Activation of AMP-Activated Protein Kinase by a Plant-Derived Dihydroisosteviol in Human Intestinal Epithelial Cell

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Our previous study has shown that dihydroisosteviol (DHIS), a derivative of stevioside isolated from *Stevia rebaudiana* (Bertoni), inhibits cystic fibrosis transmembrane conductance regulator (CFTR)-mediated transepithelial chloride secretion across monolayers of human intestinal epithelial (T84) cells and prevents cholera toxin-induced intestinal fluid secretion in mouse closed loop models. In this study, we aimed to investigate a mechanism by which DHIS inhibits CFTR activity. Apical chloride current measurements in Fisher rat thyroid cells stably transfected with wild-type human CFTR (FRT-CFTR cells) and T84 cells were used to investigate mechanism of CFTR inhibition by DHIS. In addition, effect of DHIS on AMP-activated protein kinase (AMPK) activation was investigated using Western blot analysis. Surprisingly, it was found that DHIS failed to inhibit CFTR-mediated apical chloride current in FRT-CFTR cells. In contrast, DHIS effectively inhibited CFTR-mediated apical chloride current induced by a cell permeable cAMP analog CPT-cAMP and a direct CFTR activator genistein in T84 cell monolayers. Interestingly, this inhibitory effect of DHIS on CFTR was significantly ($p<0.05$) reduced by pretreatment with compound C, an AMPK inhibitor. AICAR, a known AMPK activator, was able to inhibit CFTR activity in both FRT-CFTR and T84 cells. Western blot analysis showed that DHIS induced AMPK activation in T84 cells, but not in FRT-CFTR cells. Our results indicate that DHIS inhibits CFTR-mediated chloride secretion in T84 cells, in part, by activation of AMPK activity. DHIS therefore represents a novel candidate of AMPK activators.

Key words chloride channel; AMP-activated protein kinase; T84 cell; stevioside; dihydroisosteviol

Cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-activated Cl^- channel expressed in epithelial cells of several organs including lung, intestine, pancreas, and testes. CFTR is predominantly located in the apical membrane of epithelial cells and provides a principal route for cAMP-mediated Cl^- secretion, a process that can be stimulated by humeral, neuronal and bacterial factors.[1)] Loss-of-function mutations of CFTR cause cystic fibrosis, a common inherited disease characterized by recurrent pulmonary infection and deterioration of lung function.[2)] In addition, cAMP-mediated stimulation of transepithelial Cl^- secretion *via* CFTR is involved in the pathogenesis of other diseases such as secretory diarrhea and polycystic kidney disease.[3,4)] CFTR inhibitors have been shown to effectively inhibit fluid loss in rodent models of cholera and to reduce renal cyst progression in mouse models of polycystic kidney disease.[5,6)]

CFTR exists in a macromolecular complex consisted of several interacting partners including regulatory proteins such as protein kinase A (PKA) and adenosine monophosphate (AMP)-activated protein kinase (AMPK).[7,8)] Activation of a cAMP-dependent PKA stimulates CFTR activity *via* phosphorylation at a regulatory (R) domain. On the other hand, it has recently been shown that a metabolic sensor AMPK inhibits CFTR function in heterologous expression systems, polarized epithelial cells and mouse intestine.[8–11)] Recent studies demonstrated that AMPK phosphorylates at serine 737 and serine 768 in the R domain, resulting in inhibition of both basal and PKA-stimulated CFTR activities.[12)]

Stevioside is a natural sweetener extracted from *Stevia rebaudiana*. Stevioside has been shown to possess a number of health benefits including anti-hypertension, anti-hyperglycemia, anti-tumor and anti-inflammation.[13)] Interestingly, we recently showed that steviol, an aglycone of stevioside, inhibited cAMP-mediated Cl^- secretion across monolayers of human intestinal epithelial (T84) cells.[14)] Structural modifications of stevioside have identified dihydroisosteviol (DHIS) (Fig. 1) as the most potent steviol derivative that inhibited cAMP-activated Cl^- secretion across T84 cell monolayers (IC_{50} 9.6 μM) by mechanisms involving inhibition of CFTR Cl^- channel activity.[14)] Importantly, intraluminal administration of DHIS reduced cholera toxin-induced intestinal fluid secretion by approximate 90%.[14)] However, the exact mechanism by which DHIS inhibits CFTR remains unexplored. The present study, therefore, aimed to investigate the mechanism of CFTR inhibition by DHIS using both heterologous expression systems (Fisher rat thyroid cells stably transfected with wild-type human CFTR/FRT-CFTR cells) and T84 cells. The results showed that DHIS inhibits CFTR *via* activation of AMPK activity in T84 cells.

