAMP stimulated Cl⁻ secretion.

Epac1 protein depletion resulted mislocalization of KCNN4c channels in T84WT cells- Because depletion of Epac1 protein unexpectedly caused a significant reduction of I_{K(ap)} even with CCH stimulation, experiments were performed to determine physical localization of KCNN4c proteins in Epac1KDT84 cells by confocal microscopy. As shown in Fig. 4A(bottom panel), reconstructed confocal image in the xz plane of Epac1KDT84 monolayers revealed redistribution of KCNN4c proteins into a sub-apical intracellular compartment detected underneath the apical surface marker WGA. KCNN4c staining overlapped significantly with WGA (Figure 4A, top panel) demonstrating that KCNN4c localizes to the apex of T84 epithelia. We speculated that the depletion of Epac1 might lower the surface amount of KCNN4c and thus I_{K(ap)}. Therefore, we compared the surface amounts of KCNN4c in T84WT and Epac1KDT84 cells using biotinylation assay. As shown in Fig. 4B & 4C, Epac1 depleted cells have ~83% lower surface membrane expression of KCNN4c as compared with T84WT cells. The reduced surface amount of the apical version of KCNN4 protein [KCNN4c, (37kDa band)], in biotinylation assay may explain, in part, the decrease of I_{K(ap)} in response to both FSK and CCH. These results further support the hypothesis that Epac1 is required for insertion of KCNN4c to the apical most cell surface. Additionally, these results are also consistent with FSK stimulation of I_{K(ap)} in Epac1KDT84 cells or that 10µM TRAM-34 had insignificant effect (data not shown), even though this concentration inhibited the activity of FSK-stimulated Isc or I_{K(ap)} in T84WT cells.

FSK, 8-pCPT-2'-O-Me-cAMP stimulated Rap1 and depletion of Rap1A resulted redistribution of KCNN4c into sub-apical region- The role of Epac1-Rap1 signaling in endogenous regulation of KCNN4 was further assessed in T84WT cells by Rap1 activation using Rap1-GTP pull-down assays. We utilized FSK and the novel analog of cAMP, 8-pCPT-2'-O-Me-cAMP, a potent activator of the Epac1-Rap1 pathway. 8-pCPT-2'-O-Me-cAMP and FSK induced GTP-loading of Rap1 in T84WT cells (Fig. 5A). To determine whether Rap1 which has two isoforms Rap1A and Rap1B, are involved in the regulation of I_{K(ap)}, we used a knockdown approach employing lentivirus shRNA in T84WT cells as previously described (27). Quantitative real-time PCR was performed on cDNA from RNA preparation of Rap1A and Rap1B knockdown cells and the mRNA levels were quantified and normalized against GAPDH considered as an endogenous control. We achieved effective knockdown (Fig. 5b), with marked reduction of Rap1A and Rap1B transcript levels by construct TRC29784 and TRC29177, respectively. When Isc or I_{K(ap)} were measured in these knockdown cells grown in a transwell insert, a significant reduction of Isc or I_{K(ap)} in Rap1AKDT84 cells was observed (Fig. 5C and 5D), while depletion of Rap1B alone had no significant effect on FSK-induced Isc (Fig. 5C). This observation substantiates the notion that the Epac1-Rap1A signaling pathway may be involved in mobilization of KCNN4c to the cell apical membrane. To test directly the role of Rap1A in the mobilization of KCNN4c to the surface of T84WT cells, confocal microscopy was
performed. Confocal images (xy and xz planes) showed a co-localization of KCNN4c protein and WGA at the apical surface of Rap1BKDT84 cells. On the other hand, co-localization for KCNN4c and WGA was almost absent or found to be low in Rap1AKDT84 cells, whereas KCNN4c localization in the sub-apical intracellular compartment was increased (Fig. 5E, top panel). Colocalization signal was quantified by pearson correlation value using Zeiss LSM 510 software. There was a significant reduction of colocalization between KCNN4c and WGA in Rap1AKDT84 cells compared to T84WT or Rap1BKDT84 cells as shown in Fig. 5E (right panel, bottom). These finding suggests that stimulation of I_K(ap) required the presence of Rap1A which might have a role in the redistribution of KCNN4c from sub-apical intracellular compartment to the apical plasma membrane.

