Pharmaceutical Research, Vol. 27, No. 3, March 2010 (© 2009)
DOI: 10.1007/s11095-009-0040-y

Research Paper

A Plant-Derived Hydrolysable Tannin Inhibits CFTR Chloride Channel: A Potential Treatment of Diarrhea

Nisa Wongsamitkul,[1] Lalida Sirianant,[1] Chatchai Muanprasat,[2,3] and Varanuj Chatsudthipong[2,3]

Received September 23 2009; accepted December 14 2009; published online January 7, 2010

Purpose. The present study examined the effects and mechanisms of actions of penta-*m*-digalloyl-glucose (PDG), a hydrolysable tannin extracted from Chinese gallnut, on cystic fibrosis transmembrane conductance regulator protein (CFTR).
Materials and Methods. Fisher rat thyroid cells stably expressing human CFTR (FRT cells) and human intestinal T84 cells were used as cell models to investigate the effects of PDG on chloride secretion using short-circuit current analysis. The mechanisms by which PDG affected chloride secretion were also examined. Finally, *in vivo* antidiarrheal efficacy and effects of PDG on intestinal fluid absorption were evaluated in mouse closed-loop models.
Results. In FRT cells, apical chloride current induced by forskolin, CPT-cAMP and apigenin were reversibly inhibited by PDG (IC50 ~ 10 μM) without effects on intracellular cAMP content and cell viability. Similarly, in T84 cells, PDG effectively inhibited chloride secretion induced by forskolin and cholera toxin. However, it had no effect on calcium-induced chloride secretion. In mice, a single intraluminal injection of PDG (0.6 mg/kg) reduced cholera toxin-induced intestinal fluid secretion by 75% with no effect on intestinal fluid absorption.
Conclusions. PDG represents a new class of CFTR inhibitors. Further development of this class of compounds may provide a new therapeutic intervention for diarrhea.

KEY WORDS: CFTR; chloride secretion; diarrhea; tannin.

INTRODUCTION

Secretory diarrhea continues to be a leading cause of morbidity and mortality worldwide with an estimated death of 2.5 million per year, approximately 60% of which result from intestinal infection with enterotoxin-producing bacteria, especially *Vibrio cholera* (1,2). This strain of bacteria produces cholera toxins, which act on enterocyte ganglioside 1 (GM_1) receptors to stimulate cAMP-mediated chloride secretion, providing a driving force for fluid secretion far exceeding the reabsorbing capacity of the intestine, thus leading to fluid loss, dehydration and death if appropriate rehydration therapy is not initiated. At present, there are considerable attempts at developing new pharmacological therapies for cholera, and one of the promising approaches is by inhibition of transport proteins involved in cAMP-activated chloride secretion (3).

Multiple transport proteins function in concert to facilitate cAMP-activated intestinal chloride secretion. Chloride ions are taken up into enterocytes via Na^+-K^+-$2Cl^-$ cotransporters located at the basolateral membrane and exit across the apical membrane via cAMP-activated chloride channels, among which is cystic fibrosis transmembrane conductance regulator protein (CFTR). Mutations of CFTR cause cystic fibrosis, a genetic disease characterized by pancreatic insufficiency and chronic lung infection (4). In addition, cAMP-activated chloride secretion requires the functions of basolateral cAMP-activated K^+ channels and Na^+-K^+ ATPases to maintain its sustainability (5). Among these transport proteins, CFTR is recognized as the most attractive target protein, whose inhibitors are expected to possess therapeutic values in the treatment of cholera (6). As a consequence, high throughput screenings of small molecules have been conducted, and several new classes of CFTR inhibitors have been identified, which have the potential to be developed for treating cholera (7,8). Recently, our group reported that steviol and its analogues inhibit cAMP-activated chloride secretion in human intestinal cells by targeting CFTR, with dihydroisosteviol being the most potent analog, capable of reducing cholera toxin-induced intestinal fluid secretion in mice by 90% (9).

Hydrolysable tannins are plant polyphenols commonly found in a variety of plants (10). They are composed of a

[1] Toxicology Graduate Program, Faculty of Science, Mahidol University, Bangkok, Thailand.
[2] Department of Physiology, Faculty of Science, Mahidol University, 272 Rama VI Road, Ratchathewi District, Bangkok, 10400, Thailand.
[3] To whom correspondence should be addressed. (e-mail: scchatchai@staff2.mahidol.ac.th; scvcs@mahidol.ac.th)

ABBREVIATIONS: CFTR, cystic fibrosis transmembrane conductance regulator; $CFTR_{inh}$-172, 3-[(3-trifluoromethyl) phenyl]-5-[(4-carboxyphenyl) methylene]-2-thioxo-4-thiazolidinone; DMSO, dimethyl sulfoxide; CPT-cAMP, 8-cholorophenyl-thio-cAMP; FRT, Fisher rat thyroid; MTT, 3-(4, 5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; PBS, phosphate-buffered saline; PDG, penta- *m*- digalloyl- glucose.

central carbohydrate (usually glucoses) esterified with phenolic groups, such as gallic acid (gallotannin) and ellagic acid (ellagitannin). Hydrolysable tannins have been shown to have antimutagenic, anticarcinogenic and antioxidant activities as well as inhibitory effects on gastric H^+-K^+-ATPase activity (11–14). However, their effects on CFTR chloride channels have hitherto remained unknown.

Based on previous findings that condensed tannins exhibit inhibitory effects on CFTR chloride channels (15,16), we hypothesized that hydrolysable tannins also are inhibitory against CFTR chloride channels. In the present study, we demonstrated that a hydrolysable tannin isolated from Chinese gallnuts, penta- *m*- digalloyl- glucose (PDG) (Fig. 1) inhibited CFTR function in Fisher rat thyroid cells expressing human CFTR protein (FRT cells). Its efficacy in reducing enterotoxin-induced intestinal fluid secretion was confirmed in human intestinal T84 cells and in an *in vivo* rodent model of acute diarrhea.