MATERIALS AND METHODS

Cell Lines and Chemicals T84 cells were obtained from

The authors declare no conflict of interest.

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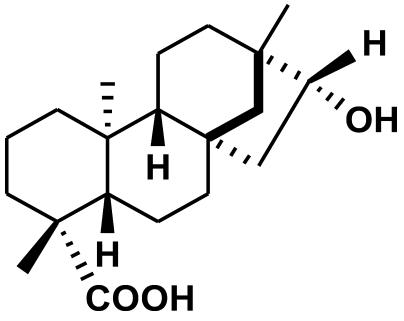

Fig. 1. Chemical Structure of Dihydroisosteviol (DHIS)

American Culture Type Collection (Manassas, VA, U.S.A.). FRT-CFTR cells were a gift from Professor A. S. Verkman, University of California, San Francisco, CA, U.S.A.[15] T84 cells were cultured in a mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 Medium (1 : 1) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. FRT-CFTR cells were cultured in Coon's Modification Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. DHIS was prepared from stevioside as previously described.[14] Purity of DHIS was >99% using thin layer chromatography and nuclear magnetic resonance spectroscopy analyses. $CFTR_{inh}$-172 was purchased from Calbiochem (San Diego, CA, U.S.A.). Trypsin–ethylenediaminetetraacetic acid (EDTA), fetal bovine serum, penicillin and streptomycin were from HyClone (Logan UT, U.S.A.). AMPKα (product code of 2532), phospho-AMPKα (Threonine 172, product code of 2535) and β actin (product code of 4970) antibodies (rabbit as host animal) were purchased from Cell Signaling Technology (Danvers, MA, U.S.A.). Other chemicals including compound C (6-[4-(2-piperidin-1-yl-ethoxy)-phenyl)]-3-pyridin-4-yl-pyrrazolo[1,5-*a*]-pyrimidine) were obtained from Sigma Aldrich (St. Louis, MO, U.S.A.).

Apical Chloride Current (I_{Cl^-}) Measurements FRT-CFTR and T84 cells grown on Snapwell supports for 7 and 14 d, respectively, were mounted in an Ussing chamber setup. A basolateral-to-apical Cl^- gradient was established by using buffers containing different concentrations of Cl^-. Basolateral high Cl^- buffer contained 130 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM Na-*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.3) and 10 mM glucose. In apical low Cl^- buffer, 65 mM NaCl was replaced with 65 mM Na-gluconate, and the concentration of $CaCl_2$ was increased to 2 mM. Thirty minutes prior to I_{Cl^-} measurements, amphotericin B (250 μg/mL) was added into the basolateral solution to allow complete basolateral membrane permeabilization. The solutions were continuously bubbled with 100% O_2 and maintained at 37°C. I_{Cl^-} was recorded using a DVC-1000 voltage-clamp (World Precision Instruments, Sarasota, FL, U.S.A.) with Ag/AgCl electrodes and 1 M KCl agar bridges.

Western Blot Analysis of AMPK Activation T84 cells or FRT-CFTR cells were seeded in 6-well plates and grown overnight in a humidified CO_2 incubator. Cells were then treated with a known AMPK activator 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide (AICAR) (1 mM) or DHIS (50 μM) for 10, 20, 30 and 60 min, and homogenized in lysis buffer containing 50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1% Triton X-100 and cocktail of protease inhibitors (pH 7.4). After centrifugation of the homogenates (10000×***g***) for 20 min, protein concentrations were determined by Lowry method, and samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. The membrane was then incubated for 1 h with 5% non-fat milk and overnight with rabbit antibodies to AMPKα, phosphorylated AMPKα (phosphorylation at threonine 172; p-AMPK) and β actin (all at 1000-fold dilution) followed by three washes with TBST and incubation for 1 h with horseradish peroxidase-conjugated goat antibody to rabbit immunoglobulin G at room temperature. The immunoblot was detected by using chemiluminescence assay kits (PerkinElmer, Waltham, MA, U.S.A.).

