CHAPTER

EPAC1 REGULATE PKA
INDEPENDENT cAMP MEDIATED
INTESTINAL CI⁻ SECRETION
ABSTRACT

The second messenger cAMP and calcium ([Ca^{2+}]_i) provoke intestinal Cl^− secretion. Recent investigation confirmed exchange protein directly activated by cAMP (Epac) as new cAMP effector apart from classical PKA. Therefore, we examine the function of Epac1 in cAMP stimulated Cl^− secretion. Human intestinal T84 cells and mouse small intestine were used to measure short circuit current (Isc) in response to agonist stimulated Cl^− secretion. cAMP agonist, forskolin stimulated Cl^− secretion was completely suppressed by additive addition of PKA inhibitor (H89) and [Ca^{2+}]_i chelator BAPTA-AM. We also observe Epac1 agonist, 8-pCPT and forskolin activate Rap2, elevate [Ca^{2+}]_i and stimulate Cl^− secretion in T84 cells and mouse intestine. Interestingly, the effect of 8-pCPT was completely inhibited by BAPTA-AM and insensitive to H89. In contrast, in Epac1 silenced T84 cells, forskolin effect was reduced and was completely inhibited by H89 but was insensitive to PLC inhibitor U73122 or BAPTA-AM. 8-pCPT induced Cl^− secretion was not inhibited by CFTR inhibitor 172 or glibenclamide signifying CFTR channels are not involved. Furthermore, biophysical characterization of Epac1 dependent Cl^− conductance of T84 cells suggests that this conductance was hyperpolarization activated and inwardly rectifying. Taken together we conclude that Epac-Rap-PLC-[Ca^{2+}]_i signal transduction pathway mediate cAMP stimulated Cl^− secretion by a novel, previously unknown Cl^− channel.
INTRODUCTION

Epithelial ion transport is a dynamic process driven by electrochemical gradients that are established by the Na\(^+\)/K\(^+\)-ATPase pump located in the basolateral membrane of polarized epithelial cells. This allow entry of Na\(^+\), Cl\(^-\) and K\(^+\) through the basolateral membrane via the NKCC1 cotransporter and exist through apical CFTR Cl channel and Ca\(^{2+}\) - activated Cl\(^-\) channel (CaCC) while K\(^+\) is recycled through basolateral K\(^+\) channels for sustained Cl\(^-\) secretion into the lumen. The universal second messenger cAMP plays a crucial role in maintaining synchronous activity of these epithelial ion channels and perturbation of cAMP mediated signaling pathways caused multiple gastrointestinal diseases such as cholera toxin mediated secretory diarrhea [34, 35].

When cholera toxin is secreted from Vibrio cholerae binds to the GM1 ganglioside receptor on the enterocyte surface, its trigger endocytosis of the toxin within the cells whereby the enzymatic A1 fragment of the toxin A subunit enters the cytosol to activates the G\(s_\alpha\) protein through an ADP-ribosylation reaction that acts to lock the G protein in its active GTP-bound form, thereby continually activating adenylate cyclase to generate abnormally high cyclic adenosine monophosphate (cAMP). High cAMP levels activate PKA dependent major apical Cl\(^-\) channel cystic fibrosis transmembrane conductance regulator (CFTR), causing a dramatic efflux of vital electrolyte and fluid from infected enterocytes, leading to secretory diarrhea [36]. This mechanism of cholera toxin illustrate major role of PKA as immediate downstream target of cAMP to modulate intestinal ion channel function to cause secretory diarrhea. Interestingly, several recent studies reported major role of Epac proteins in the mediating various PKA independent cAMP dependent signaling cascade during various physiological and pathophysiological processes [37]. In this context, several studies
unequivocally established that PKA may not be the only target of cAMP because intracellular calcium \([\text{Ca}^{2+}]_{\text{i}}\) can be altered in response to FSK and/or cAMP. For instance, Merlin group previously showed that FSK elevates \([\text{Ca}^{2+}]_{\text{i}}\) by unknown mechanisms in T84 cells [38]. Similarly, FSK/cAMP increases \([\text{Ca}^{2+}]_{\text{i}}\) in chicken enterocytes [39]. These observations suggest that FSK elicits Cl\(^/-\) secretion by activating not only PKA, but also other signaling pathways such as \([\text{Ca}^{2+}]_{\text{i}}\). Furthermore, Epac has been shown to crosstalk cAMP into \([\text{Ca}^{2+}]_{\text{i}}\) signaling via PLC through PKA independent mechanism [40]. Because cAMP and \([\text{Ca}^{2+}]_{\text{i}}\) are major regulators of intestinal Cl\(^/-\) secretion, we investigate whether Epac plays a role in FSK- or cAMP-stimulated Cl\(^/-\) secretion in intestinal epithelial cells, and whether Epac links intracellular cAMP signaling to \([\text{Ca}^{2+}]_{\text{i}}\) elevation, thus allowing cAMP to induce Cl\(^/-\) secretion in a PKA-dependent and-independent manner. Additionally, Cl\(^/-\) channels are a functionally and structurally varied group, and some function in normal fluid transport across various epithelia [41-43]. The CFTR channel is inhibited by CFTR inhibitor 172 (CFTRinh-172), and only the CFTR channel has been implicated in cAMP-activated Cl\(^/-\) secretion in a variety of epithelia, including the airway and intestine [44]. However, the Epac-activated Cl\(^/-\) secretion in the intestine was insensitive to CFTRinh-172, and thus the present study also characterizes this new Cl\(^/-\) conductance biophysically as well as pharmacologically.
MATERIALS AND METHODS