MATERIALS AND METHODS

Cell Lines and Chemicals

Fisher rat thyroid cells stably expressing human CFTR were generated as previously described (17). Human intestinal T84 cells were obtained from American Type Culture Collection (Virginia, USA). $CFTR_{inh}$-172 was purchased from Calbiochem (San Diego, CA), cholera toxin was from List Biological Laboratories, Inc. (California, USA) and trypsin-EDTA, fetal bovine serum, penicillin and streptomycin were from HyClone (Utah, USA). Other chemicals were obtained from Sigma-Aldrich (Missouri, USA).

Cell Culture

FRT cells were cultured in Coon's F-12 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. T84 cells were cultured in 50% DMEM and 50% Ham's F-12 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were maintained at 37°C in a humidified incubator under an atmosphere of 95% O_2 and 5% CO_2. For apical chloride current and short-circuit current measurements, cells were subcultured in 0.05% trypsin-EDTA solution and seeded on Snapwell filters (1 cm^2 surface area; Corning-Corstar Corp., Corning, New York, USA) at the density of 500,000 cells/filter. Prior to electrophysiological experiments, cells were grown on the Snapwell support for 7 days in order to obtain an electrical resistance of more than 1,000 $\Omega.cm^2$. Culture media were freshly replaced daily.

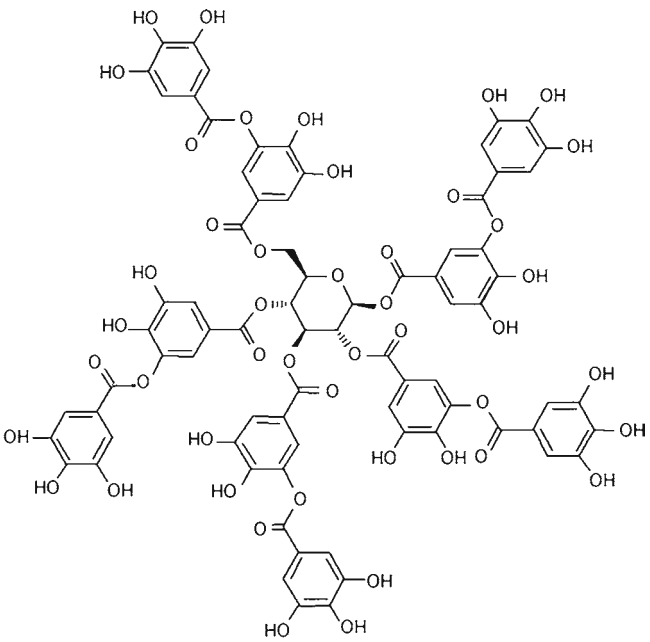

Fig. 1. Chemical structure of penta-*m*-digalloyl-glucose (PDG).

Apical Chloride Current and Short-Circuit Current Measurements

Current measurements were measured in FRT and T84 cells grown on Snapwell filters using an Ussing chamber protocol (7). For apical chloride current measurements in FRT cells, the basolateral membrane was permeabilized by prior treatment with amphotericin B (250 µg/ml) for 30 min. The Ringer's solution in the basolateral hemichamber contained (in mM) NaCl (130), KCl (2.7), KH_2PO_4 (1.5), $CaCl_2$ (1), $MgCl_2$ (0.5), Na-HEPES (10) pH 7.3 and glucose (10). To establish a chloride gradient, 65 mM NaCl of apical solution was replaced with 65 mM sodium gluconate, and concentration of $CaCl_2$ was increased to 2 mM. Solutions bathing FRT cells were bubbled with 100% O_2 and maintained at 37°C throughout the duration of experiment. For short-circuit current measurements, both hemichambers were filled with Kreb's solution (pH 7.3) containing (in mM) NaCl (120), $NaHCO_3$ (25), KH_2PO_4 (3.3), K_2HPO_4 (0.8), $MgCl_2$ (1.2), $CaCl_2$ (1.2) and glucose (10). Solutions were bubbled continuously with 95% O_2/5% CO_2 and maintained at 37°C. Apical chloride/short-circuit current was recorded using a DVC-1000 voltage-clamp (World Precision Instruments, Sarasota, Florida, USA) with Ag/AgCl electrode and 3M 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 by intraperitoneal injection of thiopenthal sodium (50 mg/kg). Body temperature was maintained between 36 and 37°C using an electrical lamp. After making a small abdominal incision, 3 closed mid-jejunal loops (2–3 cm. in length) were generated by sutures. Loops were injected with 100 µl of phosphate-buffered saline (PBS) or PBS containing cholera toxin (1 µg) with or without PDG (0.6 mg/kg). Abdominal incisions were closed with sutures, and mice were allowed to recover from anesthesia. After 4 h, mice were anesthetized, intestinal loops were removed, and loop lengths and weights were measured after removal of mesentery and connective tissues to quantify net fluid secretion. For intestinal absorption studies, loops were injected with 200 µl of PBS with or without PDG (0.6 mg/kg). Following 30 min of recovery from anesthesia, mice were re-anesthetized, and intestinal loops were removed

to measure loop weight/length ratio. Mice were euthanized with an injection of thiopenthal sodium (150 mg/kg). All animal protocols were approved by the Laboratory Animal Ethical Committee of Mahidol University, Thailand.