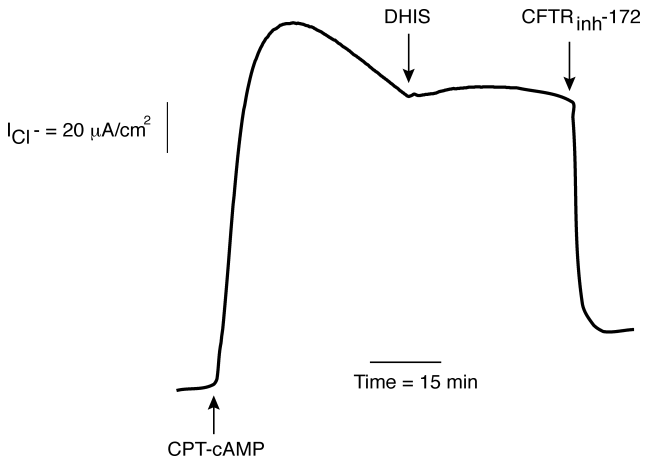

Fig. 2. Effect of DHIS on CFTR-Mediated Apical Cl^- Current in FRT-CFTR Cells

CFTR-mediated apical Cl^- current across FRT-CFTR cell monolayers was stimulated by CPT-cAMP (100 μM in both apical and basolateral solutions). Subsequently, DHIS (50 μM) and $CFTR_{inh}$-172 (5 μM) were added into both apical and basolateral solutions (n=3).

Statistical Analysis Data were expressed as means±S.E. Statistical analysis was made by Student's *t*-test or one-way analysis of variance (ANOVA), where appropriate. $p<0.05$ was considered statistically significant.

RESULTS

DHIS Did Not Inhibit CFTR Activity in CFTR-Transfected Fisher Rat Thyroid Cells (FRT-CFTR Cells) Our previous study indicates that DHIS inhibits cAMP-activated chloride secretion across T84 cell monolayers through its inhibitory effect on CFTR Cl^- channel activity.[14] As Fisher rat thyroid (FRT) cells exhibit low baseline Cl^- transport and no cAMP-activated Cl^- current, FRT cells stably transfected with wild-type human CFTR (FRT-CFTR cells) represent an appropriate cell model for studying effects of DHIS on CFTR function.[15] To determine the effects of DHIS on CFTR function, we performed apical Cl^- current analysis of FRT-CFTR cell monolayers. This technique allows a direct measurement of Cl^- transport function of CFTR, which is expressed in the apical membrane of FRT-CFTR cells. To our surprise, DHIS (50 μM, a concentration which was previously found to completely inhibit cAMP-activated apical Cl^- current in T84 cells) had no effect on CFTR-mediated apical Cl^- current stimulated by CPT-cAMP, a specific PKA activator, in FRT-CFTR cell

