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Running Title: Berberine inhibition of KCNQ1 channels

Key words: Berberine, KCNQ1 K+ channels, CFTR, chloride secretion, colon ion transport, T84 cells
Abstract

Berberine is a plant alkaloid with multiple pharmacological actions, including antidiarrhoeal activity and has been shown to inhibit Cl- secretion in distal colon. The aims of this study were to determine the molecular signalling mechanisms of action of berberine on Cl- secretion and the ion transporter targets. Monolayers of T84 human colonic carcinoma cells grown in permeable supports were placed in Ussing chambers and short-circuit current measured in response to secretagogues and berberine. Whole-cell current recordings were performed in T84 cells using the patch-clamp technique. Berberine decreased forskolin-induced short-circuit current in a concentration-dependent manner (IC_{50} 80 ± 8 μM). In apically permeabilized monolayers and whole-cell current recordings, berberine inhibited a cAMP-dependent and chromanol 293B-sensitive basolateral membrane K+ current by 88%, suggesting inhibition of KCNQ1 K+ channels. Berberine did not affect either apical Cl- conductance or basolateral Na+-K+-ATPase activity. Berberine stimulated p38 MAPK, PKCα and PKA, but had no effect on p42/p44 MAPK and PKCδ. However, berberine pre-treatment prevented stimulation of p42/p44 MAPK by epidermal growth factor. The inhibitory effect of berberine on Cl- secretion was partially blocked by HBDDE (~65 %), an inhibitor of PKCα and to a smaller extent by inhibition of p38 MAPK with SB202190 (~15 %). Berberine treatment induced an increase in association between PKCα and PKA with KCNQ1 and produced phosphorylation of the channel. We conclude that berberine exerts its inhibitory effect on colonic Cl- secretion through inhibition of basolateral KCNQ1 channels responsible for K+ recycling via a PKCα-dependent pathway.

Introduction

Berberine is a benzodioxoquinolone plant alkaloid isolated from several species of Berberis and Coptis. In Chinese and Hindu medicine, berberine has been used to treat gastroenteritis, abdominal pain and diarrhoea for over two millennia. Various pharmacological actions have been described for berberine, including antimicrobial (Iwasa et al., 1998), anti-inflammatory (Ivanovska and Philipov, 1996) and cardiovascular effects (Lau et al., 2001). In several small-scale clinical trials, berberine has proven effective in the treatment of secretory diarrhoea (Rabbani et al., 1987; Tang and Eisenbrand, 1992). Although its therapeutic benefit has been attributed in part to its antimicrobial and antimotility properties (Yamamoto et al., 1993), berberine has been shown to prevent epithelial electrolyte secretion in vitro in rabbit and rat intestine (Guandalini et al., 1987; Taylor and Baird, 1995).

Diarrheal diseases continue to be a major cause of morbidity and mortality in children and elderly people throughout the world. Oral rehydration therapy remains the mainstay of treatment for diarrhoea (Taylor and Greenough, 1989). However, in recent years significant effort has been made in the search for antisecretory drugs that will directly inhibit secretory processes within the enterocytes (Farthing, 2006; Ma et al., 2002).

Activated Cl- secretion from the intestinal crypt is thought to play a major role in secretory diarrhoea of several aetiologies (Field, 2003). The generation of the electrochemical driving force required for Cl- secretion by crypt epithelial cells depends on their ability to accumulate intracellular Cl- ions to concentrations greater than their electrochemical equilibrium (Kunzelmann and Mall, 2002; Barrett and Keely, 2000). Cl- enters the cell across the basolateral membrane through the activity of Na+-K+-2Cl- cotransporters. The cotransporter is, in turn, driven by a strong inwardly directed electrochemical Na+ gradient established by the basolaterally located Na+-K+-ATPase. In order to maintain the membrane potential at rest and during Cl- secretion, both Na+ and K+ must be recycled out of the cell through the basolateral membrane. The Na+-K+-ATPase serves to recycle Na+, while
basolateral potassium channels recycle K⁺ (Schultheiss and Diener, 1998). The basolateral K⁺
conductance in intestinal epithelial cells is formed by at least two different types of K⁺
channels, one activated by Ca²⁺-mobilizing secretagogues and the other by cAMP-dependent
agonists (Heitzmann and Warth 2008). Electrophysiological studies have revealed the latter to
be KCNQ1, a low conductance (1-3 pS) basolateral K⁺ channel, which is activated during
cAMP-stimulated Cl⁻ secretion and inhibited by chromanol 293B and HMR-1556 (Schroeder
et al., 2000; Robbins 2001). As in all secretory epithelia, the channels and transporters of the
crypt epithelial cell must operate in concert to achieve vectorial ion transport. Therefore,
blockade of specific basolateral K⁺ conductance would be expected to inhibit the Cl⁻ secretory
process.