Intracellular cAMP Assay

cAMP level was measured using a BIOTRAK enzymatic immunoassay (GE Healthcare, Buckinghamshire, UK) according to manufacturer's instructions. In brief, FRT cells, 5×10^5 cells/well, were cultured in 24-well microplate overnight at 37°C under an atmosphere of 95% O_2 and 5% CO_2. After removal of culture medium and preincubation with PBS for 20 min, cells were incubated for 30 min with PBS containing 0.1% (v/v) DMSO, forskolin (10μM), forskolin (10μM) plus PDG (100μM) or PDG (100μM) alone. Then, cells were lysed with 1B reagent provided by the manufacturer, and lysates were assayed for cAMP content in four separate experiments conducted in duplicate. Within 30 min of addition of 100μl of 1 M sulfuric acid, absorbance was measured at 450 nm (Thermo Labsystems Instrument, Helsinki, Finland), and cAMP concentration was read from a calibration curve.

Cell Viability Assay

Cell viability was determined using a 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. In brief, FRT cells were seeded in 96-well plate at the density of 2×10^4 cells/well. After growth for 24 h in a humidified 5% CO_2/95% O_2 incubator at 37°C, cells were incubated for 24 h with a culture medium containing various concentrations of PDG. Medium was removed, and cells were incubated with 20μl of 5 mg/ml MTT for 4 h at 37°C. Then, a 100μl aliquot of 100% DMSO was added, and cells were incubated for further 3 h before measurements of an absorbance at 590 nm (BMG LABTECH, Victoria, Australia).

Statistical Analysis

Results are presented as mean ± S.E. Statistical difference between control and treatment groups was determined using Student's t test, with p value <0.05 considered statistically significant.

RESULTS

Inhibition by PDG of CFTR-Mediated Apical Chloride Current in FRT Cells

The effect of PDG on CFTR function was investigated by measuring apical chloride current in FRT cells stably expressing human wild-type CFTR. FRT cells were basolaterally permeabilized by pretreatment with amphotericin B in the presence of apically directed chloride gradient. CFTR-mediated apical chloride current was stimulated by CFTR agonists, forskolin (an adenylate cyclase activator), CPT-cAMP (a cell-permeable cAMP) and apigenin (a flavone-type direct activator of CFTR). PDG inhibited CFTR-mediated apical chloride current induced by all agonists in a dose-dependent fashion, with an IC50 of ~10μM (Fig. 2). Complete inhibition was obtained at 100μM PDG. Control experiments (without treatments with PDG) showed that an increase in CFTR-mediated apical chloride current was stable over the time course of experiments (Fig. 2A–C, inset).

Reversibility of PDG Inhibitory Effect on CFTR-Mediated Apical Chloride Current and Cytotoxicity Evaluation in FRT Cells

To determine if the inhibitory effect of PDG on CFTR function in FRT cells was reversible, apical chloride current

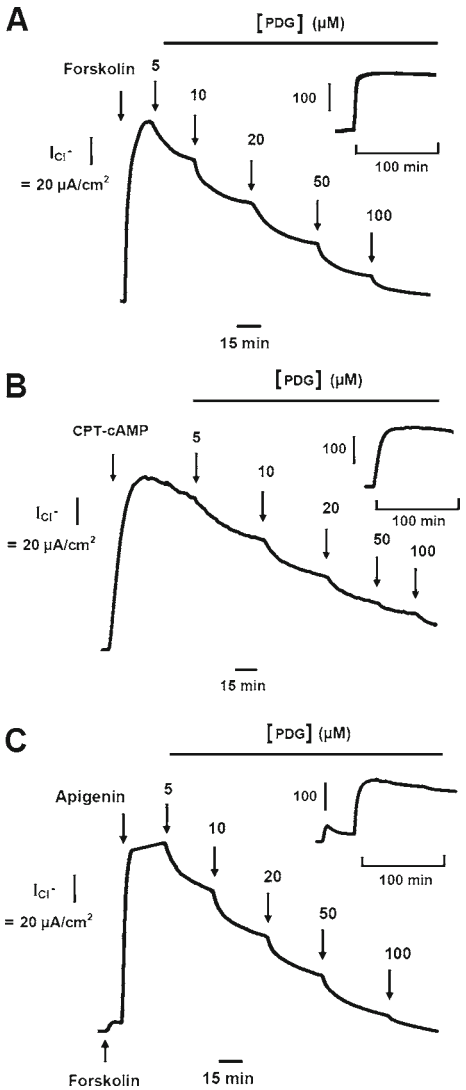

Fig. 2. Inhibitory effect of PDG on apical chloride current induced by CFTR agonists in FRT cells. Forskolin (**A**), CPT-cAMP (**B**) and apigenin (**C**) at a final concentration of 10, 100 and 20μM, respectively, were added into both hemichambers to elicit CFTR-mediated apical chloride current. After stabilization of stimulated current, PDG was added accumulatively into both apical and basolateral solutions at indicated concentrations. Data shown are representative of 3–5 separate experiments. An inset in (**A**), (**B**) and (**C**) shows a time control (no PDG) tracing of apical chloride current stimulated by forskolin (10μM), CPT-cAMP (100μM) and apigenin (20μM), respectively.

was measured after removal of PDG from the bathing solutions. Current was restored to more than 90% (as analyzed from percent $CFTR_{inh}$-172 sensitive current) of forskolin-stimulated current in untreated cells (Fig. 3A). Exposure of FRT cells to 5–100 μM PDG for 24 h produced no cytotoxicity compared to control cells, as examined using MTT assay (Fig. 3B).