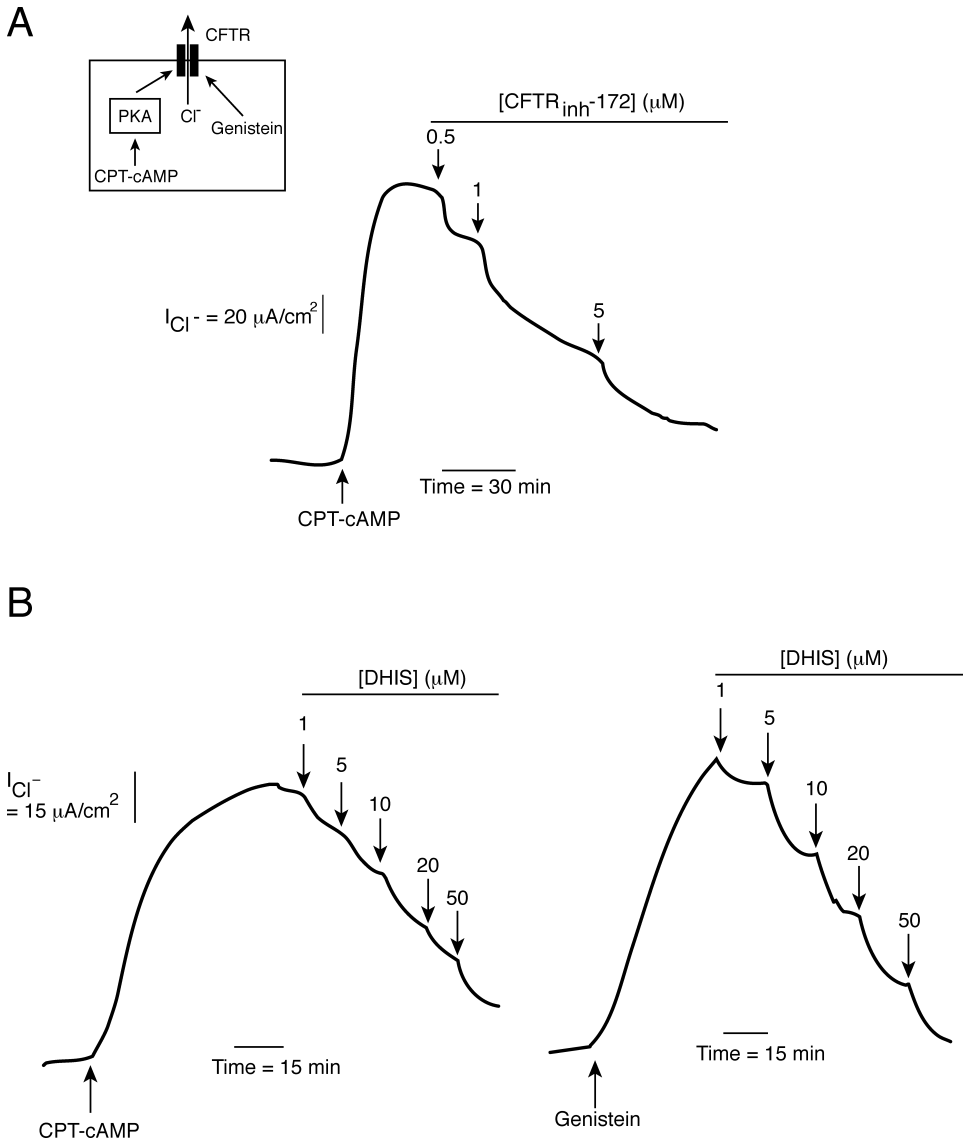

Fig. 3. Effect of DHIS on CFTR-Mediated Apical Cl^- Current in T84 Cells Was PKA-Independent

(A) CFTR as a principal Cl^- channel mediating CPT-cAMP-activated apical Cl^- current in T84 cell monolayers. CPT-cAMP (100 μM) and $CFTR_{inh}$-172 (5 μM) were added into both apical and basolateral solutions (n=3). Inset, A schematic diagram shows mechanism of CFTR activation by CPT-cAMP and genistein. (B) Left: Effect of DHIS on CPT-cAMP-activated apical Cl^- current. A representative current tracing obtained from the study of effect of DHIS on CPT-cAMP-activated apical Cl^- current is shown (n=3). CPT-cAMP (100 μM) and DHIS at indicated final concentrations were added into both apical and basolateral sides of T84 cell monolayers. Right: Effect of DHIS on genistein-activated apical Cl^- current. Genistein (50 μM) and DHIS at indicated final concentrations were added into both apical and basolateral sides of T84 cell monolayers. A representative current tracing of 3 separate experiments is shown.

monolayers (Fig. 2). On the other hand, the remaining CPT-cAMP-induced apical Cl^- current was completely abolished by $CFTR_{inh}$-172 (5 μM), a specific CFTR inhibitor, confirming that the CPT-cAMP-induced apical Cl^- current in FRT-CFTR cells was solely mediated by CFTR. The results indicate that DHIS does not directly inhibit CFTR in FRT-CFTR cells.

Inhibitory Effect of DHIS on CFTR-Mediated Apical Cl^- Current Induced by Different Types of CFTR Agonists in T84 Cells We then studied the mechanisms by which DHIS inhibits CFTR activity in T84 cells using apical Cl^- current measurements, in which basolateral membrane was permeabilized by amphotericin B and buffers with basolateral-to-apical Cl^- gradient were used. CPT-cAMP-activated apical Cl^- current was completely abolished by a specific CFTR inhibitor, $CFTR_{inh}$-172, confirming that the CPT-cAMP-induced Cl^- current in T84 cells was solely mediated by CFTR (Fig. 3A). It was found that DHIS inhibited CPT-cAMP-activated apical Cl^- current in T84 cell monolayers in a dose-dependent manner with an IC_{50} of approximate 10 μM (Fig. 3B). In addition, we investigated the effect of DHIS on CFTR-mediated apical Cl^- current stimulated by genistein (50 μM), which has previously been shown to directly activate CFTR activity.[16] Interestingly, DHIS inhibited genistein-stimulated apical Cl^- current with an IC_{50} of approximate 10 μM (Fig. 3B). The similar potency (IC_{50} approximate 10 μM) between DHIS inhibition of CPT-cAMP and genistein-stimulated Cl^- current indicates that the mechanism of CFTR inhibition by DHIS may not be through PKA inhibition, but *via* direction inhibition and/or modulation of other proteins that regulate CFTR activity.