Cell culture and Ussing chamber set up: Wild type human colonic T84 intestinal epithelial cells (T84WT) were obtained from the Gastroenterology Division, The John Hopkins University. T84WT cells was routinely maintained in a 1:1 ratio of Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F-12 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. 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, catalogue number 3407) and grown for 10–14 days during which time the media were changed every 48 h. Monolayer resistance was determined using an EVOM ohmmeter with STX2 electrodes (World Precision Instruments, Inc.). Monolayers were considered polarized and mounted in an Ussing chamber when resistance was equal to or greater than 1,500 ohms/cm². Polarized T84WT was mounted in an Ussing chamber, and transepithelial potential differences were clamped to 0 mV using a VCC MC6 multichannel voltage current clamp amplifier (Physiologic Instruments). The Isc was continuously recorded using Ag-AgCl electrodes in 3 M KCl agar bridges. Apical and basolateral solutions were maintained at 37 °C by heated water jackets and separately perfused and oxygenated with 100% O2. The bath Ringer’s solution contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 10 mM glucose, and 10 mM HEPES adjusted to pH 7.4. The change in Isc induced by the treatment was expressed as the difference from the base line to the steady state.

Measurement of Apical Chloride Current (ICl) in permeabilized T84 cells—ICl(ap) was assessed by applying a high basolateral to low apical [Cl⁻] gradient across the T84
monolayers followed by permeabilization with the monovalent ionophore nystatin used previously [45, 46]. Briefly, both apical and basolateral hemichamber of Ussing Chamber were initially filled with a Cl⁻ free/high K⁺ solution containing 10 mM sodium gluconate, 140 mM potassium gluconate, 1 mM calcium gluconate, 10 mM glucose, and 10 mM HEPES, pH 7.4. After equilibration, the basolateral solution was replaced with a high Cl⁻/high K⁺ solution containing 10 mM NaCl, 140 mM KCl, 1 mM MgSO₄, 1 mM CaCl₂, 10 mM glucose, and 10 mM HEPES, pH 7.4. 50μg/ml nystatin was then added to permeabilize the basolateral membrane to potassium and chloride. 35μM ouabain was added to the basolateral hemichamber to inhibit the Na⁺-K⁺-ATPase. Under these conditions, there was an agonist-induced ICₜ as Cl ions from the cell moved down the concentration gradient through the Cl channels in the apical membrane.

Current-Voltage (I-V) Relationship Study: For the generation of an I-V relationship, the voltage across the T84 monolayer was sequentially stepped from a holding voltage of 0 mV to values between +100 and -100mV in 20mV increments with a pulse duration of 5 s, and corresponding currents were recorded. A 50-s interval between each pulse was sufficient for recovery from activation. The protocol was performed after sustained stimulation with forskolin or 8-pCPT-2’-O-Me-cAMP.