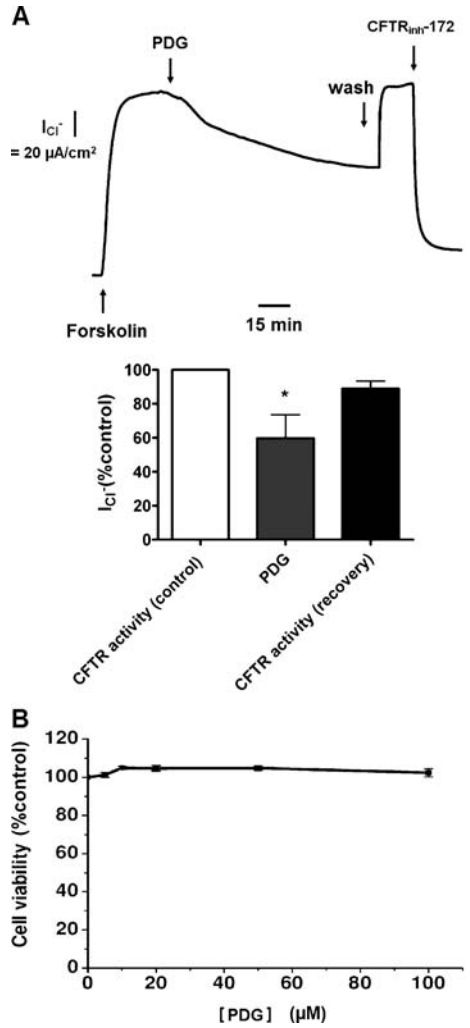

Fig. 3. Reversibility of PDG inhibition of CFTR function and PDG cytotoxicity in FRT cells. (**A**) *Top*, a representative current tracing showing reversibility of PDG on CFTR inhibition. After stimulation of CFTR-mediated apical chloride current by forskolin (10 μM), PDG (20 μM) was applied to the apical solution. After stabilization of the current, apical solution was removed and replaced with the same solution containing forskolin. Finally, $CFTR_{inh}$-172 (5 μM), a direct CFTR inhibitor, was added into the apical compartment to verify chloride current. *Bottom*, summary of the data from reversibility experiments. Absolute values of forskolin-stimulated current before and after additions of PDG and $CFTR_{inh}$-172 sensitive recovered current were 95.93±17.9, 53.92±12.5 and 86.82±19.1, respectively. Data are mean of % control ± S.E.; $n=3$; * $p<0.05$. (**B**) Effect of PDG on viability of FRT cells as measured by MTT assay. FRT cells were incubated with PDG for 24 h, and then MTT reagent (10% v/v) was added and incubated for 4 h. Following treatment with 100% DMSO, absorbance at 490 nm was measured. Data are expressed as mean of % cell viability compared to vehicle-treated group ± S.E.; $n=3$.

Lack of PDG Effect on Intracellular cAMP Level

To determine whether PDG inhibition of CFTR function in FRT cells involved an alteration in intracellular cAMP level, an enzymatic immunoassay was employed. Treatment with 100 μM PDG did not change cAMP level compared to that of control (7.6±3.0 and 7.9±3.5 fmol/μg protein respectively). In addition, PDG had no effect on the increase in intracellular cAMP induced by forskolin (23.6±1.9 and 18.6±4.2 fmol/μg protein for forskolin treated and forskolin plus PDG treated group, respectively) (Fig. 4). These results indicated that PDG inhibition of CFTR function in FRT cells does not involve change in intracellular cAMP level.

Inhibition by PDG of CFTR-Mediated Chloride Secretion Across Human Colonic Epithelial Cells

The effectiveness of PDG in inhibiting CFTR-mediated chloride secretion in human epithelial cells was assessed using short-circuit current analysis of human intestinal T84 cell line. These cells are widely used to study intestinal chloride secretion, as their epithelial transport properties are similar to native intestinal cells residing in the crypt where chloride secretion mostly takes place (5). The study was performed in the presence of amiloride (10 μM) in the apical solution to inhibit any contribution of the epithelial sodium transport to the current measured, and forskolin (10 μM) was employed as an agonist of cAMP-activated chloride secretion. PDG inhibited forskolin-induced active chloride secretion in a dose-dependent manner, with an IC50 of 100 μM and more than 90% inhibition at 1 mM (Fig. 5). Control experiments (without treatments with PDG) showed that an increase in cAMP-activated short-circuit current was stable over the time course of measurements (Fig. 5, inset).

Lack of PDG Effect on Calcium-Activated Chloride Secretion in T84 Cells

In addition to cAMP-activated pathway, intestinal chloride secretion is activated by a calcium-mediated signaling

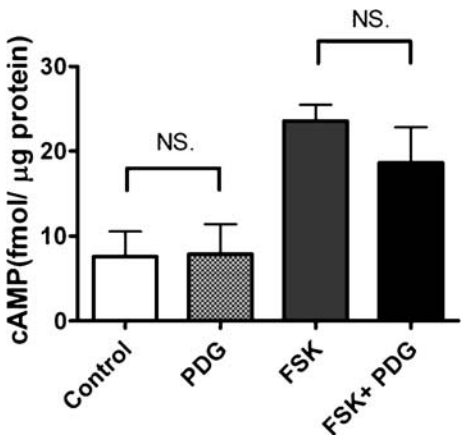

Fig. 4. Effect of PDG on intracellular cAMP level. Intracellular cAMP content in FRT cells after pretreatment with DMSO (0.1%v/v), PDG (100 μM), forskolin (10 μM) or forskolin (FSK) (10 μM) plus PDG (100 μM) were analyzed using an enzymatic immunoassay. Data are shown as mean±S.E.; $n=4$. NS, no statistical significance.