DHIS Inhibited CFTR-Mediated Cl^- Current in an AMPK-Dependent Manner in T84 Cells Previous *in vitro* and *in vivo* studies demonstrated that AMPK co-localizes with CFTR in intestinal epithelial cells and AMPK activation inhibited CFTR-mediated Cl^- secretion in both cultured

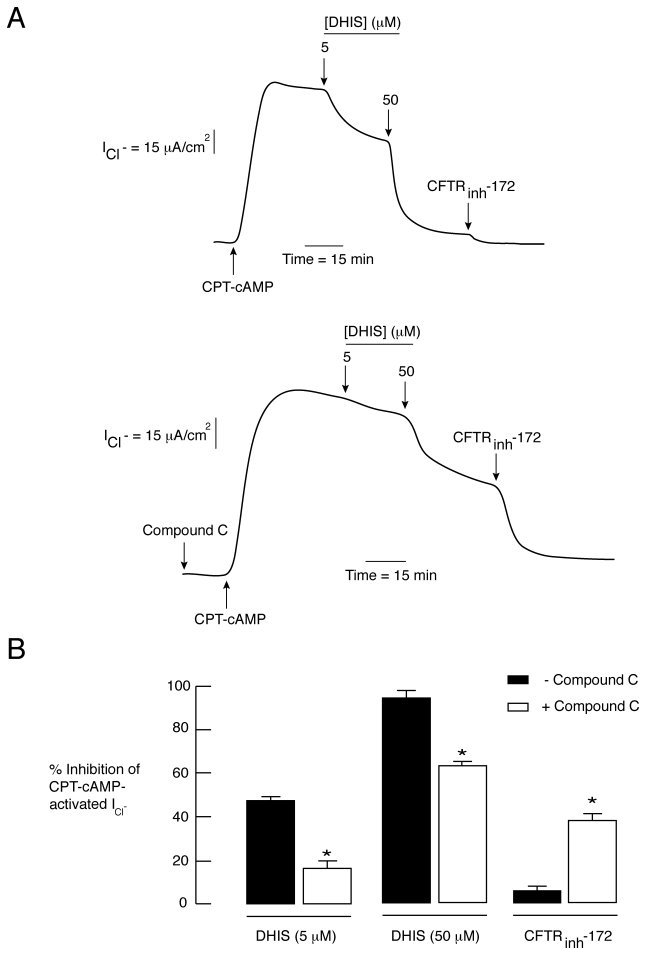

Fig. 4. Involvement of AMPK in the DHIS Inhibition of CFTR-Mediated Apical Cl^- Transport in T84 Cells

(A) Representative current tracings of CFTR-mediated apical Cl^- current. With basolateral membrane permeabilization by amphotericin B (250 μg/mL) and in the presence of basolateral-to-apical Cl^- gradient, T84 cell monolayers were pretreated for 15 min with vehicle (DMSO) or compound C, a specific AMPK inhibitor, (80 μM, added into both apical and basolateral solutions), after which CPT-cAMP (100 μM), DHIS (5, 50 μM), and $CFTR_{inh}$-172 (5 μM) were subsequently added into both apical and basolateral solutions. (B) Summary of the data. Data were shown as means % inhibition of CPT-cAMP-activated apical Cl^- current±S.E. (n=3–5) *p<0.05 compared with control (same concentration of DHIS/$CFTR_{inh}$-172 but no compound C).