RhoA, ROCK and KCNN4 inhibitors reduced FSK stimulated current and CT induced intestinal fluid accumulation - Epac1-Rap1 signaling was shown previously to be necessary for translocation of functional α2c-adrenoceptor to the cell surface involving RhoA and Rho-associated kinase ROCK (41). To better understand the specific involvement of these kinases in KCNN4 regulation in intestinal Cl secretion, I_K(ap) was measured in T84WT cells. Finding that both RhoA and ROCK kinase inhibitors significantly reduced FSK stimulated I_K(ap) (Fig. 6A), suggests cAMP stimulated I_K(ap) conductance requires RhoA and ROCK which are a downstream target of the Epac1-Rap1 signaling pathway. To determine whether activation of Epac1-Rap1A-RhoA signaling alters the distribution of KCNN4c, cell monolayers were fixed and imaged after stimulation with 8-pCPT-2′-O-Me-cAMP either in presence or absence of ROCK inhibitor H1152. Reconstructed xz plane through representative regions are shown in Fig. 6B. The apical distribution of KCNN4c is clearly enhanced following 8-pCPT-2′-O-Me-cAMP stimulation as evident by the increased co-localization with WGA (Fig. 6B, bottom) whereas, following H1152 treatment, overall co-localization was lower and KCNN4c congregated immediately below the plasma membrane (Fig. 6B, top).

To provide proof of concept that blockade of KCNN4c channels or its regulator Epac1-Rap1A signaling could be used in a therapeutic setting, we tested the effect of KCNN4 inhibitor TRAM-34, Rap1A inhibitor GGTI298 on CT induced diarrhea in a mouse model (42). In ligated ileal loops, CT treatment stimulated fluid accumulation which was significantly reduced by TRAM-34 or GGTI298 in a dose dependent manner (Fig. 7). Additionally, we tested ROCK-mediated inhibition of fluid accumulation in mice that was stimulated by CT. A single dose of ROCK inhibitor H1152 effectively prevented fluid accumulation in the intestinal loop. These results clearly demonstrate that the apical KCNN4c channel requires Rap1A and its downstream signaling RhoA and ROCK in CT-induced fluid accumulation.

DISCUSSION:
In these present experiments we present a novel function for Epac1: the regulation of KCNN4c channels and cAMP-stimulated Cl secretion. We hypothesized that the non-CFTR mediated component of Cl secretion stimulated by Epac1 resulted from a cAMP-induced increase of [Ca^{2+}]_i through the Epac1-Rap2-PLC-[Ca^{2+}]_i signaling cascade and required the opening of apical KCNN4c channels to facilitate transepithelial Cl secretion by increasing the electrochemical driving force. Our data are consistent with the coupling of apical KCNN4c and non-CFTR Cl channels in mediating the Cl secretory effect of Epac1. We found 8-pCPT-2′-O-Me-cAMP-induced a moderately inwardly rectifying Ca^{2+} activated I_K(ap) response and that the cAMP stimulated Isc response was inhibited by the KCNN4 specific inhibitor TRAM-34 in T84WT cells. In contrast to T84WT cells, Epac1KDT84 cells almost lacked this inwardly rectified I_K(ap) and the application of TRAM-34 blocked the inwardly rectification in T84WT cells, agreeing with the absence of a TRAM-34 sensitive I_K(ap). Furthermore, KCNN4c does express on the apical membrane of T84WT cells and mouse intestine as shown in Fig. 2B. Indeed much of the KCNN4c co-localizes with plasma membrane marker WGA in T84WT cells, indicating that the primary site of membrane-inserted KCNN4c in these cells is the apical plasma membrane as shown in Fig 2A. Regulation of apical membrane Cl channels is often considered to be the primary factor determining...
the rate of Cl\(^-\) secretion (43). Our data show that regulation of apical membrane KCNN4c channels can also determine the rate of Cl\(^-\) secretion. This conclusion was best illustrated in Figure 1B & 3D where FSK activated apical KCNN4c (via an increase in [Ca\(^{2+}\)]\(_i\) by Epac1), resulted in stimulation of apical Cl\(^-\) secretion which was 7 times greater (30 µA vs. 4.1 µA) than in the presence of apical TRAM-34. Similarly, addition of apical DC-EBIO activated apical KCNN4c stimulating Cl\(^-\) secretion 10 times greater (44 µA vs. 4.1 µA) than in the presence of apical TRAM-34. Thus, the rate of Cl\(^-\) secretion can be determined by regulation of either channel (Cl\(^-\) or K\(^+\)) at the apical membrane. Recently, we have shown that Epac1, in response to cAMP, stimulates intestinal Cl\(^-\) secretion through a Cl\(^-\) channel other than CFTR in a Rap2-PLC\(\epsilon\)-[Ca\(^{2+}\)]\(_i\) dependent manner (27). However, activation of Rap1 by cAMP is also achieved by the binding of cAMP to Epac proteins, which function as G protein exchange factors for Rap1 (44-46). The role of Rap1 in intestinal epithelial ion transport remains relatively unexplored.