Calcium imaging: T84 cells were grown on glass coverslips were incubated with 2 μM Fura2-AM in HBSS for 30–45 min in the dark at 37°C, followed by 30 min de-esterification in HBSS containing 5μM indomethacin at room temperature. Fluorescence images were monitored at a 510-nm emission with alternating excitation at 340 and 380 nm as described
previously [47]. Individual Fura-2 loaded T84 cells were selected and [Ca\(^{2+}\)]\(_i\) values were calculated using Origin software (OriginLab). All measurements shown are representative of a minimum of four independent experiments with no fewer than 50 cells calculated in each study.

**Reverse transcription (RT)-PCR:** Using TRIZOL reagent (Invitrogen) total RNA was isolated from T84 cells. cDNA was synthesized from total RNA using random hexamers and superscript II RT. 1 μl of the first-strand cDNA product was then used as template for PCR amplification with Taq DNA polymerase by 35 thermocycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min using oligonucleotides specific for Epac1 (sense: TTCCTCCAGCAACTCTCAG; antisense: TCAGCTCTTGCGCTTCTTG; size of PCR product is 460 bp) and Epac2 (sense:CTCATTGGACCTACGTTCC; antisense: AGTCATCTACTTCATGCAG; size of PCR product is 480 bp).

**Western blot analysis:** T84WT and Epac1KDT84 cells grown on Transwell permeable support (75-mm diameter; Costar, catalogue number 3419) were washed and scraped in PBS solution and then homogenized by sonication in RIPA buffer with protease inhibitor mixture (1:100; Sigma) to obtain cell lysate. Total cell lysate from different region of mouse intestine were prepared as reported previously [48]. These cell lysates 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 overnight with primary antibody at 4 °C. The primary antibodies used were Epac1 mouse monoclonal. IRDye 800- or 680-conjugated anti-mouse or anti-rabbit IgG (Rockland Immunochemicals, Gilbertsville,
PA) was used as a secondary antibody for 1 h followed by three washes. The fluorescence signal was analyzed using the Odyssey infra red system at 700- and 800-nm wavelengths (LI-COR Biosciences) as described previously.

**In Vitro Activation of Rap2:** Activation of Rap2 was determined by the EZ-Detect Rap activation kit (Thermo Fisher Scientific) as described previously with slight modification (27). Serum deprived cells were stimulated with or without forskolin (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 mixture. After lysis, samples were centrifuged at 14,000 X g at 4°C for 10 min, and the protein concentrations of the lysates were measured. 500ug of cell lysates were immediately affinity precipitated at 4 °C for 1 h with 20 µg of glutathione S-transferase-tagged Ras-binding domain of the Ral guanine nucleotide dissociation stimulator (RalGDS-RBD) following the manufacturer’s protocol. Activated Rap2 (Rap2-GTP) was eluted in 50ul of Laemmli sample buffer and detected by Western blotting using an antibody specific to Rap2.

**RNA interference:** Epac1 Knockdown by Lenti shRNA—The RNA Interference Consortium (TRC) lentivirus-based short hairpinRNAs (shRNAs) were used to knock down Epac1 inT84WT cells according to our previously described method [45]. In brief, gene sequence-specific shRNA clones constructed within the lentivirus plasmid vector pLKO.1-puromycin were obtained from Sigma-Aldrich (TRC47228, TRC47230, and TRC47231). Stable cell lines of T84WT with expression of Epac1 knocked down were generated by infecting cells with respective gene-specific lenti-shRNA particles, and positively
transduced cells were selected with 10\(\mu\)g/ml puromycin-containing medium. mRNA expression in transduced cells was evaluated by quantitative PCR as well as by measuring Isc. The lenti-shRNA construct specific for green fluorescence protein (GFP) was transduced in T84WT cells and served as a negative control.

**Immunocytochemistry**: Confluent T84 cells grown on 25-mm tissue culture inserts (Thermo Fisher Scientific) were fixed on ice in 3% paraformaldehyde solution in PBS (EM grade; Electron Microscopy Sciences). Cells were washed in ice-cold PBS and quenched with 50 mM NH4Cl in PBS for 15 min on ice. Nonspecific staining was blocked in PBS containing 1% BSA and 0.075% saponin for 30 min on ice. An anti–Epac 1 antibody (1:10) was then added for 1 h at room temperature, after which excess antibody was washed in 0.1% BSA in PBS with 0.075% saponin. The cells were then incubated with a goat anti–mouse secondary antibody conjugated to Alexa 568 for an additional hour. After extensive washing of excess secondary antibody, cells were incubated with Hoechst 33324 (Invitrogen) and mounted on microscope slides. Microscopy was performed using an LSM 510 (Carl Zeiss, Inc.).
RESULT

