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A Natural Plant-Derived Dihydroisosteviol Prevents Cholera Toxin-Induced Intestinal Fluid Secretion

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ABSTRACT

Stevioside and its major metabolite, steviol, have been reported to affect ion transport in many types of tissues, such as the kidney, pancreas, and intestine. The effect of stevioside, steviol, and its analogs on intestinal Cl^- secretion was investigated in a human T84 epithelial cell line. Short-circuit current measurements showed that steviol and analogs isosteviol, dihydroisosteviol, and isosteviol 16-oxime inhibited in a dose-dependent manner forskolin-induced Cl^- secretion with IC_{50} values of 101, 100, 9.6, and 50 μM, respectively, whereas the parent compound stevioside had no effect. Apical Cl^- current measurement indicated that dihydroisosteviol targeted the cystic fibrosis transmembrane regulator (CFTR). The inhibitory action of dihydroisosteviol was reversible and was not associated with changes in the intracellular cAMP level. In addition, dihydroisosteviol did not affect calcium-activated chloride secretion and T84 cell viability. In vivo studies using a mouse closed-loop model of cholera toxin-induced intestinal fluid secretion showed that intraluminal injection of 50 μM dihydroisosteviol reduced intestinal fluid secretion by 88.2% without altering fluid absorption. These results indicate that dihydroisosteviol and similar compounds could be a new class of CFTR inhibitors that may be useful for further development as antidiarrheal agents.

Secretion of fluid and electrolytes by the gastrointestinal tract is important in lubricating the epithelial surface, thereby protecting mucosa from physical damage as a food bolus passes along the length of the intestinal tract (Sidhu and Cooke, 1995). Intestinal fluid secretion involves a number of channels and transporters including basolateral K^+ channel, Na^+-K^+-$2Cl^-$ cotransporter, Na^+-K^+ ATPase, apical CFTR-Cl^- channel, and other Cl^- channels (Barrett and Keely, 2000). Abnormal stimulation of secretory processes resulting in diarrhea can be induced by infection with bacteria, parasites, and viruses (Farthing, 2001).

Several bacteria produce enterotoxins that can have profound effects on chloride secretion. *Vibrio cholera* induces a severe secretory diarrhea by producing a toxin that elevates intracellular cAMP and subsequently activates Cl^- secretion into the intestinal lumen (Lencer et al., 1995). The principal methods of managing diarrhea are oral-rehydration therapy and antibiotic drugs. However, oral-rehydration therapy cannot reduce the severity and duration of cholera-induced diarrhea, and antibiotic resistance becomes more prevalent. Therefore, several efforts have been made to search for promising drugs that could be used to treat cholera more efficiently. These methods include inhibitors of cholera toxin receptor binding (Pickens et al., 2004), 5-hydroxytryptamine receptor antagonists (Mourad et al., 1995; Turvill and Farthing, 1997), somatostatin receptor agonists (Farthing, 1996), enkephalinase inhibitors (Cézard et al., 2001), and CFTR inhibitors (Muanprasat et al., 2004, 2007).

Several natural products isolated from plants have also been used as sources of pharmaceuticals and as ingredients of traditional medicines (Phillipson, 1994). *Stevia rebaudiana* is a family member of chrysanthemum, originating from Paraguay (Bridel and Lavieille, 1931). A major extract from *S. rebaudiana* is stevioside, which can be degraded to its major metabolite, steviol, by human intestinal microflora (Fig. 1) (Gardana et al., 2003). Stevioside is approximately

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ABBREVIATIONS: CFTR, cystic fibrosis transmembrane conductance regulator; $CFTR_{inh}$-172, 3-[(3-trifluoromethyl) phenyl]-5-[(4-carboxyphenyl) methylene]-2-thioxo-4-thiazolidinone; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; AC, adenylate cyclase; 8-cpt-cAMP, 8-cholorophenyl-thio-cAMP; FSK, forskolin; CaCC, calcium-activated chloride channel; INH 1, 2-[*N*-(3-hydroxy-4-carboxyphenyl)amino]-4-(4-methylphenyl)-thiazole; GlyH-101, *N*-(2-naphthalenyl)-[3,5-dibromo-2,4-dihydroxyphenyl)methylene]glycine hydrazide.