We were also interested in determining the role of KCNN4c channels to support cAMP- and/or Epac1-stimulated Cl\(^-\) secretion? It appears that KCNN4c is required for cAMP-stimulated Epac1-dependent Cl\(^-\) secretion. First, FSK stimulated Isc was significantly abolished by apical application of the KCNN4 specific inhibitor TRAM-34 but not by BK or SK channel inhibitors. It has been demonstrated before that BK channels are not involved in stimulated electrogentic Cl\(^-\) secretion which is consistent with our present finding (24). Second, KCNN4c channel opener DC-EBIO failed to increase Isc or FSK stimulated I_{K(ap)} in Epac1 depleted cells. This notion was further supported by our previous study that only the cAMP-stimulated Epac1 dependent Cl\(^-\) conductance (I_{Cl}) and Isc was abolished in Epac1 depleted cells without any effect on PKA dependent CFTR mediated I_{Cl} (27). It is unlikely that the effect of Epac1 depletion caused reduction of Calcium-activated Chloride Channel (CaCC) protein expression which resulted in complete abolition of I_{Cl} because calcium agonist, CCH, stimulated I_{Cl} was essentially unaffected (only a moderate reduction was observed, data not shown). In contrast to the increasing confusion surrounding the role of a non-CFTR channel in Epac1 stimulated Cl\(^-\) secretion, a role for CaCC has progressed. This, of course, does not exclude the possibility that Epac1 and its downstream signaling may activate Ca\(^{2+}\) dependent apical KCNN4c channels, hyperpolarize the cells, and result in Cl\(^-\) secretion.