FSK stimulated Cl⁻ secretion is PKA independent and Ca²⁺ dependent in T84 cells

We first examined if cAMP stimulated Cl⁻ secretion in T84 cells was mediated exclusively by PKA dependent manner. Polarized T84 cells were mounted in Ussing chambers containing an HCO₃⁻ free Cl⁻ solution. The effect of FSK was partially inhibited by the conventional PKC inhibitor Gö6976, the PLC inhibitor U73122, PKA inhibitor H89 or the intracellular Ca²⁺ chelator BAPTA-AM (Fig.1). Interestingly, inhibiting both PKA and PKC activity by high concentration of H89, completely inhibited FSK stimulated Cl⁻ secretion [49, 50]. Therefore, we further tested whether the combination of H89 and other kinase inhibitors could completely inhibit the FSK-stimulated Cl⁻ secretion. The effects of H89 and Gö6976, of H89 and BAPTA-AM, or of H89 and U73122 were additive. These results imply that FSK-stimulated Cl⁻ secretion was both PKA and Ca²⁺ dependent, and that Ca²⁺ effect was additive to the effect of PKA.

Figure 1. Effect of protein kinase modulators and BAPTA-AM on FSK-stimulated Cl⁻ secretion. T84 cells were pretreated with H89, Gö6976, U73122, BAPTA-AM, H89 plus Gö6976, H89 plus BAPTA-AM, or H89 plus U73122 for 30 min with the indicated concentrations. FSK-stimulated Isc for each condition was derived from the Isc values before and after the addition of FSK. Statistical comparisons between means were performed using Student’s t test. *, P < 0.05; compared with FSK control group.
**FSK elevate [Ca\(^{2+}\)]_i in T84 cells**

Next we determined the change of [Ca\(^{2+}\)]_i in T84 cells upon FSK stimulation. As observed in Fig.2A, FSK caused a sustained rise in [Ca\(^{2+}\)]_i immediately after its addition. To determine the source of this [Ca\(^{2+}\)]_i increase by FSK treatment, we measure [Ca\(^{2+}\)]_i in the absence of extracellular Ca\(^{2+}\) and then after the introduction of Ca\(^{2+}\) to the extracellular medium. Both Ca\(^{2+}\) release and entry were observed after FSK addition (Fig. 2B). The sustained rise in [Ca\(^{2+}\)]_i depended on extracellular Ca\(^{2+}\), whereas the initial rise did not. This increase in [Ca\(^{2+}\)]_i was blocked by preincubation with the PLC inhibitor U73122 (Fig. 2C). Together, these preliminary results show that Ca\(^{2+}\) release from IP3-sensitive internal Ca\(^{2+}\) stores and entry from the extracellular milieu may have a role in FSK-stimulated Cl\(^{-}\) secretion.

**Figure 2. Measurement of [Ca\(^{2+}\)]_i in T84 cells.** (A) Cells were loaded with Fura2-AM at 37°C in the presence of 1.2 mM of extracellular Ca\(^{2+}\). After 30 min of de-esterification in the presence of 5 μM indomethacin, cell monolayers were used for [Ca\(^{2+}\)]_i measurement at room temperature in the presence of 1.8 mM of extracellular calcium and challenged with 10 μM FSK as indicated (n = 3). (B) [Ca\(^{2+}\)]_i was measured in cells challenged with 10 μM FSK initially in the absence of extracellular Ca\(^{2+}\), followed by reintroduction of 1.8 mM of extracellular Ca\(^{2+}\). (C) [Ca\(^{2+}\)]_i was measured in cells in the absence or presence of extracellular Ca\(^{2+}\) by preincubating with 10 μM U73122 during the 30 min of deesterification. FSK was then added (n = 3).
Epac express in T84 cells and in mouse intestine

As part for investigating role of Epac1 in FSK stimulated Cl⁻ secretion, we next examined whether Epac1 protein expresses in human colonic adenocarcinoma T84 and HT29 cell monolayers. Immunoblot in Fig 3A reveals a band around 105 kDa corresponding to expression of Epac1 protein in T84 and HT29 cells. Similarly, Epac1 expression was confirmed in different region of mouse intestine with highest expression observed in colon followed by ileum (Fig. 3B). We further studied distribution of endogenous Epac1 in T84 cells by immunostaining. Fig 3C shows Epac1 staining (red) of T84WT cells mostly intracellular relative to apical surface marker WGA (green).