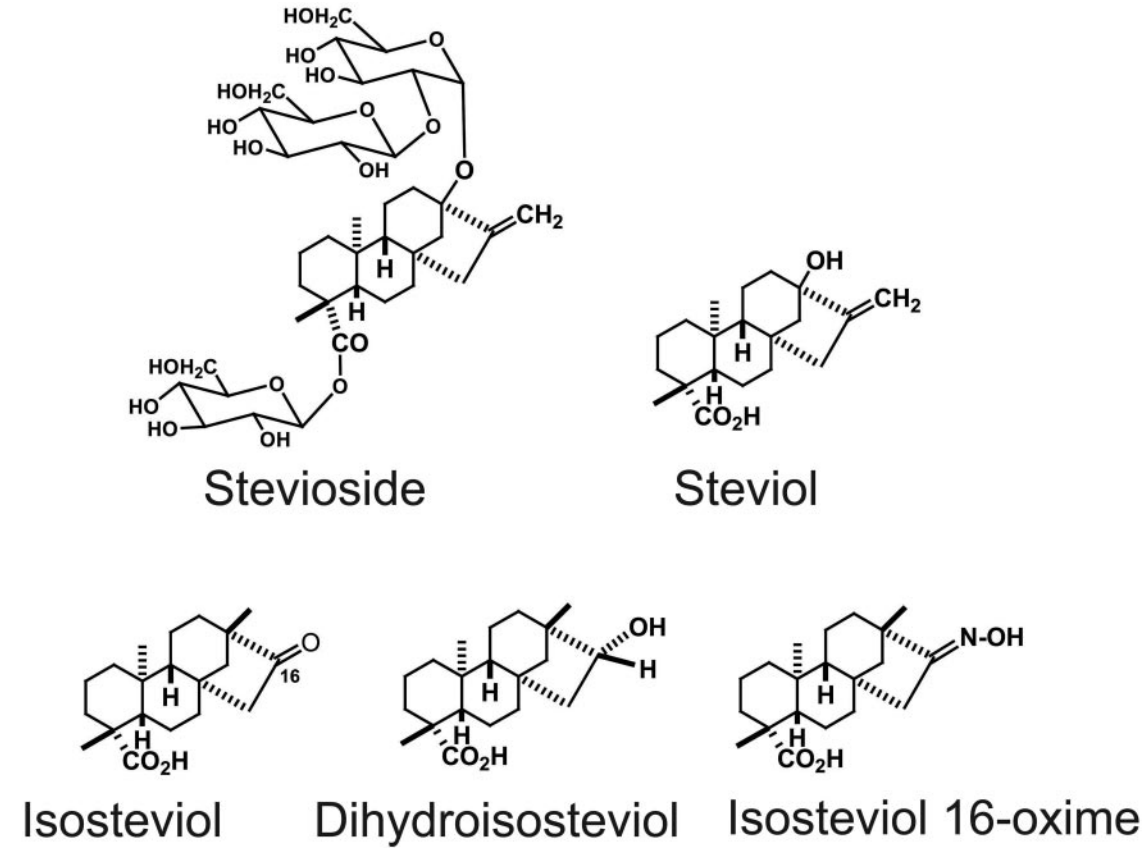

Fig. 1. Chemical structure of stevioside, steviol, isosteviol, dihydroisosteviol, and isosteviol 16-oxime.

300 times sweeter than sucrose and is widely used as a noncaloric sugar substitute in many kinds of food and as a food supplement in many countries in Asia and South America (Koyama et al., 2003). In addition to their sweetening properties, stevioside and steviol have been used to treat metabolic syndromes, such as hypertension (Lee et al., 2001), hyperglycemia, dyslipidemia, and diabetes (Jeppesen et al., 2006). Its analog, isosteviol, produces vasodilation of rat aorta (Wong et al., 2004). The effects of stevioside and steviol on membrane transporters also have been examined. Stevioside and steviol have been shown to inhibit renal *para*-aminohippurate transport by rabbit and human organic anion transporters (Chatsudthipong and Jutabha, 2001; Srimaroeng et al., 2005). It is interesting to note that an extract of the *Stevia* leaf has been shown to have activity against the rotavirus that induces secretory diarrhea (Takahashi et al., 2001).

The present study investigated the effects of stevioside, steviol, and its analogs (Fig. 1) on Cl^- secretion using a human T84 epithelial cell line and a mouse closed-loop system, and we demonstrated that these natural compounds acted as CFTR inhibitors.

Materials and Methods

Cell Culture. The T84 human colonic carcinoma cell line was purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (HyClone, Logan, UT) and nutrient mixture F-12 Ham (Sigma-Aldrich, St. Louis, MO) supplemented with 5% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin. For short-circuit current measurements, T84 cells were cultured on Snapwell inserts (1-cm^2 surface; Corning Life Sciences, Acton, MA) in a humidified 95% O_2-5% CO_2 atmosphere at 37°C. Cells were plated at a density of 5×10^5 cells/well, with fresh medium being replaced every 24 h. Monolayers were grown with medium on both the apical and basolateral sides, and inserts were used for Ussing experiments 10 to 14 days after seeding.

Chemicals and Compounds. Stevioside and steviol were kindly provided by Prof. Chaiwat Toskulkao (Mahidol University, Bangkok, Thailand). Isosteviol was synthesized from stevioside according to the method of All et al. (1992). Dihydroisosteviol was synthesized by reduction of isosteviol in methanol according to the method of Al'fonsov et al. (2000), with some modifications. Isosteviol 16-oxime was synthesized by treating isosteviol ketone with hydroxylamine hydrochloride in pyridine (Al'fonsov et al., 2003). Spectroscopic (proton nuclear magnetic resonance and mass spectra) data of the synthesized compounds were consistent with reported values (Al'fonsov et al., 2003). $CFTR_{inh}$-172 was purchased from Calbiochem (San Diego, CA). Trypsin-EDTA, fetal bovine serum, penicillin, and streptomycin were purchased from HyClone; cholera toxin was obtained from List Biological Laboratories, Inc. (Campbell, CA); and carbachol, amiloride, amphotericin B, Ham F-12, and other chemicals were obtained from Sigma-Aldrich.

Short-Circuit Current and Apical Cl^- Current Measurements. For short-circuit current measurements in nonpermeabilized T84 cells, Snapwell inserts (1-cm^2 surface area with resistance $>1000\ \Omega cm^2$) containing cell monolayers were mounted in an Ussing chamber system as described by Ma et al. (2002). Both hemichambers were filled with Krebs-bicarbonate solution (pH 7.4) containing 120 mM NaCl, 25 mM $NaHCO_3$, 3.3 mM KH_2PO_4, 0.8 mM K_2HPO_4, 0.5 mM $MgCl_2$, 10 mM Na-HEPES, and 10 mM glucose, and the chambers were continuously bubbled with a 95% O_2-5% CO_2 gas mixture. For apical Cl^- current measurements, the basolateral hemichamber was filled with buffer containing 130 mM NaCl, 2.7

mM KCl, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM MgCl, 10 mM Na-HEPES, and 10 mM glucose (pH 7.4) and permeabilized with amphotericin B (250 μg/ml) for 30 min before measurement. In the apical solution, 65 mM NaCl was replaced by 65 mM sodium gluconate, and $CaCl_2$ concentration was increased to 2 mM. Apical Cl^- and short-circuit current were recorded using a DVC-1000 voltage clamp (World Precision Instruments, Sarasota, FL) with Ag/AgCl electrodes and 1 M KCl agar bridge.