These several observations support the interpretation that there is a coupling between KCNN4c and Epac1 stimulated Cl\(^-\) secretion. However, our results further described a novel effect of Rap1 in intestinal epithelial cells: that the stimulation of KCNN4c is apparently caused by the activation of a Rap1-dependent pathway, dependent on Epac1, and leading to a RhoA-ROCK-mediated rapid incorporation of functional KCNN4c into the apical surface. Several lines of evidence support this hypothesis. First, depletion of Epac1 decreased KCNN4c protein abundance in the cell surface but not KCNN4b protein as evident by surface biotinylation study (Fig. 4B). Second, CCH, FSK or 8-pCPT-2’-O-Me-cAMP and DC-EBIO failed to stimulate the I_{K(ap)} or Isc or inwardly rectifying Ca\(^{2+}\)-activated K\(^+\) current in Epac1KDT84 cells, with the reduction of Cl\(^-\) secretion by CCH stimulation in Epac1KDT84 cells clearly explaining the loss of functional KCNN4c in the apical surface. Third, FSK and the Epac activator 8-pCPT-2’-O-Me-cAMP both increased active Rap1, GTP-Rap1 in T84WT cells. Fourth, H1152, a ROCK inhibitor, significantly reduced cAMP-stimulated I_{K(ap)} in Epac1KDT84 cells, with the reduction of Cl\(^-\) secretion by CCH stimulation in Epac1KDT84 cells clearly explaining the loss of functional KCNN4c in the apical surface. In most cell types, RhoA acts as an upstream signal molecule to activate ROCK, which, in turn, is involved in the translocation of protein to the cell surface (47, 48). A similar signaling module has been described in microvascular smooth muscle cells (49). In line with previously described ROCK functional data, we have observed a significant reduction in colocalization of KCNN4c with WGA in T84WT cells pretreated with ROCK inhibitor, while 8-pCPT-2’-O-Me-cAMP induces this colocalization to the apical surface in the absence of this inhibitor. The findings described above demonstrate for the first time rapid KCNN4c mobility in T84 epithelia as a dynamic process that is regulated by Epac1 stimulation with a time scale of 30 minutes that involves ROCK and remain to be investigated. We found that the co-
localization of KCNN4c and WGA markedly increased in T84WT cells, in contrast to the marked decrease in Epac1KDT84 cells. This is consistent with our observations that surface amount of KCNN4c was significantly reduced in Epac1KDT84 cells while KCNN4b levels in the basolateral membrane were unaffected (Fig. 4B). The decreased amount of surface KCNN4c and increased amount of KCNN4c in the sub-apical compartment in Epac1KDT84 cells are in good agreement with a predominate role of apical KCNN4c in mediating the effect of Epac1 in Cl secretion. These several observations indicate that Epac1 may play a critical role in the distribution of KCNN4c and its activation via the Rap1A-RhoA-ROCK signaling pathway. Epac1-Rap1 signaling could enhance the protein abundance in the cell membrane either by insertion of KCNN4c in the apical surface or by delaying the retrieval of this channel protein from the membrane, which of these two possibilities is not readily evident at present and will require future experiments. Finally, to explore the therapeutic potential of KCNN4c channel blockade in the regulation of active Cl secretion and its regulation by Epac1 signaling, we tested the effect of the Rap1A inhibitor, GGTI298, the ROCK inhibitor, H1152, and the KCNN4c inhibitor, TRAM-34, in a closed loop mouse model. A significant decrease in intestinal fluid accumulation was observed in mice that received any one of these compounds. These results clearly demonstrated that inhibiting Epac1-Rap1-RhoA-ROCK signaling may represent a novel, potential therapeutic approach for the treatment of diarrhea that is mediated by cAMP by interfering with the recruitment of KCNN4c to the apical membrane.

In conclusion, we have demonstrated for the first time that apical KCNN4c channel expression and activity are markedly decreased due to Epac1 depletion involving Rap1A-RhoA-ROCK signaling and provides a molecular framework for new understanding of the regulation of epithelial Cl secretion. More importantly, the identification of TRAM-34 and its effective anti-secretory role in both cultured cell monolayers and in vivo in mice represents a promising new platform upon which a new therapy for diarrhea and other gastrointestinal disorders could be developed.

**FOOTNOTES**

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**Author Contribution**

Shiekh IA and KMH performed research, design experiments and analysis data. HK helped to perform ileal loop experiments. MKC gave inputs for preparation of the manuscript. KMH & Shiekh IA wrote the manuscript, KMH interpret data.

**Conflict of Interest**

The authors declare that they have no conflict of interest.
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FIGURE LEGENDS:

FIGURE 1. Effect of K⁺ channel blockers on FSK stimulated currents of the human intestinal T84WT cell monolayers grown on transwell insert. A: Summary of calculated Isc in the presence of various K⁺ channel blockers in intact T84WT cells. FSK stimulated Isc in the absence of K⁺ channel inhibitor was set to 100% (control). B: Representative trace of the effect of apical TRAM-34(10µM) on I_K(ap) of T84WT monolayer with basolaterally permeabilized membrane. I_K(ap) was measured as described in the method section. TRAM-34 was applied either apically before 30 min of FSK stimulation or after as indicated. The inset figure shows the summary of the mean effect of TRAM-34 on I_K(ap) from three independent experiments. Single cell illustration (top) indicates the direction of K⁺ gradient and the dashed line indicates the permeabilization of the basolateral membrane with nystatin. C: Summary of the data from six monolayers of T84WT cells permeabilized by basolateral nystatin described in reference (28) showing TRAM-34 did not have any inhibitory effect on FSK stimulated I_Cl(ap). 10µM FSK was added to the basolateral membrane. Illustration of the cell (top) indicates the direction of the Cl⁻ gradient and the dashed line indicates the permeabilization of the basolateral membrane with nystatin. ΔI_K(ap) or ΔI_Cl(ap) was derived from values before and after FSK stimulation. Values are mean ± SEM. Asterisks indicates significant difference *P<0.001 and **P<0.05 when compared with control N = 6-10.