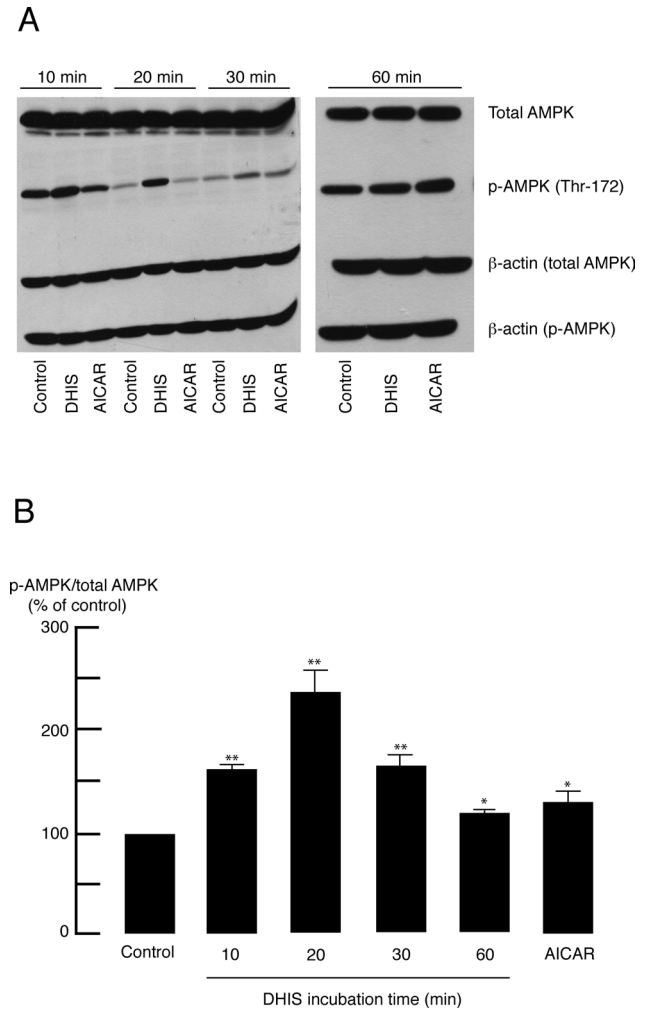

Fig. 5. AMPK Activation by DHIS in T84 Cells

(A) Representative Western blot analyses of AMPK activation. T84 cells were treated with AICAR (1 mM) or DHIS (50 μM) for 10, 20, 30 or 60 min. The protein-transferred membrane was incubated for 1 h with 5% non-fat milk and overnight with rabbit antibodies to AMPKα, phosphorylated AMPKα (phosphorylation at threonine 172; p-AMPK) and β actin before incubation for 1 h with horseradish peroxidase-conjugated goat antibody to rabbit immunoglobulin G. The immunoblot was detected by using chemiluminescence assay kits. (B) Summary of the data. Data for AICAR were from experiments with 60 min of incubation. Data were expressed as means of % control of p-AMPK/total AMPK±S.E. (n=3–4). *p<0.05; **p<0.01 compared with control.

T84 monolayers and mouse intestine.[8,10] We, therefore, hypothesized that DHIS inhibited CFTR-mediated Cl^- secretion in T84 cells *via* activation of AMPK. To test this hypothesis, we investigated the involvement of AMPK in the inhibition by DHIS of CFTR-mediated apical Cl^- current in T84 cells. In this experiment, % inhibition of CFTR-mediated apical Cl^- current by DHIS (at two concentrations, 5, 50 μM) in the presence or absence of compound C, an AMPK inhibitor, was compared. As shown in Fig. 4, DHIS inhibited CFTR-mediated apical Cl^- current (stimulated by CPT-cAMP) in a concentration-dependent manner with almost complete inhibition at 50 μM (% inhibition at 5, 50 μM were 47.4±1.7, 95.4±2.9%, respectively). Pretreatment with compound C (80 μM) significantly (p<0.05) reduced the inhibitory effect of DHIS (% inhibition at 5, 50 μM were 14.4±5.3, 64.2±2.0%, respectively). These findings suggest that AMPK is involved in the CFTR inhibition by DHIS in T84 cells.

Activation of AMPK in T84 Cells In order to confirm that DHIS inhibited CFTR by activating AMPK activity in T84 cells, the effect of DHIS on AMPK activation was investigated using Western blot analysis of phosphorylated AMPKα (Thr-172), an index of AMPK activation. As shown in Fig. 5, treatment with DHIS (50 μM) induced a significant increase in phosphorylated AMPKα by 10 min, and the effect peaked at 20 min after stimulation. The DHIS-induced AMPKα phosphorylation was sustained for 60 min. Similarly, treatment with AICAR, a known AMPK activator, induced AMPKα phosphorylation after 30 min of incubation.