Intestinal Fluid Secretion and Absorption Measurements in Mice. Mice (30–35 g, ICR strain; The National Laboratory Animal Center, Bangkok, Thailand) were fasted for 24 h and anesthetized with an i.p. injection of ketamine (40 mg/kg) and xylazine (8 mg/kg). Body temperature was maintained between 36 and 38°C using a heating pad. After a small abdominal incision, three closed midjejunal loops (2–3 cm in length) were isolated by sutures. Loops were injected with 100 μl of phosphate-buffered saline (PBS) or PBS containing cholera toxin (1 μg) with or without test compounds. In some experiments, the test compound was prepared in 100 μl of dimethyl sulfoxide (DMSO) and injected i.p. before abdominal closure. Abdominal incisions were closed with sutures, and mice were allowed to recover from anesthesia. At 4 h, the mice were anesthetized again, intestinal loops were removed, and loop lengths and weights were measured after removal of mesentery and connective tissue to quantify net fluid secretion. For intestinal absorption, loops were injected with 200 μl of PBS or PBS containing test compound for 30 min before measuring the loop weight/length ratio. Mice were euthanized by an overdose injection of ketamine and xylazine. All animal protocols were approved by the Laboratory Animal Ethical Committee of Mahidol University.

Cell Viability Assay. Cell viability was determined using a sulforhodamine B assay (Skehan et al., 1990). In brief, T84 cells (6×10^4/well) were seeded in a 96-well microplate and incubated at 37°C in medium containing dihydroisosteviol for 3 days. Medium was removed, and the adherent monolayer of cells was fixed with 20% (w/v) trichloroacetic acid for 30 min at 4°C. Cells were then washed five times with distilled water and air-dried. Afterward, cells were stained for 30 min at room temperature with 0.4% (w/v) sulforhodamine B dissolved in 1% acetic acid, and they were rinsed quickly five times with 1% acetic acid. Cellular protein was solubilized with 200 μl of 10 mM Tris base, and absorbance at 515 nm was measured in a spectrophotometer (EL 312, Bio-Kinetics Reader; Bio-Tek Instruments Inc., Helsinki, Finland).

Intracellular cAMP Assay. cAMP activity was measured using BIOTRAK enzymatic immunoassay (GE Healthcare, Little Chalfont, Buckinghamshire, UK). In brief, T84 cells (10^6/ml) were cultured in 96-well microplates overnight at 37°C under an atmosphere of 5% CO_2 and 95% O_2. After the medium was removed and incubated in PBS for 20 min, cells were incubated for 30 min with 0.1% DMSO (as control), 10 μM forskolin (FSK), or FSK plus 100 μM dihydroisosteviol and lysed with one B reagent (provided by the manufacturer) with shaking for 10 min. Lysates were assayed for cAMP content in duplicate in three separate experiments and titrated so that the data were within the linear range of the standard curve according to manufacturer's instructions. The yellow color that was