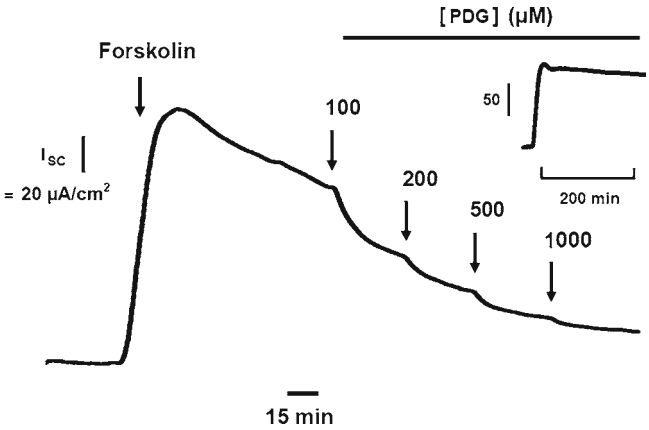

Fig. 5. Inhibitory effect of PDG on cAMP-activated chloride secretion in human intestinal T84 cells. In the presence of amiloride (10μM) in the apical solution, PDG was added accumulatively into both bathing solutions after stimulation of chloride secretion with forskolin (10μM). The recording of the current is a representative of three similar sets of measurements. An inset shows a time-control (no PDG) tracing of forskolin-activated short-circuit current.

cascade (18). Stimulation of this transport process has been implicated in pathophysiology of diarrhea during rotavirus infection and that induced by anti-HIV drugs (19,20). The effect of PDG on this chloride secretory process was evaluated using short-circuit current measurement in T84 cells. Carbachol (100μM), a muscarinic receptor agonist that elevates intracellular calcium, was used to stimulate calcium-activated chloride secretion in the presence of 10 μM amiloride and $CFTR_{inh}$-172 (20μM), the latter added to prevent any contribution of epithelial sodium transport and CFTR chloride channel, respectively, to the current measured. PDG (100μM) had no effect on carbachol-induced chloride secretion (1.71±0.5μA/cm^2) compared to control (1.81±0.6μA/cm^2) (Fig. 6).

Inhibition by PDG of Cholera Toxin-Induced Chloride Secretion in Human Intestinal Cells and Mouse Closed-Loop Model of Cholera

It is well-documented that CFTR chloride channels provide a principal route for apical chloride exit during enterotoxin-induced intestinal chloride secretion (21). Thus, compounds possessing CFTR inhibitory effects are expected to have potential therapeutic utility in the treatment of secretory diarrhea, including cholera. We evaluated the antidiarrheal efficacy of PDG using both *in vitro* and *in vivo* measurements of chloride secretion induced by cholera toxin, a well-characterized bacterial enterotoxin. *In vitro* measurements of chloride current across T84 cells indicated that cholera toxin (1μg/ml) stimulated gradual chloride secretion, which was completely abolished by treatment with 200μM PDG (Fig. 7A). Control experiments (without treatments with PDG) showed that an increase in cholera toxin-induced short-circuit current was stable over the time course of measurements (Fig. 7A, inset). *In vivo* efficacy of PDG was determined using a mouse closed-loop model of cholera. Fluid secretion was quantified from measurements of loop weight/length ratio. Concomitant intraluminal administration of PDG (0.6 mg/kg) with cholera toxin (1 μg/loop) reduced cholera toxin-induced intestinal fluid secretion by 75% (Fig. 7B). Weight/length ratio of loop injected with saline, cholera toxin and cholera toxin plus PDG was 0.06±0.01, 0.18±0.02 and 0.09±0.02 g/cm, respectively. PDG did not affect intestinal fluid absorption as measured from the remaining fluid in closed intestinal loop after 30 min of injection of saline (with or without PDG) (Fig. 7C).

DISCUSSION

The present studies identified a novel effect of penta-*m*-digalloyl- glucose (PDG), a hydrolysable tannin extracted from Chinese gallnut, on CFTR chloride channel function. Using FRT cells stably expressing human wild-type CFTR, it was shown that PDG reversibly inhibited CFTR chloride transport activity without altering intracellular cAMP level or causing any cytotoxic effect. Furthermore, studies in human intestinal cells and in an *in vivo* mouse model of cholera revealed that PDG was effective in inhibiting chloride secretion induced by cAMP and cholera toxin, without effecting intestinal fluid absorption or calcium-activated chloride secretion.

Tannins are polyphenols commonly found in plants such as apple fruit, pine bark, grape seed, tea and oak (22) and possess a variety of biological effects, including anticarcinogenic (23), antimutagenic (12), antimicrobial (24) and antioxidative activities (11,13). Condensed tannins (proanthocyanidins) inhibit CFTR chloride channel in intestinal epithelial cells, and the proposed mechanism involves direct interaction with CFTR protein at the extracellular site of the channel (16,25). We therefore hypothesized that hydrolysable tannins should be able to exert an inhibitory effect on CFTR-mediated chloride transport. Fisher rat thyroid (FRT) cells stably expressing human CFTR were used to study CFTR function as they have

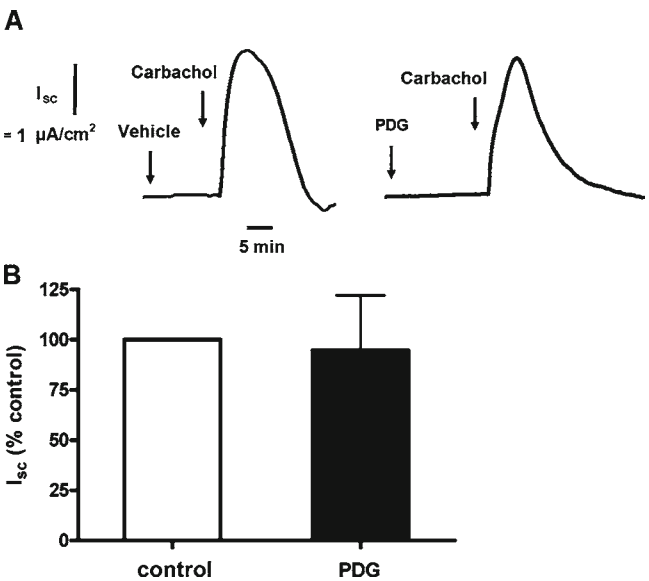

Fig. 6. Effect of PDG on calcium-activated chloride secretion in T84 cells. (**A**) Representative recordings of chloride secretion current induced by carbachol (100μM) after pretreatment with PDG (100μM) or vehicle. Amiloride (10 μM) and $CFTR_{inh}$-172 (20 μM) were present in apical solution. (**B**) Summary of the data. Absolute values of carbachol-stimulated chloride current after pretreatment with PDG (100μM) or vehicle were 1.71±0.5 and 1.81±0.6, respectively. Data are shown as % control ± S.E.; n=3–8.