![Immunoblot of Epac1 expression in T84 & HT29 and fibroblast cell COS. (B) Epac1 localized intracellularly in T84 cells as shown by XZ scanning with confocal microscopy. Epac1 (red), nucleus (Hoechst, blue) and apical surface staining (wheat germ agglutinin, green) AP, apical and BL basolateral. (C) Immunoblot of Epac1 expression in different region of mouse intestine.](image)

Figure 3: Expression of Epac1 in T84 cells and mouse intestine. (A) Immunoblot of Epac1 expression in T84 & HT29 and fibroblast cell COS. (B) Epac1 localized intracellularly in T84 cells as shown by XZ scanning with confocal microscopy. Epac1 (red), nucleus (Hoechst, blue) and apical surface staining (wheat germ agglutinin, green) AP, apical and BL basolateral. (C) Immunoblot of Epac1 expression in different region of mouse intestine.

Function of Epac in FSK stimulated Cl⁻ secretion in T84 cells

We next determined whether 8-pCPT-2’-O-Me-cAMP, an Epac agonist, could stimulate Cl⁻ secretion in T84 cells so as to establish a link from cAMP to PLC-[Ca²⁺]-PKC signaling and to support a role for Epac in epithelial Cl⁻ secretion. As shown in Fig. 4A, 50 μM 8-
pCPT-2’-O-Me-cAMP stimulated Cl− secretion which was significantly inhibited by pretreatment of T84WT cells with BAPTA-AM, but not with H89. Therefore, [Ca^{2+}]i, but not PKA, provoke the effect of 8-pCPT-2’-O-Me-cAMP. We hypothesized that if the Epac1-PLC-[Ca^{2+}]i-PKC pathway contributed to the PKA independent component of FSK stimulated Cl− secretion, the combined effects of PKA and Epac1 on Cl− secretion in T84WT cells would be additive. As shown in Fig. 4B, the PKA agonist Sp-8-pCPT-cAMP (20 μM) robustly rise Isc which further rises due to addition of Epac activator in T84WT cells. This stimulatory effect of Sp-8-pCPT-cAMP and 8-pCPT-2’-O-Me-cAMP on Cl− secretion was additive.

Figure 4. Direct activation of Epac with 8-pCPT-2’-O-Me-cAMP–stimulated Cl− secretion in T84 cells. (A) 50 μM 8-pCPT-2’-O-Me-cAMP was added to the basolateral membrane of control cells or cells preincubated with 1 μM H89 or 25 μM BAPTA-AM for 30 min. 8-pCPT-2’-O-Me-cAMP–stimulated Isc was derived from the Isc values before and after the addition of 8-pCPT-2’-O-Me-cAMP. Values are means ± SEM (n = 5). (B) Additive effects of Sp-8-pCPTcAMP and 8-pCPT-2’-O-Me-cAMP on Cl− secretion in T84 cells. 20 μM Sp-8-pCPT-cAMP was added to the basolateral membrane. This induced a sustained Cl− secretion. 50 μM 8-pCPT-2’-O-Me-cAMP was subsequently added, and this caused a further increase in Isc. Values are means ± SE (n = 6).

Next we examine whether FSK or 8-pCPT-2’-OMe-cAMP able to activate Rap2 in T84 cells because activation of Rap2 results in PLC activation and increased [Ca^{2+}]i [51]. As
shown in Fig. 5A, both FSK and 8-pCPT-2’-O-Me-cAMP increased the amount of active GTP Rap2 in T84WT. Together this result suggest that Epac was activated by both FSK or 8-pCPT-2’-O-Me-cAMP and activated Epac consequently led to Rap2 activation and elevation of [Ca²⁺]i, the later event caused by selective activation of Epac1 with 8-pCPT-2’-O-Me-cAMP, leading to arise of [Ca²⁺]i (Fig. 5B).