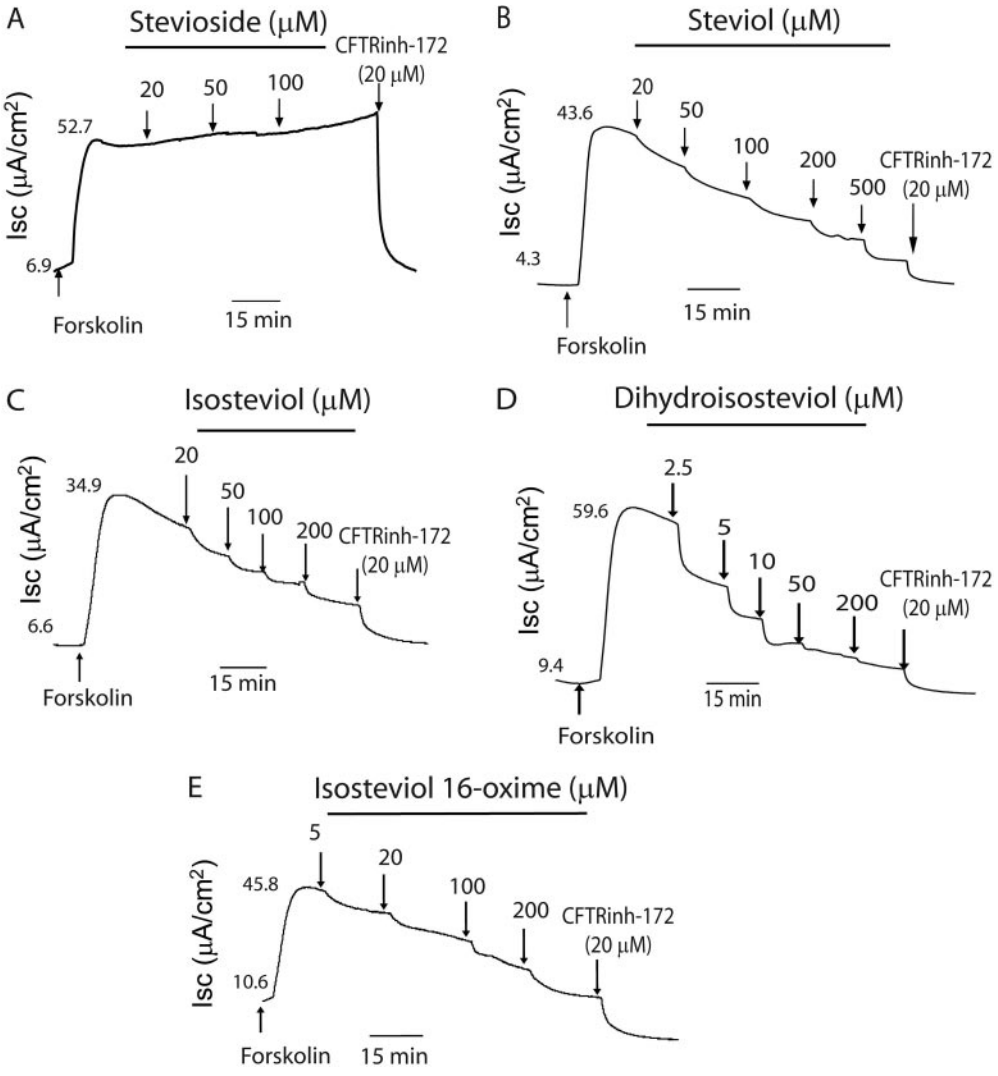

Fig. 2. Dose-response relationship of steviol, isosteviol, dihydroisosteviol, and isosteviol 16-oxime determined by short-circuit current measurement in intact T84 cells. Representative currents were recorded at the indicated concentrations of stevioside (n = 3) (A), steviol (n = 4) (B), isosteviol (n = 3) (C), dihydroisosteviol (n = 4) (D), or isosteviol 16-oxime (n = 3) (E). Data were fitted to the Hill equation to show an inhibitory potency (IC_{50}) of 101, 100, 9.6, and 50 μM, respectively. At the end of the experiments, $CFTR_{inh}$-172 (20 μM) was added. The current value inhibited by $CFTR_{inh}$-172 represents the remaining Cl^- secretion unaffected by the test compound.

developed by the addition of 100 μl of 1 M sulfuric acid was measured at 450 nm within 30 min in a spectrophotometer.

Statistical Analysis. The results of all of the experiments were presented as the mean ± S.E.M. The statistical difference between control and treatment was determined using the Student's paired or unpaired t test when appropriate. A p value <0.05 was considered statistically significant. n represents the number of experiments.

Results

Inhibition by Steviol of cAMP-Stimulated Chloride Secretion across T84 Monolayers. FSK activates adenylate cyclase (AC), resulting in an increase in intracellular cAMP level and a stimulation of active Cl^- secretion in T84 cells (Soriani et al., 2002). The addition of 10 μM FSK to both apical and basolateral sides of T84 cells resulted in an increase in short-circuit current (I_{sc} = 43.86 ± 7.7 μA/cm^2) above the baseline current. This forskolin-stimulated current reached a maximum within 5 min, which was maintained throughout the experimental period (~2–3 h) (data not shown). After stabilization of forskolin-stimulated Cl^- secretion, stevioside, at concentrations ranging from 20 to 100 μM, was added into either apical or basolateral solution, or both. There was no significantly change in chloride current (Fig. 2A). However, chloride secretion stimulated by forskolin (10 μM) was inhibited in a stepwise manner by sequential additions of steviol to the apical solution (Fig. 2B). At the end of the experiments, $CFTR_{inh}$-172 (20 μM) was added. The amount of current inhibited by $CFTR_{inh}$-172 represents the remaining Cl^- secretion unaffected by the test compound. The data were fitted to the Hill equation and yielded an inhibitory potency (IC_{50}) of 101 μM. Isosteviol, dihydroisosteviol, and isosteviol 16-oxime inhibited forskolin-induced Cl^- secretion with IC_{50} values of 100, 9.6, and 50 μM, respectively (Fig. 2, C–E). Because dihydroisosteviol was the most potent isosteviol derivative, it was used in additional experiments.

Inhibition by Dihydroisosteviol of Apical Chloride Secretion in Permeabilized T84 Cells. Addition of dihydroisosteviol into the solution-bathing basolateral membrane did not have an effect on cAMP-activated chloride secretion, whereas addition to the apical side produced a pronounced effect (Fig. 3A), suggesting that cAMP-activated apical chloride channel (i.e., CFTR) is a target of dihydroisosteviol. To test this notion, the effect of dihydroisosteviol on CFTR using apical chloride current measurements in amphotericin B-permeabilized T84 cells in the presence of a chloride gradient was determined. Because the previous study by Muanprasat et al. (2004) showed the presence of amiloride-sensitive current in T84 cells, amiloride (10 μM) was then added into the bathing solution to avoid the contribution of amiloride-sensitive current. The addition of 10 μM forskolin led to an increase in short-circuit current by 23.43 ± 1.13 μA/cm^2. Dihydroisosteviol (Fig. 3B, top) inhibited forskolin-stimulated Cl^- current in a dose-dependent manner, with more than 90% inhibition observed with 100 μM dihydroisosteviol. The data were fitted to the Hill equation and yielded an IC_{50} value of 5 μM (Fig. 3B, bottom).

Inhibition by Dihydroisosteviol of 8cpt-cAMP-Induced Chloride Secretion in T84 Cells without Alteration of Intracellular cAMP Content. To determine whether the inhibitory effect of dihydroisosteviol involves inhibition of AC, short-circuit current measurements were performed in intact T84 cells using a membrane-permeable cAMP, 8cpt-