Target of DHIS Action AMPK activation is known to inhibit PKA-stimulated CFTR Cl^- current in both heterologous expression systems and native epithelial cells.[8] Failure of DHIS to inhibit CFTR in FRT-CFTR cells despite its effective CFTR inhibition in T84 cells led us to hypothesize that target(s) of DHIS is (are) upstream of AMPK. As shown in Fig. 6A, a known AMPK activator AICAR significantly inhibited CFTR-mediated apical Cl^- current in both FRT-CFTR and T84 cells. With no addition of AICAR, the stimulated

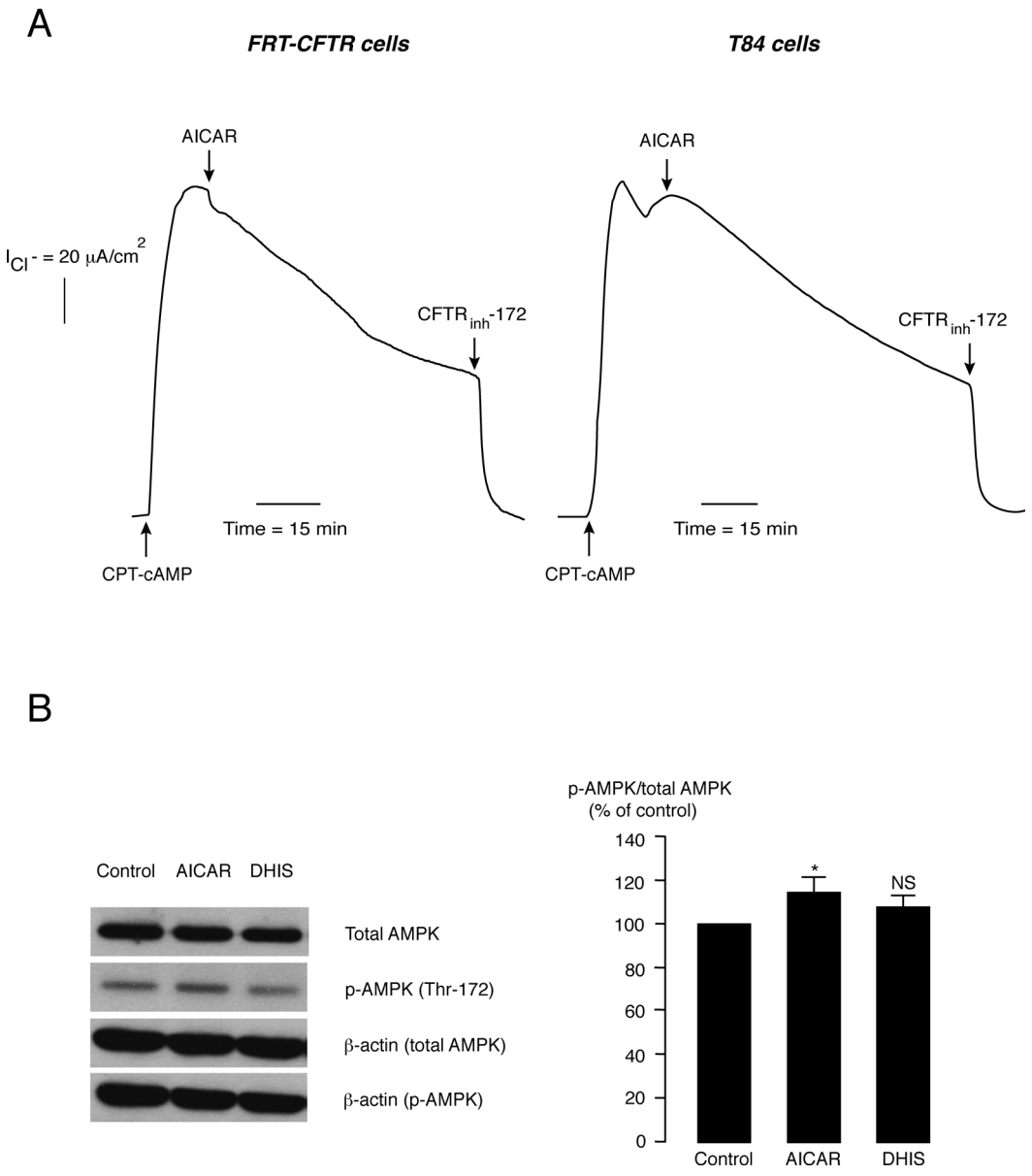

Fig. 6. Target of DHIS Action

(A) Effect of a known AMPK activator AICAR on CFTR-mediated Cl^- current in FRT-CFTR (Left) and T84 (Right) cells. Under basolateral membrane permeabilization by amphotericin B in the presence of basolateral-to-apical Cl^- gradient, after which AICAR (1 mM) and $CFTR_{inh}$-172 (5 μM) were subsequently added into both apical and basolateral solutions (n=3). (B) AMPK activation by DHIS in FRT-CFTR cells. FRT-CFTR cells were treated with AICAR (1 mM) or DHIS (50 μM) for 20 min. A representative (Left) and summary of data (Right) from Western blot analysis of AMPKα, phosphorylated AMPKα (phosphorylation at threonine 172; p-AMPK) and β actin were shown. Data were expressed as means of % control of p-AMPK/total AMPK±S.E. (n=3). NS, not statistically significant; *p<0.05 compared with control.