**Figure 5. Activation of Rap2 by FSK and 8-pCPT-2’-O-Me-cAMP in T84 cells.** (A) Cells were stimulated for 20 min without (control) or with either 10 μM FSK or 50 μM 8-pCPT-2’-O-Me-cAMP, followed by extraction of GTP-loaded Rap2 with glutathione-S transferase-RALGDS-RBD fusion protein. Samples were analyzed by Western blotting with an anti-Rap2 antibody. A representative Western blot of three experiments is shown. (B) 8-pCPT-2’-O-Me-cAMP elevated [Ca²⁺]i in T84 cells. Cells were loaded with Fura2- AM at 37°C in the presence of extracellular Ca²⁺. After 30 min of de-esterification, cell monolayers were subjected to [Ca²⁺]i measurement in the presence of 1.8 mM of extracellular Ca²⁺ and challenged with 50 μM 8-pCPT-2’-O-Me-cAMP as indicated (n = 3).
Role of Epac on apical Cl conductance in T84 cells

Next we investigate function of Epac on apical Cl channels and whether Epac stimulated Cl− secretion is enhanced in T84 cells with the basolateral membrane permeabilized by nystatin [52]. Under this condition, treatment with FSK caused robust and sustained increase in ICl, which symbolize activation of apical chloride channels in T84WT cells (Fig. 6A). Effect of FSK was partially blocked by BAPTA-AM (Fig. 6A). 8-pCPT-2’-O-Me-cAMP also amplify apical ICl in basolaterally permeabilized T84WT cells. In contrast to FSK, we observe 8-pCPT-2’-O-Me-cAMP induced apical ICl was completely suppressed by BAPTA-AM (Fig. 6 B).

Figure 6. Effect of FSK and 8-pCPT-2’-O-Me-cAMP on apical Cl conductance in T84 cells. After equilibration, when the steady state was reached, a basolateral to apical Cl− gradient was imposed and nystatin was added to permeabilize the basolateral membrane. After the basal ICl subsided, FSK (A) or 8-pCPT-2’-O-Me-cAMP (B) was added to the permeabilized basolateral membrane. These induced a robust increase of ICl. Cells were incubated with BAPTA-AM for 30 min before the addition of FSK or 8-pCPT-2’-O-Me-cAMP. A representative tracing is shown for each experiment. The increase in ICl was derived from the ICl value before and after the addition of FSK (n = 3) or 8-pCPT-2’-O-Me-cAMP (n = 5). Values are means ± SEM. Statistical comparisons between means were performed using Student’s t test. **, P < 0.01; ***, P < 0.001 compared with control group.
**Epac1 depletion reduces Cl secretion in T84 cells**

To further confirm the functional role of Epac1 in FSK stimulated Cl⁻ secretion, lentivirus containing shRNA against Epac1 was employed to specifically knockdown Epac1 protein expression in T84 cells. Of the three lent shRNA constructs used to transduce T84 cells, only the constructs 228 significantly reduce Epac1 expression in T84 cells (fig. 7A). The functional consequence of Epac1 knockdown with respect to FSK stimulated Cl⁻ secretion was investigated using this Epac1KD T84 cell lines. As shown in Fig. 7B, Epac1KD T84cst228 reveal only partial (44–51%) of FSK stimulated Cl⁻ secretion as compared with T84WT and vector transduced T84 cells. In contrast, Epac1KD T84cst230

![Figure 7](image-url)

**Figure 7.** Epac1 silencing in T84 cells. (A) Western blot analysis of Epac1 expression in total cell lysate of wild-type T84 (T84-WT), vector control, and Epac1 shRNA-transduced cells (Epac1KD T84CST228, EpacKD T84CST230, and EpacKD T84CST231) (B) Epac1KD T84CST228 and Epac1KD T84CST231 cells responded to FSK with a stimulated Cl⁻ secretion having only half the magnitude as that of wild-type and vector-transduced cells (n = 5) (C) FSK-stimulated Cl⁻ secretion in Epac1KD T84CST228 cells was inhibited by 1 μM H89, but not by U73122 and BAPTA (n = 5). Values are means ± SEM. Statistical analysis was performed using Student’s t test. *, P < 0.05 compared with control.

cells which was unable to deplete Epac1 protein, exhibit similar rise in Isc as observed in T84WT and vector transduced cells when charge with FSK. U73122 and BAPTA-AM has
no effect but H89 completely blocked FSK stimulated Cl⁻ secretion in Epac1KDT84cst228 cells (Fig. 7C), confirming that in the loss of Epac1 protein, FSK stimulated Cl⁻ secretion was completely PKA dependent in T84 cells.