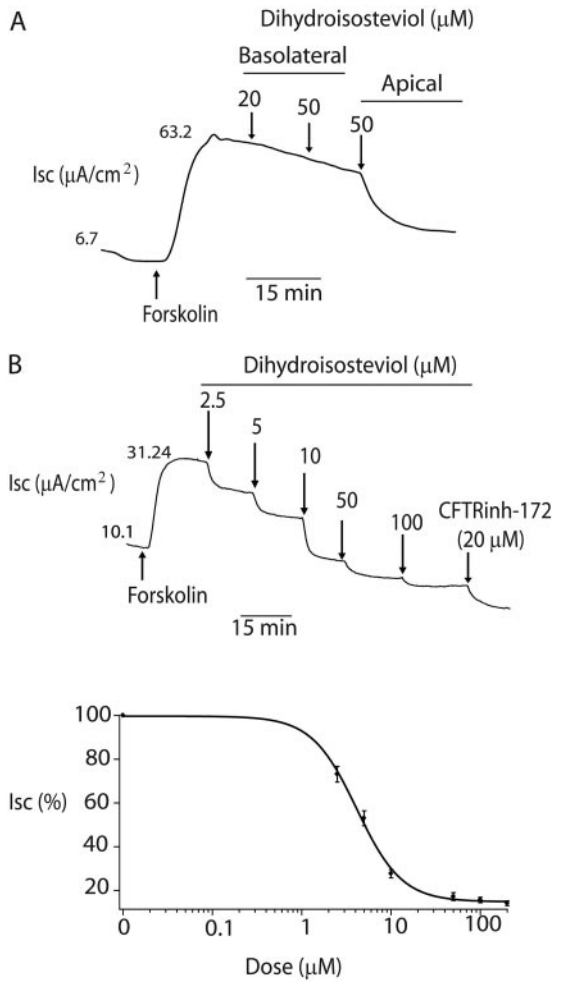

Fig. 3. A, differential effects of dihydroisosteviol in intact T84 cells. After activation of short-circuit current by forskolin (10 μM), dihydroisosteviol (50 μM) was added to basolateral and apical solution as indicated, respectively. B, top, a representative current recording at indicated concentrations of dihydroisosteviol in permeabilized T84 cells is shown; and bottom, the summary of results is shown (mean ± S.E., n = 4). Data were fitted to the Hill equation showing an inhibitory potency (IC_{50}) of 5 μM.

cAMP, as an activator of chloride secretion. Dihydroisosteviol inhibited 8cpt-cAMP-induced chloride secretion in a dose-dependent manner with an IC_{50} value of 14 μM (Fig. 4A). The measurement of intracellular cAMP concentration using an enzymatic immunoassay showed no significant difference in cAMP concentration after incubation for 30 min with 100 μM dihydroisosteviol combined with 10 μM forskolin (6.40 ± 1.08 fmol/μl) or with10 μM FSK alone (6.53 ± 0.97 fmol/μl) (Fig. 4B). This result suggests that dihydroisosteviol acts at a site distal to that causing cAMP elevation.

Reversibility of Action and in Vitro Toxicity of Dihydroisosteviol. The reversibility of the dihydroisosteviol inhibitory CFTR effect in T84 cells was assessed using apical-chloride-current measurements. The inhibitory effect of dihydroisosteviol on the forskolin response was reversed when dihydroisosteviol was removed from the bathing solution ($p < 0.01$) (Fig. 5, A and B). As in controls, the recovered current was completely inhibited by 20 μM $CFTR_{inh}$-172. To test for dihydroisosteviol toxicity, T84 cell viability was measured after exposure for 3 days to doses ranging from 1 to 200 μM. As shown in Fig. 6, cell viability of T84 cell after

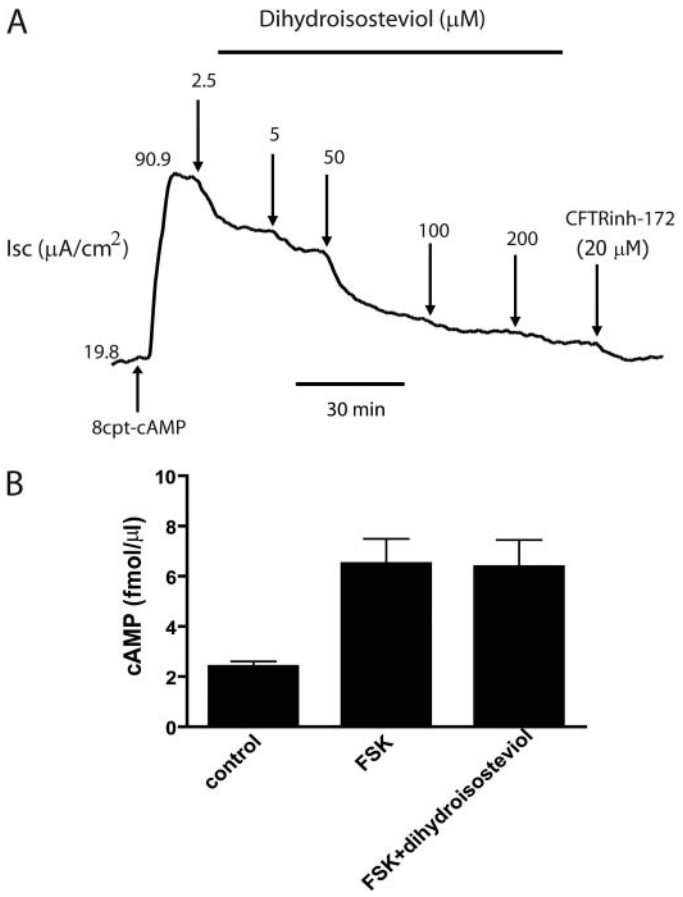

Fig. 4. A, representative current recordings of the dose-response relationship of dihydroisosteviol on chloride secretion induced by 8cpt-cAMP (100 μM) in intact T84 cells. B, the measurement of intracellular cAMP concentration in T84 cell lysate after incubation with FSK (10 μM) and dihydroisosteviol (100 μM) (mean ± S.E., $n = 3$) is shown.