CFTR-mediated apical Cl^- current declined by less than 5% per an hour (data not shown). To investigate whether DHIS activated AMPK in FRT-CFTR cells, Western blot analysis of phosphorylated AMPKα (Thr-172) was performed. As depicted in Fig. 6B, AICAR treatment significantly increased AMPKα phosphorylation in FRT-CFTR cells. In contrast, DHIS had no effect on AMPKα phosphorylation in these cells. These results suggest that DHIS targets in T84 cells might be upstream of AMPK.

DISCUSSION

DHIS has recently been found to potently inhibit CFTR-mediated Cl^- secretion in T84 cells and to reduce cholera toxin-induced intestinal fluid secretion *in vivo*.[14] In the present study, we determined the mechanisms by which DHIS inhibits CFTR function in T84 cells. Using both FRT-CFTR and T84 cells, we provided evidence that DHIS inhibits CFTR-mediated Cl^- secretion in T84 cells by activating AMPK.

In our previous study, it was hypothesized that DHIS inhibits cAMP-activated Cl^- secretion in T84 cells by targeting CFTR.[14] In this study, we tested this hypothesis using FRT-CFTR cells. This cell model represents an appropriate heterologous expression system for studying CFTR function since FRT cells can form monolayer when grown on permeable supports, allowing short-circuit current analysis.[17] In addition, FRT null cells have been shown to have low baseline Cl^- transport and exhibit no cAMP-activated Cl^- secretion.[17] This cell model has been extensively employed to identify novel CFTR modulators and to characterize their mechanisms

of action.[18–21] To our surprise, apical Cl^- current analysis of FRT-CFTR cell monolayers demonstrated that DHIS had no effect on CFTR-mediated Cl^- transport. This result led us to speculate that the inhibition of CFTR-mediated Cl^- current by DHIS in T84 cells is not due to the direct effect on CFTR, but rather a result of action on other proteins that regulate CFTR function.

AMP-activated protein kinases, energy sensor proteins playing important roles in the maintenance of cellular metabolic homeostasis, have been accepted as an important regulator of CFTR Cl^- transport function. A number of *in vitro* and *in vivo* studies have shown that activation of AMPK inhibits PKA-activated CFTR activity *via* a mechanism involving AMPK-mediated phosphorylation at inhibitory sites within CFTR regulatory domain.[12] Our experiments in T84 cells demonstrated that the inhibitory effect of both high and low doses of DHIS on CFTR-mediated apical Cl^- current was markedly reduced by pretreatment with compound C, a specific AMPK inhibitor (Fig. 4). In addition, it was found in T84 cells that DHIS acutely activated AMPK with a maximal degree of activation by 20 min, at which time the inhibition by DHIS of CFTR-mediated apical Cl^- transport was completed. These findings suggest that DHIS inhibits CFTR-mediated Cl^- transport in T84 cells, at least in part, by activating AMPK. It should be noted that the pretreatment with compound C did not completely abolish effect of DHIS on CFTR-mediated apical Cl^- current in T84 cells. Therefore, it is possible that DHIS inhibits CFTR function by multiple mechanisms. Interestingly, AICAR, a known AMPK activator, was found in this study to inhibit CFTR Cl^- current in both FRT-CFTR and T84 cells. In addition, we found that DHIS failed to activate AMPK in FRT-CFTR cells. Lack of the effect of DHIS on CFTR Cl^- transport activity and AMPK activation in FRT-CFTR cells suggests that target(s) of DHIS might be at the upper cascade of AMPK.

In summary, our study indicates that DHIS inhibits CFTR-mediated transepithelial Cl^- secretion in human intestinal epithelial cells *via* mechanisms involving AMPK activation. This study was the first to identify a small-molecule CFTR inhibitor that exerts its effect, at least in part, by activating the cellular energy sensor AMPK. This study also indicates that DHIS represents a novel class of AMPK activators. Future studies of AMPK activation by DHIS and related compounds will shed light on the therapeutic promise of DHIS and related compounds in the treatment of diseases/conditions, in which AMPK activation may be beneficial such as diabetes, cancer and inflammatory diseases.

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