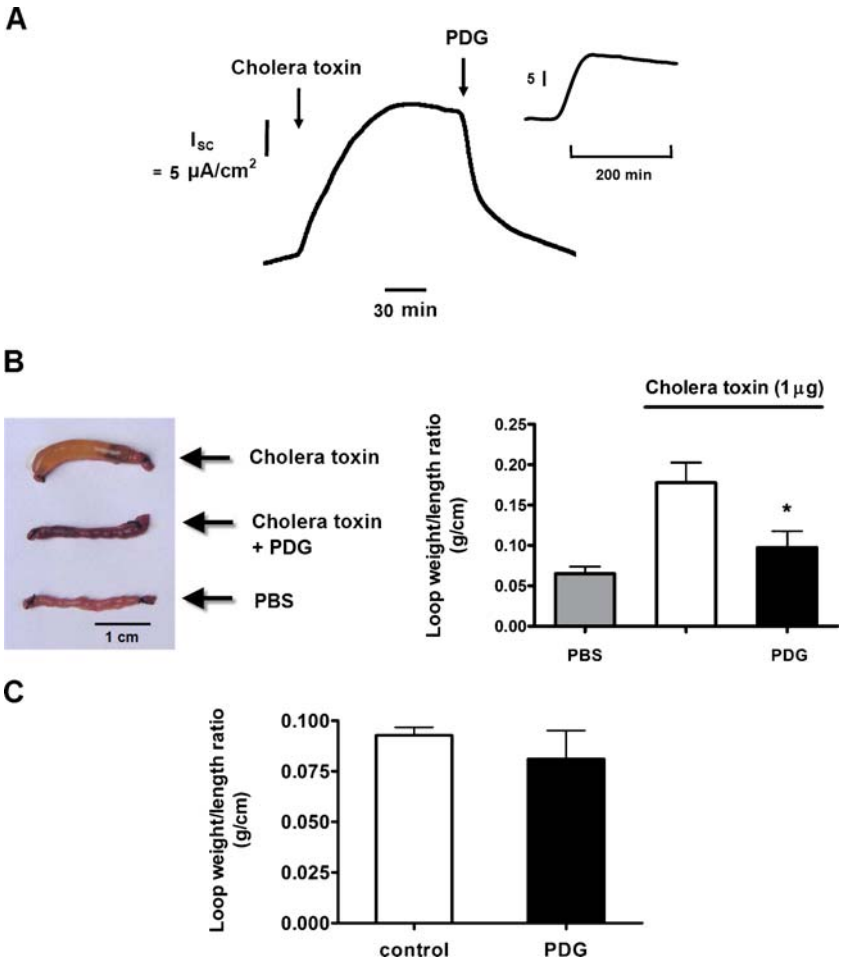

Fig. 7. Antidiarrheal efficacy of PDG in human intestinal cells and mouse closed-loop model of cholera. (**A**) Inhibitory effect of PDG on cholera toxin-induced active chloride secretion across monolayers of T84 cells. In the presence of amiloride (10μM), PDG (200μM) was added after stimulation of active chloride secretion by addition of cholera toxin (1μg/ml) to apical solution. An inset shows a time-control (no PDG) tracing of cholera toxin-induced short-circuit current. (**B**) *In vivo* efficacy of PDG in mouse closed-loop model of cholera toxin-induced intestinal fluid secretion. *Left*, a photograph of isolated mouse mid-jejunal loops at 4 h after luminal injection of cholera toxin (1μg), cholera toxin (1μg) plus PDG (0.6 mg/kg) and saline control. *Right*, summary of loop weight/length ratios. Data are expressed as mean ± S.E.; $n=5$ and * $p<0.05$. (**C**) Effect of PDG on intestinal fluid absorption in mouse closed-loop model. Saline, with or without PDG (0.6 mg/kg), was injected into intestinal loops 30 min before assessment of loop weight/length ratio. Data are shown as mean ± S.E.; $n=3$.

negligible expression of non-CFTR chloride channels (26). In addition, they can form epithelial monolayers on Snapwell filters with electrical resistance high enough for use in electrophysiological studies. Our data showed that in basolaterally permeabilized FRT cells, PDG inhibited cAMP-mediated apical chloride conductance activated by forskolin (an adenylate cyclase activator) with an IC50 of ~ 10μM. Similar results were obtained for apical chloride current measurements using CPT-cAMP (a cell permeable and hydrolysis-resistant cAMP analog) and apigenin (a direct CFTR agonist) as activators of apical chloride current. These results indicated that the target of PDG inhibitory actions should be downstream of cAMP production. In support of this notion, PDG, at a concentration completely inhibiting CFTR-mediated chloride current (100μM), produced no effect on intracellular cAMP levels. In addition, the inhibitory effect of PDG on CFTR function was fully reversible and did not cause any reduction in cell viability. As PDG is a bulky polar molecule (MW. 1701.2), containing 25 hydroxyl groups, we speculated that PDG can not enter cells and blocks CFTR function by binding to the extracellular side of the channel, similar to that proposed for condensed tannins (16).