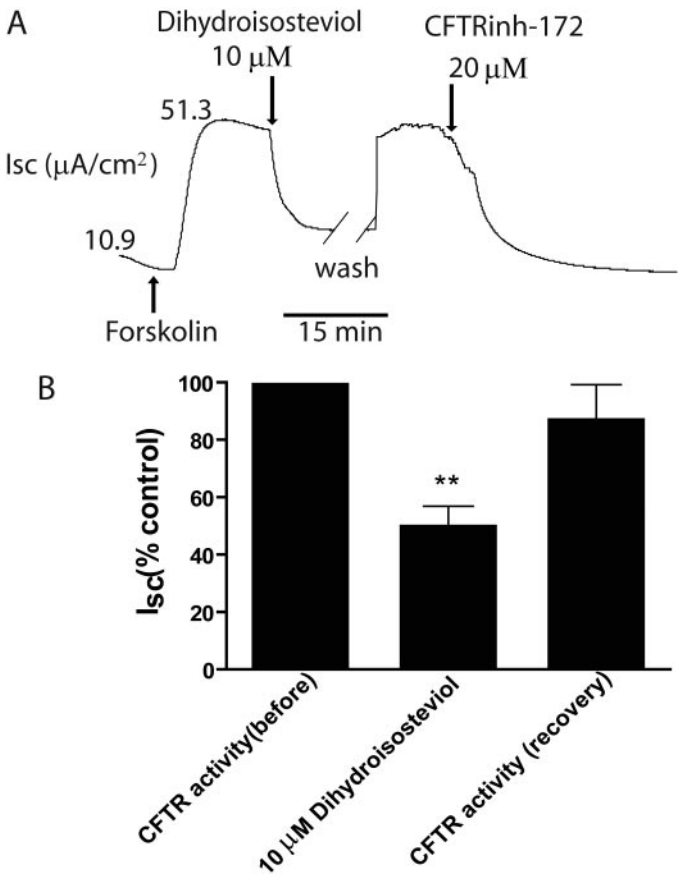

Fig. 5. Reversibility of the dihydroisosteviol effect on T84 cells. Under the permeabilized condition with a chloride gradient, 10 μM dihydroisosteviol was added to the apical solution after CFTR activation by 10 μM forskolin. The broken line indicates the time when the apical-bathing solution was removed, and the chamber was washed before adding fresh buffer containing forskolin (10 μM) but without dihydroisosteviol. After stable-recovered current was achieved, $CFTR_{inh}$-172 (20 μM) was added into the apical-bathing solution. A, a representative current recording of one of three experiments is shown. B, a summary of the data (mean ± S.E., $n = 3$) is shown. **, $p < 0.01$.

exposure to dihydroisosteviol was not significantly different from control ($p > 0.05$).

Lack of Inhibition by Dihydroisosteviol on Calcium-Activated Chloride Channel Activity. Chloride secretion at the apical membrane of intestinal lumen involves a number of channels, including CFTR chloride channel and calcium-activated chloride channel (CaCC). To examine whether dihydroisosteviol could have any effect on CaCC in an epithelial intestinal cell, nonpermeabilized T84 cells were treated with 10 μM amiloride, and 20 μM $CFTR_{inh}$-172 were added to the apical solution during the entire experimental period to eliminate contributions from epithelial sodium channel and CFTR to the short-circuit current measurements. Afterward, 0.1% DMSO (as control) or 100 μM dihydroisosteviol was added to the apical solution, and cells were incubated for 10 min. The acetylcholine agonist, carbachol (100 μM), was added to the basolateral side to stimulate calcium-sensitive chloride channels by elevating intracellular calcium concentration. Figure 7 shows that carbachol-stimulated chloride secretion was not affected by 100 μM dihydroisosteviol.

Inhibition by Dihydroisosteviol of Cholera Toxin-Induced Intestinal Fluid Secretion in an in Vivo Model. Antidiarrheal efficacy of dihydroisosteviol was examined using a mouse intestinal closed-loop model. Loops were injected with PBS or PBS containing cholera toxin (1 μg/100 μl) with and without test compounds, and intestinal fluid secretion (loop weight/length ratio) was measured 4 h after the injection. Intraluminal injection of 50 μM dihydroisosteviol decreased cholera toxin-induced intestinal fluid secretion by

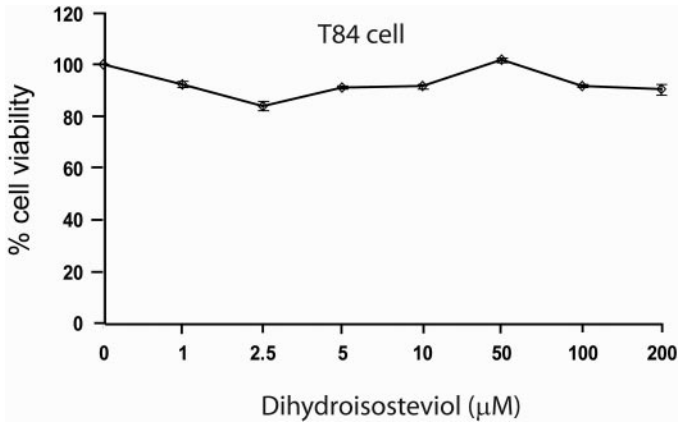

Fig. 6. Effect of dihydroisosteviol on T84 cell viability. T84 cells were cultured in 96-well plates with various concentrations of dihydroisosteviol. The cell viability was measured after 3 days of incubation. Each point represents the percentage of cell viability in the absence of dihydroisosteviol (mean ± S.E.) of three separate experiments.