**Biophysical properties of the Epac1 stimulated apical Cl⁻ conductance (ICl)**

Next to characterize the biophysical properties of the secretory currents activated by Epac1, we plotted I-V curve of basolaterally permeabilized T84 cells in presence of 50 μM 8-pCPT-2’-O-Me-cAMP. The recorded currents had significant inward rectification with a reversal potential of 39 mV. Fig. 8B reveals that outwardly directed FSK activated currents (black squares) had large amplitudes and linear, but the apical application of CFTRinh172 reduced the current amplitude and modify the shape of the I-V curve. The FSK plus CFTRinh172 currents display inwardly rectifying, similar to the currents activated by 8-pCPT-2’-O-Me-cAMP (Fig. 8B).

Figure 8: Biophysical characterization of FSK-activated, Epac-mediated Isc current. (A) I-V plot of the 8-pCPT-2’-O-Me-cAMP–stimulated current of nystatin-permeabilized T84 cells with basal to apical Cl⁻ gradient. (B) I-V plot of current recordings of nystatin permeabilized T84 cells treated with 10 μM FSK (black squares) or 10 μM FSK plus 5 μM CFTRinh172 (red circles) under symmetrical Cl⁻ gradient. (data are means ± SEM; n = 2–8).
To characterize the apical Cl channel involves in Epac1 mediated Cl secretion, we established a pharmacological profile using various inhibitors of Cl channels on apical Cl conductance in basolaterally permeabilized T84WT cells. As shown in Fig. 9A, 8-pCPT-2’-O-Me-cAMP–stimulated ICl was insensitive to 5 μM of the CFTRinh-172 [53] or 100 μM glibenclamide [54]. Other inhibitors added were 100 μM niflumic acid, which inhibits the calcium-activated Cl channel (CaCC), 100 μM zinc (Zn²⁺), and 1 mM cadmium chloride (Cd²⁺), which inhibits the voltage-gated Cl channel isoform 2 (ClC2). The lack of effect of these inhibitors suggested that CFTR, CaCC, and ClC2 were not responsible for Epac-mediated Cl secretion (Fig. 9A).

**Figure 9. Pharmacological profile of the Cl channel blockers in basolaterally permeabilized T84 cells.** (A) Effect of channel blockers on 8-pCPT-2’-O-Me-cAMP–stimulated apical Cl conductance. CFTRinh-172, glibenclamide, niflumic acid, or cadmium chloride (CdCl2) was applied after maximum Cl currents in the presence of 8-pCPT-2’-OMe-cAMP (data are mean ± SEM; n = 3–5). NS, not significant. (B) Effects of DIDS or NPPB on FSK-stimulated ICl in the presence of CFTRinh-172. Representative traces showing that DIDS and NPPB inhibited the FSK-stimulated, CFTRinh-172 insensitive Cl current (Epac mediated) in basolaterally permeabilized T84 cells. (C) Effect of GlyH-101 on FSK-stimulated ICl in basolaterally permeabilized T84 cells. Representative trace showing that GlyH-101 completely inhibited FSK-stimulated apical ICl (n = 3).
To prove that cAMP-stimulated Cl\(^-\) secretion is the result of two different Cl\(^-\) channels, we determine ICl in basolaterally permeabilized T84WT cells in presence of either CFTRinh-172 plus DIDS or CFTRinh-172 plus NPPB. Fig. 9B shows that the combined effect of CFTRinh-172 along with either DIDS or NPPB was greater compared with CFTRinh-172 alone. Thus, these two inhibitors partially blocked a component of the cAMP stimulated Cl\(^-\) secretion in intact T84 cells. Interestingly, we have found that the cAMP-stimulated apical ICl was completely inhibited by GlyH-101 (Fig. 9C), a previously considered CFTR blocker but now known to also block CaCCs [55].
DISCUSSION:

In modulating CFTR function in the apical membrane of intestinal and airway cells, classical PKA has been considered as the only effector of cAMP [56-58]. The observation that cAMP agonist FSK which mediates Cl\textsuperscript{−} secretion additionally cause elevation of [Ca\textsuperscript{2+}] suggest [Ca\textsuperscript{2+}] might play a role in epithelial Cl\textsuperscript{−} secretion. However the signal transduction pathway through which cAMP elevates [Ca\textsuperscript{2+}] is unclear. Recently, Epac1 was discovered as an additional cAMP binding protein that transduces various cAMP signaling pathway that was previously attributed solely due to PKA activity [59]. Because both PKA and Epac1 are activated by cAMP with similar affinity and are expressed in many tissues, this present study aim to decipher the role of Epac1 in the crosstalk between cAMP and Ca\textsuperscript{2+} signaling in the context of epithelial Cl\textsuperscript{−} transport mechanism.