FIGURE 2. Expression and polarization of KCNN4c on the apical membrane of intestinal epithelial cells and mouse intestine. A: Proteins from total lysate of mouse intestinal tissues and human colonic cell lines were resolved in 10% SDS-PAGE as described under “Method Section” and immunoblotted with KCNN4abc antibody. KCNN4 proteins migrate in all cases as two major bands with apparent molecular weights of 37kDa (KCNN4c) and 40kDa (KCNN4b) respectively. B: Colocalization of KCNN4c (red) with marker of apical membrane wheat germ agglutinin (WGA, green) in T84WT cells. Polarized T84WT cells were grown on transwell inserts and analyzed in xy and xz scans by confocal microscopy as described in the method section. xy confocal section of T84WT cells showing clear apical membrane staining of KCNN4c co-localized with WGA. Colocalization is reflected by a yellow signal of the merged composite in xy and xz section (bottom), the culture surface is at the bottom of image, the apical surface of the cells at the top. The merged image is shown on the bottom panel. Insets show enlarged regions within the white box. XZ plane of region marked in white square is shown in bottom. The scale bar in panel represents 10µm. Cellular illustration (bottom left) indicates the presence of functional KCNN4c to the apical most membrane but not beneath the subapical membrane in T84WT cells. C: KCNN4c in mouse colon. Paraffin sectioned of mouse colonic tissues were fixed, stained for KCNN4c (green), and xy image were collected by confocal microscopy as described in the method section.

FIGURE 3. Inhibition of I_K(ap) by FSK, CCH and I_sc by DC-EBIO stimulation in Epac1KDT84 cells with basolaterally permeabilized membrane. A: representative I_K(ap) recordings in response to FSK(10µM) in T84WT and Epac1KDT84 cells with imposed basolateral (145mM) to apical (5mM) K⁺ gradient. Inset shows the summary of the data from six such monolayers. Values are mean ± SEM, N = 6. B: CCH stimulated I_K(ap) in T84WT and Epac1KDT84 cells. Values are mean ± SEM. N = 6. C: current(I)-voltage(V) relationship in symmetric K⁺ concentration in the presence or absence of TRAM-34 in T84WT or Epac1KDT84 cells using step voltage protocol described under “Method Section”. The records are averaged from four independent experiments. The inset figure shows the stimulation of I_K(ap) by 8-pCPT-2’-O-Me-2cAMP in the presence or absence of BAPTA-AM. Values are mean ± SEM, N = 4. D: Effect of apical DC-EBIO (100µM) on change in I_sc in intact T84WT or Epac1KDT84 cells. T84WT cells were pre-incubated with or without apical TRAM-34 for 30 min thereafter DC-EBIO was added apically. ΔI_K(ap) or I_sc was derived from values before and after FSK or CCH or DC-EBIO stimulation. Values are mean ± SEM, N = 6.

FIGURE 4. Epac1 depletion affects the KCNN4c protein abundance within the apical plasma membrane of T84WT cells. A: Effect of Epac1 depletion on KCNN4c(red) co-localization with WGA(green) in apical membrane showing loss of co-localization with WGA (xz plane, bottom)
compared to T84WT cells (xz plane, top). The scale bar in panel represents 10µm. B: cell surface expression of KCNN4c (37kDa) in T84WT and Epac1KDT84 cells by surface biotinylation experiments described under “Method section”. Representative Western blot were quantified with multiple dilutions of total and surface fractions probed with infrared fluorescence IRDye secondary antibody as described under “Method Section. C: summary of surface KCNN4c levels (as percentage of total) of T84WT and Epac1KDT84 cells. The cellular illustration demonstrates the mislocalization of KCNN4c immediately beneath the subapical membrane due to depletion of Epac1 (right). Experiments were repeated at least three times and results are shown as mean ± SEM. Asterisk (*) indicates significant difference (P<0.05) from T84WT cells. N = 3.