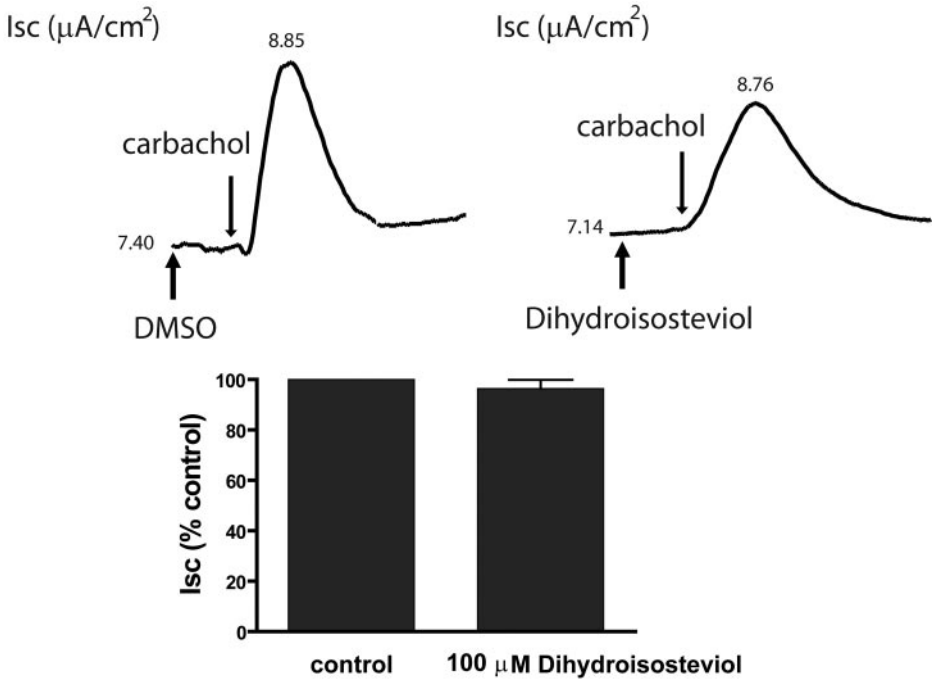

Fig. 7. Effect of dihydroisosteviol on CaCC. In the presence of $CFTR_{inh}$-172 (20 μM), DMSO (0.1% as a control) or dihydroisosteviol (100 μM) was applied to the apical solution. Carbachol (100 μM) was then added to basolateral solution to increase intracellular Ca^{2+} and subsequently activate CaCC activity (mean ± S.E., $n = 3$).

88.2% (160.3 ± 8.7 mg/cm for the cholera toxin-treated group versus 63.3 ± 9.1 mg/cm for the dihydroisosteviol-treated group, $p < 0.01$) (Fig. 8A), whereas a single i.p. injection of 5 mg/kg body weight dihydroisosteviol did not have any effect (data not shown). An intraluminal injection of 50 μM dihydroisosteviol, which strongly inhibited cholera toxin-induced fluid secretion, did not significantly alter the intestinal fluid absorption (109.4 ± 3.1 mg/cm) compared with the control (120.7 ± 6.8 mg/cm) (Fig. 8B).

Discussion

The effects of stevioside and its analogs, steviol, isosteviol, dihydroisosteviol, and isosteviol 16-oxime, on cAMP-regulated Cl^- secretion were studied in human T84 colonic epithelial cells and in vivo for their antidiarrheal efficacy. Using short-circuit current measurements, we found that the parent compound, stevioside, did not change chloride secretion in intact T84 cells, whereas its major aglycone derivative, steviol, and its analogs, inhibited cAMP-activated Cl^- secretion in intact T84 cells in a dose-dependent manner. The ineffectiveness of stevioside could be due to its molecular bulkiness (Fig. 1), which makes it relatively impermeable to cell membranes, thus impeding its interaction with target proteins.

The inhibitory effect of dihydroisosteviol, the most potent of the stevioside analogs tested, on the cAMP-activated Cl^- secretion was observed only when it was added to the apical-bathing solution but not to the basolateral side. We hypothesized that dihydroisosteviol may act on a cAMP-activated Cl^- channel of the intestinal cell apical membrane. If the basolateral membrane is permeabilized by amphotericin B in the presence of a chloride gradient, any current stimulated by forskolin would reflect chloride transport through the cAMP-activated chloride channel in the apical membrane. Dihydroisosteviol effectively inhibited cAMP-activated Cl^- current under this condition in a dose-dependent manner with an apparent IC_{50} value of 5 μM. In T84 cells, CFTR provided the principal route for chloride exit across the apical membrane in the process of cAMP-activated chloride secretion (Huflejt et al., 1994), and thus these findings indicate that dihydroisosteviol may act at the apical CFTR-Cl^- channel to inhibit cAMP-activated chloride secretion. This finding is supported by the results showing that dihydroisosteviol inhibited chloride secretion induced by a cell-permeable cAMP analog (8cpt-cAMP). Moreover, intracellular cAMP content did not change after dihydroisosteviol treatment in confluent T84 cell monolayers. The exact mechanism by which this compound acts on CFTR will require additional experiments.

We previously reported that renal proximal tubule cells that were stably transfected with hOAT1 and hOAT3 were less viable after their exposure to steviol for 3 days at concentrations ranging between 25 and 100 μM (Srimaroeng et al., 2005). However, up to 200 μM dihydroisosteviol did not affect T84 cell viability. Furthermore, the inhibitory effect of dihydroisosteviol on cAMP-activated Cl^- secretion was only temporal. It was almost fully reversible after the removal of dihydroisosteviol from the solution. Therefore, it is likely that dihydroisosteviol affected Cl^- secretion from outside the cell.