Compounds possessing CFTR inhibitory activities have been expected to offer novel effective options for treatment of human diseases such as secretory diarrhea and polycystic kidney diseases. cAMP-activated chloride secretion is central to the pathogenesis of secretory diarrhea, including cholera (3,27), and cyst formation and enlargement in polycystic kidney diseases (28). Recent studies conducted with small-molecule CFTR inhibitors identified by high-throughput screening provide proof-of-concept in treatment of cholera

and polycystic kidney diseases using CFTR inhibitors (7,8,29). As demonstrated by short-circuit current analysis in human intestinal T84 cells, PDG was able to inhibit transepithelial active chloride secretion induced by cAMP, while it had no effect on that induced by elevation of intracellular calcium. The latter process involves apical chloride and basolateral potassium channels, which are physiologically and pharmacologically distinct from those involved in the cAMP-activated process. Accordingly, our results indicated that the action of PDG is specific to cAMP-activated transepithelial chloride secretion in human intestinal cells. Nevertheless, the discrepancy in IC50 of PDG obtained from experiments using FRT cells stably expressing human CFTR (~10μM) and T84 cells (~100μM) was found. Indeed, this observation is consistent with previous studies reporting that potency of impermeant CFTR inhibitors such as divalent polyethylene glycol-malonic acid hydrazides was lower in T84 cells (30).

In vivo studies in mice showed that concomitant administration of PDG significantly reduced cholera toxin-induced intestinal fluid secretion, whereas intestinal fluid absorption was unaffected. As transepithelial chloride secretion across a monolayer of T84 cells activated by pretreatment with cholera toxin was effectively inhibited by PDG, this confirmed its direct effect on CFTR function. This rules out the possibility that PDG may precipitate cholera toxin or disrupt binding of the cholera toxin to its ganglioside receptor on enterocyte apical membrane. These experiments suggest that PDG could have potential applications in therapy of secretory diarrhea whose pathogenesis involves cAMP, such as cholera and intestinal infection with enterotoxic *Escherichia coli*.

Compared to other natural product-derived inhibitors of CFTR chloride channel, such as dihydroisosteviol, flavonoids and proanthocyanidins, PDG and related hydrolysable tannins present an attractive chemical option amenable to further development of novel treatment for diarrhea. The presence of multiple hydroxyl groups confers PDG high water solubility (>1 mM). PDG has a potency (IC50 of 10μM) comparable to dihydroisosteviol, proanthocyanidin and flavonoid-related compounds, which have IC50 of 20–100μM (9,15,16,25). In addition, hydrolysable tannins, including PDG, possess antioxidative properties (11,13). Elevated levels of reactive oxygen species have been implicated in the pathological processes leading to deranged intestinal fluid and electrolyte transport in infection and inflammation-associated diarrhea such as inflammatory bowel disease (31). Of note, during infectious and inflammatory processes in intestine, nitric oxide released from immune cells is capable of stimulating CFTR-mediated chloride secretion (31–33). Moreover, CFTR expression is increased up to five-fold in colonic mucosa of ulcerative colitis patients (33).

In summary, the present study identified for the first time an inhibitory effect of hydrolysable tannin on CFTR chloride channel function. Further development of this class of compounds may provide a new therapeutic intervention for diarrhea and polycystic kidney and other diseases associated with CFTR hyperactivity.

ACKNOWLEDGEMENTS

This work was supported by the Thailand Research Fund (grant RMU5180029 to VC), the National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand National Science and Technology Development Agency (Grant 3-2548) and Center for Environmental Health, Toxicology and Management of Chemicals, Commission for Higher Education, Ministry of Education, Thailand.

We thank Prof. Alan S. Verkman (University of California, San Francisco, CA, USA) for providing the FRT cells and highly appreciate Prof. Prapon Wilairat (Department of Biochemistry, Mahidol University) for his valuable comments and corrections of this manuscript.