FIGURE 5. Involvement of Rap1 on FSK stimulated Isc and I_{K(ap)} through Epac1 stimulation. A: endogenous Rap1 activity was measured with FSK or 8-pCPT-2’-O-Me-cAMP or without (control). Cells were incubated in serum free medium, FSK and 8-pCPT-2’-O-Me-cAMP leads to an activation of endogenous Rap1 in T84WT cells. Western blots of total cell lysate probed for total Rap1 and Rap1B gene, which were shown mRNA levels relative to levels of GAPDH in log scale. CT, cycle threshold. Values are mean ± SEM, n = 3. Summary of the effects of Rap1A and Rap1B knockdown by gene specific lenti-shRNA on FSK stimulated Isc (C) and (D) I_{K(ap)} of Rap1AKDT84 cells. FSK stimulated Isc in T84WT cells was set to 100% (control). eGFP shRNA transduced cell monolayers were used as non-targeting control. Values are mean ± SEM, n = 4, NS= statistically not significant. E: depletion of Rap1A affected co-localization of KCNN4c with WGA of apical membrane which is reflected by significant loss of yellow signal of the merged composite (xy and xz plane, top left vs. top right). Insets show enlarged regions within the white box. Confluent Rap1AKDT84 and Rap1BKD T84 cell monolayers were stained with FITC-WGA on ice then fixed, stained for KCNN4c (red) and a stack of xz images collected by confocal microscopy. Representative xz section is shown here. The scale bar in panel represents 10µm. Bottom right: Quantification of KCNN4c-colocalized WGA in T84WT vs. Epac1KDT84 or Rap1AKDT84 cells and presented as mean ± SEM by calculating Pearson’s colocalization coefficient using Carl Zeiss LSM software (version 3.2). Cellular illustration (bottom left) indicates the redistribution of KCNN4c beneath the subapical membrane due to depletion of Epac1 or Rap1A.

FIGURE 6. Involvement of Epac1-Rap1A-RhoA-ROCK signaling in intestinal Cl− secretion. A: summary of the effects of RhoA inhibitor C3 toxin (1µg/ml) and ROCK inhibitor H1152 (1µM) on I_{K(ap)}. Confluent T84WT cell monolayers of permeable support were pretreated with C3 toxin for 4h in culture media prior to subjected in Ussing chamber experiment and H1152 for 30 min directly in the chamber, FSK was then added. The results are presented as means ± SEM, N = 4. B: 8-pCPT-2’-Me-cAMP enhances the insertion of KCNN4c (red) into the membrane (bottom) which was partially inhibited by ROCK inhibitor, H1152 (top). The scale bar in panel represents 10µm. Confluent T84WT cell monolayers were stimulated with 8-pCPT-2’-O-Me-cAMP for 30 min at 37°C prior to stained with FITC-WGA(green) on ice, then fixed, stained for KCNN4c(red).

FIGURE 7. Inhibition of Rap1A, ROCK or KCNN4c by in vivo intestinal loop experiments reduce secretory diarrhea in mice. Dose response curve of fluid accumulation (FA) ratio in closed mice ileal loops by cholera toxin (CT) stimulation in the presence of (A) KCNN4c inhibitor, TRAM34 (B) Rap1A specific inhibitor GGTI 298. Saline control (no CT) is shown for comparison. Inset shows photograph of representative mouse ileal loops at 6h after luminal injection of CT (1µg) along with or without inhibitors. (C) A single dose of ROCK inhibitor H1152 (1µM) inhibits CT stimulated FA. The bar graph shows the average FA ratio at 6h after CT challenge with or without H1152. FA ratio was calculated as described in the method section. Data represents the mean ± SEM (n = 10 mice per group).
Table 1. Lenti-shRNA constructs used for gene silencing in this study

| Protein | Construct   |
|---------|-------------|
| Rap1A   | 1-TRC29784  |
|         | 2-TRC29785  |
|         | 3-TRC29786  |
|         | 4-TRC29787  |
| Rap1B   | 1-TRC29174  |
|         | 2-TRC29175  |
|         | 3-TRC29177  |
|         | 4-TRC29178  |
Figure 1