Chloride secretion in the intestine can be activated by a rise in intracellular calcium (Cliff and Frizzell, 1990). This process involves chloride (CaCC) and potassium channels that are distinct from those participating in the cAMP-activated chloride secretion. CaCC has been found in the small intestine as well as in T84 cells (Kunzelmann et al., 2002). Several CFTR inhibitors, such as glibenclamide, 5-nitro-2-(3-phenylpropylamino)-benzoic acid, GlyH-101, and 2-[*N*-(3-hydroxy-4-carboxyphenyl)amino]-4-(4-methylphenyl)-thiazole (INH 1), have also been found to inhibit CaCC (McCarty et al., 1993; Rabe et al., 1995; Dawson et al., 1999; Muanprasat et al., 2004, 2007). Therefore, to determine its target specificity, dihydroisosteviol was tested in T84 cells as a blocker of Ca^{2+}-induced Cl^- secretion. Using carbachol, an acetylcholine agonist, as an activator of calcium-activated Cl^- secretion,

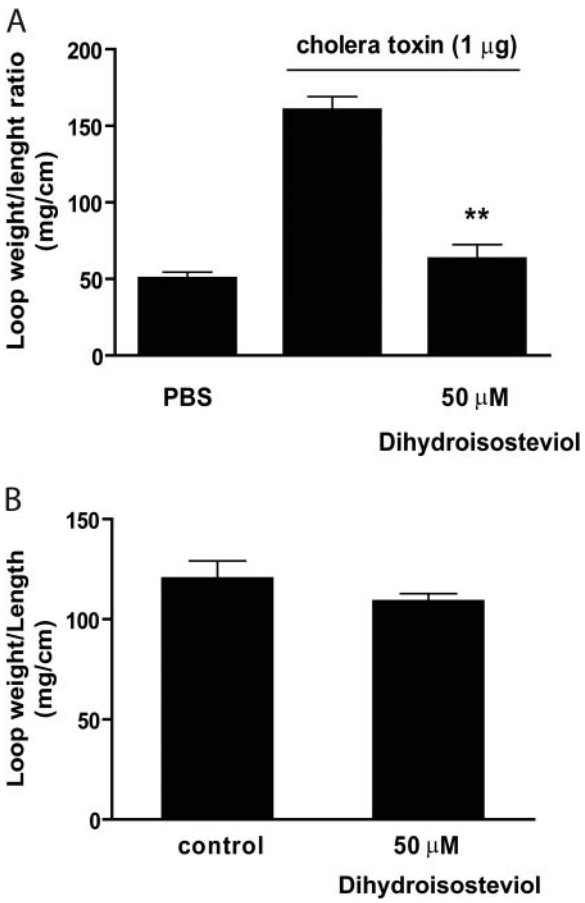

Fig. 8. In vivo efficacy of dihydroisosteviol tested in the closed-loop model of cholera toxin-induced intestinal fluid secretion. Intestinal fluid accumulation, shown as loop weight/length ratio (milligram per centimeter) was measured at 4 h after injection of cholera toxin. A, intestinal loops injected with cholera toxin (1 μg) or dihydroisosteviol (50 μM) before abdominal suture was performed (mean ± S.E.M., $n = 5$). B, intestinal fluid content is shown as the loop weight/length ratio (milligram per centimeter) measured at 30 min after injection of 200 μl of PBS or PBS containing 50 μM dihydroisosteviol into individual intestinal loops (mean ± S.E.M., $n = 5$). **, $p < 0.01$ compared with control.

we found that Ca^{2+}-activated Cl^- secretion was unaffected by dihydroisosteviol. However, it would be premature to conclude that dihydroisosteviol is specific for CFTR. The effects of dihydroisosteviol on the activity of other transport proteins, such as multidrug-resistant transporter 1 and other chloride channels (Ma et al., 2002; Muanprasat et al., 2004), will need to be investigated.

An inhibitory effect of dihydroisosteviol on cAMP-activated Cl secretion was also observed in a mouse model of cholera toxin-induced intestinal fluid secretion. An increase in intracellular cAMP causes both stimulation of Cl^- secretion in the small intestine and inhibition of Na^+ absorption in both the small intestine and colon, resulting in secretory diarrhea with massive intestinal fluid loss (Kunzelmann and Mall, 2002). The present study showed that dihydroisosteviol did not alter intestinal fluid absorption in the mouse closed-loop model, indicating that the in vivo efficacy of dihydroisosteviol might be due to inhibition of the cAMP-stimulated Cl^- secretion process.

Dihydroisosteviol inhibited cAMP-induced intestinal Cl^- secretion with potency (IC_{50} = 9.6 μM) comparable to known CFTR inhibitors that have potential applications in the treatment of secretory diarrhea, namely $CFTR_{inh}$-172 (IC_{50} = 5 μM), GlyH-101(IC_{50} = 5 μM), SP-303 (IC_{50} = 5 μM), and INH 1 (IC_{50} = 24.5 μM) (Gabriel et al., 1999; Muanprasat et al., 2004, 2007; Thiagarajah et al., 2004). Unlike GlyH-101 and INH 1, dihydroisosteviol does not affect calcium-activated chloride channel activity. In addition, it is a derivative of a natural product that can be readily synthesized at a reasonable cost.

In summary, an isosteviol analog, dihydroisosteviol, reversibly inhibited cAMP-activated Cl^- intestinal secretion by targeting CFTR chloride channels. This inhibitory effect did not involve changes in AC activity and intracellular cAMP content. Dihydroisosteviol was nontoxic to human T84 colonic epithelial cells in vitro and had no effect on Ca^{2+}-activated Cl^- secretion. Furthermore, dihydroisosteviol prevented cholera toxin-induced intestinal fluid secretion without altering intestinal fluid absorption. Thus, dihydroisosteviol and similar compounds represent a promising class of CFTR inhibitors that may be useful in the treatment of secretory diarrheas, the pathogenesis of which involves cAMP as a second messenger, such as cholera and those caused by enterotoxic *Escherichia coli*.

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