REFERENCES

1. Kosek M, Bern C, Guerrant RL. The global burden of diarrhoeal disease, as estimated from studies published between 1992 and 2000. Bull World Health Organ. 2003;81:197–204.
2. Huilan S, Zhen LG, Mathan MM, Mathew MM, Olarte J, Espejo R, et al. Etiology of acute diarrhoea among children in developing countries: a multicentre study in five countries. Bull World Health Organ. 1991;69:549–55.
3. Thiagarajah JR, Verkman AS. New drug targets for cholera therapy. Trends Pharmacol Sci. 2005;26:172–5.
4. Grubb BR, Boucher RC. Pathophysiology of gene-targeted mouse models for cystic fibrosis. Physiol Rev. 1999;79:S193–214.
5. Barrett KE, Keely SJ. Chloride secretion by the intestinal epithelium: molecular basis and regulatory aspects. Annu Rev Physiol. 2000;62:535–72.
6. Verkman AS, Galietta LJ. Chloride channels as drug targets. Nat Rev Drug Discov. 2009;8:153–71.
7. Ma T, Thiagarajah JR, Yang H, Sonawane ND, Folli C, Galietta LJ, et al. Thiazolidinone CFTR inhibitor identified by high-throughput screening blocks cholera toxin-induced intestinal fluid secretion. J Clin Invest. 2002;110:1651–8.
8. Muanprasat C, Sonawane ND, Salinas D, Taddei A, Galietta LJ, Verkman AS. Discovery of glycine hydrazide pore-occluding CFTR inhibitors: mechanism, structure-activity analysis, and *in vivo* efficacy. J Gen Physiol. 2004;124:125–37.
9. Pariwat P, Homvisasevongsa S, Muanprasat C, Chatsudthipong V. A natural plant-derived dihydroisosteviol prevents cholera toxin-induced intestinal fluid secretion. J Pharmacol Exp Ther. 2008;324:798–805.
10. Serrano J, Puupponen-Pimia R, Dauer A, Aura AM, Saura-Calixto F. Tannins: current knowledge of food sources, intake, bioavailability and biological effects. Mol Nutr Food Res. 2009;53 Suppl 2:S310–29.
11. Andrade Jr RG, Dalvi LT, Silva Jr JM, Lopes GK, Alonso A, Hermes-Lima M. The antioxidant effect of tannic acid on the *in vitro* copper-mediated formation of free radicals. Arch Biochem Biophys. 2005;437:1–9.
12. Chenand SC, Chung KT. Mutagenicity and antimutagenicity studies of tannic acid and its related compounds. Food Chem Toxicol. 2000;38:1–5.
13. Andrade Jr RG, Ginani JS, Lopes GK, Dutra F, Alonso A, Hermes-Lima M. Tannic acid inhibits *in vitro* iron-dependent free radical formation. Biochimie. 2006;88:1287–96.
14. Murakami S, Muramatsu M, Otomo S. Inhibitory effect of tannic acid on gastric H+, K(+)-ATPase. J Nat Prod. 1992;55:513–6.
15. Schuier M, Sies H, Illek B, Fischer H. Cocoa-related flavonoids inhibit CFTR-mediated chloride transport across T84 human colon epithelia. J Nutr. 2005;135:2320–5.
16. Fischer H, Machen TE, Widdicombe JH, Carlson TJ, King SR, Chow JW, et al. A novel extract SB-300 from the stem bark latex of Croton lechleri inhibits CFTR-mediated chloride secretion in human colonic epithelial cells. J Ethnopharmacol. 2004;93:351–7.
17. Galietta LV, Jayaraman S, Verkman AS. Cell-based assay for high-throughput quantitative screening of CFTR chloride transport agonists. Am J Physiol Cell Physiol. 2001;281:C1734–42.
18. Kunzelmann K, Mall M. Electrolyte transport in the mammalian colon: mechanisms and implications for disease. Physiol Rev. 2002;82:245–89.
19. Rufo PA, Lin PW, Andrade A, Jiang L, Rameh L, Flexner C, et al. Diarrhea-associated HIV-1 APIs potentiate muscarinic acti-

vation of Cl- secretion by T84 cells via prolongation of cytosolic Ca2+ signaling. Am J Physiol Cell Physiol. 2004;286:C998–1008.
20. Morris AP, Scott JK, Ball JM, Zeng CQ, O'Neal WK, Estes MK. NSP4 elicits age-dependent diarrhea and Ca(2+)mediated I(-) influx into intestinal crypts of CF mice. Am J Physiol. 1999;277: G431–44.
21. Greger R. Role of CFTR in the colon. Annu Rev Physiol. 2000;62:467–91.
22. Chung KT, Wong TY, Wei CI, Huang YW, Lin Y. Tannins and human health: a review. Crit Rev Food Sci Nutr. 1998;38:421–64.
23. Gali HU, Perchellet EM, Klish DS, Johnson JM, Perchellet JP. Hydrolyzable tannins: potent inhibitors of hydroperoxide production and tumor promotion in mouse skin treated with 12-O-tetradecanoylphorbol-13-acetate *in vivo*. Int J Cancer. 1992;51:425–32.
24. Buzzini P, Arapitsas P, Goretti M, Branda E, Turchetti B, Pinelli P, et al. Antimicrobial and antiviral activity of hydrolysable tannins. Mini Rev Med Chem. 2008;8:1179–87.
25. Gabriel SE, Davenport SE, Steagall RJ, Vimal V, Carlson T, Rozhon EJ. A novel plant-derived inhibitor of cAMP-mediated fluid and chloride secretion. Am J Physiol. 1999;276:G58–63.
26. Jayaraman S, Haggie P, Wachter RM, Remington SJ, Verkman AS. Mechanism and cellular applications of a green fluorescent protein-based halide sensor. J Biol Chem. 2000;275:6047–50.
27. Flach CF, Lange S, Jennische E, Lonnroth I. Cholera toxin induces expression of ion channels and carriers in rat small intestinal mucosa. FEBS Lett. 2004;561:122–6.
28. Sullivan LP, Wallace DP, Grantham JJ. Epithelial transport in polycystic kidney disease. Physiol Rev. 1998;78:1165–91.
29. Yang B, Sonawane ND, Zhao D, Somlo S, Verkman AS. Small-molecule CFTR inhibitors slow cyst growth in polycystic kidney disease. J Am Soc Nephrol. 2008;19:1300–10.
30. Sonawane ND, Zhao D, Zegarra-Moran O, Galietta LJ, Verkman AS. Nanomolar CFTR inhibition by pore-occluding divalent polyethylene glycol-malonic acid hydrazides. Chem Biol. 2008;15:718–28.
31. Martinez-Augustin O, Romero-Calvo I, Suarez MD, Zarzuelo A, de Medina FS. Molecular bases of impaired water and ion movements in inflammatory bowel diseases. Inflamm Bowel Dis. 2009;15:114–27.
32. Dobbins JW, Laurenson JP, Forrest Jr JN. Adenosine and adenosine analogues stimulate adenosine cyclic 3′, 5′-monophosphate-dependent chloride secretion in the mammalian ileum. J Clin Invest. 1984;74:929–35.
33. Lohi H, Makela S, Pulkkinen K, Hoglund P, Karjalainen-Lindsberg ML, Puolakkainen P, et al. Upregulation of CFTR expression but not SLC26A3 and SLC9A3 in ulcerative colitis. Am J Physiol Gastrointest Liver Physiol. 2002;283:G567–75.