Our result reveal both PKA and PLC-[Ca\textsuperscript{2+}]i-PKC pathway are involved in FSK-stimulated Cl\textsuperscript{−} secretion and this effect is additive. The use of H89 highlight the existence of two independent (PKA and PKC) signaling pathway. 1uM H89 which inhibits PKA partially block FSK stimulated Cl\textsuperscript{−} secretion while 50uM H89 which additionally block PKC caused complete inhibition of FSK stimulated Cl\textsuperscript{−} secretion. Also, FSK stimulated Cl\textsuperscript{−} secretion was observe partially inhibited by U73122, BAPTA-AM and Gö6976 whereas the effects of H89 and Gö6976, of H89 and BAPTA-AM, or of H89 and U73122 were additive. These results suggested the presence of a Ca\textsuperscript{2+}-sensitive, PKA-independent component of Cl\textsuperscript{−} secretion. The functional role of Epac1 in Cl\textsuperscript{−} secretion was reveals by Epac1 agonist 8-pCPT, which we observe to activate Rap2, rise [Ca\textsuperscript{2+}]i and Cl\textsuperscript{−} secretion in T84WT cells. Epac1 role in Cl\textsuperscript{−} secretion was confirmed by the observe reduction in FSK stimulated Cl\textsuperscript{−} secretion (~50%) as compared to T84WT and vector control T84 cells. Most notably, this reduction in Isc
response to FSK was not due to change in CFTR protein. Our studies also found that the construct TRC47230 failed to silence Epac1 therefore, Epac1KDT84cst230 cells was considered a negative control in addition to the vector-transduced cells. FSK stimulated Cl− secretion in Epac1KDT84cst228 cells was completely dependent on PKA, no longer dependent on PLC, and insensitive to BAPTA-AM treatment. This compelling result confirmed Epac1 mediates cAMP stimulated Cl− secretion.

Next to identify the identity of apical Cl channel involves in Epac1 mediated Cl− secretion, we study its I-V curve and blocker sensitivity. The Epac1 induced current was inwardly rectifying, was sensitive to BAPTA-AM and thus Ca2+ sensitive and was insensitive to Cd2+, Zn2+, CFTRinh-172, or niflumic acid, but was blocked by DIDS, NPPB, and GlyH-101. The participation of a non-CFTR Cl channel was strongly indicated by the observe specific CFTR channel blocker, CFTRinh-172, partially suppressed the cAMP-stimulated Cl− secretion but was unable to inhibit 8-pCPT-2’-O-Me-cAMP stimulated apical Cl conductance in T84WT cells. Based on the criteria of rectification and blocker sensitivity, we presume that the Epac1 stimulated apical Cl channel is not the recently identified CaCC, now called ANO1 (TMEM16A) [60]. The characteristics of the Epac-mediated Cl− current of T84 cells do not neatly fit the characteristics of any known epithelial Cl channel. There was reasonable evidence in our study to show that FSK- and 8-pCPT-2’-O-Me-cAMP-increased [Ca2+]i in T84 cells was required for some of the actions of cAMP, “the Epac1–Ca2+ pathway” in the activation of Cl conductances. It is likely this new conductance is another previously undocumented calcium-activated IC1.
These results suggest that PKA and Epac1 are independent downstream effectors of cAMP and Epac1 activates Rap2, which then activates PLC to elevate $[\text{Ca}^{2+}]_i$ in T84 cells. PLC is activated by small GTPases, such as Ras and Rho, and the involvement of PLC was indicated by the rise of $[\text{Ca}^{2+}]_i$ by FSK, which was completely blocked by U73122, and that FSK induced Cl$^-$ secretion was partially inhibited by U73122. We conclude from all of this that the final outcome of Epac activation is a Cl$^-$ secretion through a yet-to-be identified apical Cl channel. This pathway of enhanced Cl$^-$ secretion, independent of the CFTR channel, might be useful in treating cystic fibrosis where the CFTR channel is defective. Thus, Epac1 transduces cAMP signaling into Ca$^{2+}$ signaling, providing an additional pathway to manifest the effects of cAMP on intestinal Cl$^-$ secretion.