A

FSK stimulated $I_{sc}$ in the presence of K channel inhibitors (% of control)

| Inhibitor       | Control | BaCl2 (1mM) | BaCl2 (3mM) | TEA (10mM) | ZnCl2 (100uM) | IBTX (100uM) | Apamin (300nM) | CLT (20uM) | TRAM-34 (10uM) |
|-----------------|---------|-------------|-------------|------------|---------------|--------------|----------------|------------|----------------|
| Control         | 100     | 88          | 70          | 66         | 84            | 88           | 88             | 88         | 88             |
| BaCl2 (1mM)     | 90.5    | 87.5        | 64          | 70         | 83            | 87           | 87             | 87         | 87             |
| BaCl2 (3mM)     | 90.5    | 87.5        | 64          | 70         | 83            | 87           | 87             | 87         | 87             |
| TEA (10mM)      | 90.5    | 87.5        | 64          | 70         | 83            | 87           | 87             | 87         | 87             |
| ZnCl2 (100uM)   | 90      | 87.5        | 64          | 70         | 83            | 87           | 87             | 87         | 87             |
| IBTX (100uM)    | 90      | 87          | 64          | 70         | 83            | 87           | 87             | 87         | 87             |
| Apamin (300nM)  | 90      | 87          | 64          | 70         | 83            | 87           | 87             | 87         | 87             |
| CLT (20uM)      | 90      | 87          | 64          | 70         | 83            | 87           | 87             | 87         | 87             |
| TRAM-34 (10uM)  | 90      | 87          | 64          | 70         | 83            | 87           | 87             | 87         | 87             |

B

Control

TRAM-34

FSK stimulated $I_{K(ap)}$ ($\mu A/cm^2$)

Time(min)

FSK

FSK + TRAM-34
Figure 1

C

= Cl channel

ap [Cl] 5mM

bl [Cl] 145mM

P<0.01

ΔI_{Cl(ap)} (μA/cm²)

FSK

FSK + TRAM-34

FSK + CFTR-172

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Figure 2A

HT29.19a  T84WT  Mouse Jejunum  Mouse Ileum  Mouse Colon

40kDa (KCNN4b)
37kDa (KCNN4c)
GAPDH
Figure 2B

KCNN4c

WGA

Apical
Sub-Apial
Basolateral

0 = KCNN4c proteins

Overlay

XY

2C

Mouse colon
Figure 3

A

B

FSK stimulated $I_{K(ap)}$ (µA/cm²)

Time (min)

CCH stimulated $\Delta I_{K(ap)}$ (µA/cm²)

T84WT  Epac1KDT84

5mM  145mM

K⁺ gradient

bl Nystatin

WT  Epac1KD

P < 0.01

P < 0.005
Figure 4

A

XZ

T84WT

XZ

T84Epac1KD

B

7.5ul 15ul 30ul

Total

40kDa

37kDa

Surface

40kDa

37kDa

7.5ul 15ul 30ul

T84WT

Epac1KDT84

C

Total | Surface

Amount of KCNN4c (% of control)

| T84WT | T84Epac1KD |
|-------|------------|
| 110   | 88         |
| 88    | 66         |
| 66    | 44         |
| 44    | 22         |

* = KCNN4c channel

Apical

Sub-apical

Basolateral
Figure 6

A

\[ \Delta I_{K(\text{ap})} (\mu A/cm^2) \]

| Treatment                | Value       |
|--------------------------|-------------|
| FSK                     | 35          |
| FSK + 4h C3             | 25          |
| Toxin + H1152           | 20          |

P < 0.05  P < 0.01

B

XZ

-H1152 treatment

+H1152 treatment

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The Epac1 signaling pathway regulates Cl<-> secretion via modulation of apical KCNN4c channels in diarrhea
Irshad Ali Shiekh, Hemanta Koley, Manoj K. Chakrabarti and Kazi Mirajul Hoque

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