The T84 cell line is a well-differentiated intestinal human carcinoma cell line proved
to be a robust model for the study of molecular mechanisms of intestinal secretion in close to
1,200 publications since the early 1980s. (Dharmsathaphorn et al. 1984). Previous studies
using T84 cells, grown to confluence and mounted in Ussing chambers, have shown that
berberine decreased Cl⁻ secretion in a dose-dependent manner (Taylor and Baird, 1995). More
importantly, berberine attenuated the large Cl⁻ secretory current produced by agents that
increase intracellular cAMP. However, the specific transport pathways responsible for the
inhibitory effect of berberine on Cl⁻ secretion have not been identified. Herein we report on a
series of experiments designed to clarify which epithelial transport processes are affected by
berberine. Using the short circuit current technique, we studied the ability of berberine to
inhibit Cl⁻ secretion induced by cAMP in T84 cells. Using the pore-forming antibiotics
nystatin and amphotericin B to permeabilize the basolateral and apical membranes
respectively, we were able to isolate membrane currents and assess the effects of berberine on
1) the apical membrane Cl⁻ conductance, 2) the basolateral membrane Na⁺-K⁺-ATPase
activity, and 3) the basolateral membrane K⁺ conductance. Also, we explored the signalling
involved in the antisecretory action of berberine in particular the role of protein kinases such
as PKC, PKA, mitogen-activated protein kinase (MAPK) and membrane targets such as ion
channels and transporters. Our results indicate that berberine inhibits Cl⁻ secretion by
decreasing the basolateral membrane K⁺ conductance and therefore K⁺ recycling necessary for
the generation of the favourable electrochemical gradient required for Cl⁻ secretion.

Materials and methods

Cell Culture
T84 cells (American Type Culture Collection, Manassas, VA) were cultured in a 1:1 mixture
of DMEM and Ham's F-12 supplemented with 10% foetal bovine serum, 1% non-essential
amino acids, 50 U·ml⁻¹ penicillin, 0.05 mg·ml⁻¹ streptomycin and grown onto Costar Snapwell
culture inserts (Corning, Dublin Ireland) with an area of 1 cm² for short circuit current
measurements or onto 24 mm Costar Transwell filters (0.4 μm pore) for protein assays.
Experiments were conducted on confluent monolayers 8-12 days after culture onto the
permeable supports.

Transepithelial transport studies
T84 cells were grown onto Snapwell inserts and mounted in Ussing chambers (Physiologic
Instruments, San Diego, CA, USA). Transepithelial potential difference was clamped to zero
using an EVC-4000 voltage-clamp apparatus (World Precision Instruments, UK). The
transepithelial short-circuit current (Isc) was recorded using Ag-AgCl electrodes in 3M KCl
agar bridges as previously described (Condliffe et al., 2001). Transepithelial resistance (Rt)
was calculated by measuring the Isc resulting from 5-s square voltage pulses (2 or 4 mV)
imposed across the monolayer. All preparations were allowed to equilibrate for 30 min before
the experiments were performed. Amiloride (50 μM) was added at the start of the experiments
to block Na⁺ absorption. All experiments were performed at 37°C. The $I_{sc}$ was defined as
positive for anion flow from the basolateral to apical chamber.

Specific apical Cl⁻ channel conductance and basolateral Na⁺/K⁺ pump and K⁺ channel
conductances were isolated and analyzed using a well-established technique consisting of
selective membrane permeabilization using ionophores as previously described (DuVall et al.,
1998). To investigate the activity of apical Cl⁻ conductance in isolation, the basolateral
membrane was permeabilized by addition of 200μg·mL⁻¹ nystatin in the presence of an apical
to basolateral Cl⁻ gradient. Forskolin was then added to the apical and basolateral sides of the
monolayers to activate the cystic fibrosis transmembrane conductance regulator (CFTR).
Under these conditions, the $I_{sc}$ represents the Cl⁻ current ($I_{cl}$) as Cl⁻ moves down its
concentration gradient through the CFTR channels in the apical plasma membrane.

To investigate the activity of basolateral Na⁺-K⁺-ATPase activity in isolation, the
apical membrane was permeabilized by addition of 10 μM amphotericin B. Na⁺-K⁺-ATPase
activity was examined in monolayers bathed with medium in which NaCl was replaced by N-
methyl-D-glutamine chloride, such that the final bath Na⁺ concentration was 25 mM on both
sides of the monolayers. Under short-circuit conditions, the resulting current is due to the
transport of Na⁺ across the basolateral membrane by the Na⁺-K⁺-ATPase ($I_{n}$).

To investigate the activity of the basolateral K⁺ conductance in isolation, the apical
membrane was permeabilized by addition of 10 μM amphotericin B in the presence of an
apical to basolateral K⁺ gradient. Ouabain (100 μM) was added to the basolateral bath to
inhibit Na⁺-K⁺-ATPase. The resulting $I_{sc}$ is due to the movement of K⁺ through channels in
the basolateral membrane ($I_k$). The use of antibiotic-based ionophores to selectively
permeabilise epithelial membranes is a well-established electrophysiological protocol since the
1980s to gain an insight into ion conductive properties at the opposite unpermeabilised
membrane. Antibiotic ionophores used for transport studies are permselective for ions of
similar size with the exception of valinomycin for K⁺. The establishment of large
transepithelial (transmembrane) ionic gradients for the ion under study and the use of
selective channel blockers aid in extracting the conductive properties of the particular
rheogenic ion transport pathway as was done in this study.

Patch-clamp recordings
A small aliquot of Krebs solution containing single T84 cells was transferred into a 1 ml
superfusion chamber mounted on the stage of an inverted microscope (Nikon Diaphot). Patch
pipettes were prepared from capillary glass (GC150 F-10, Harvard Apparatus Ltd,
Edenbridge, UK) using a programmable horizontal puller (DMZ-Universal Puller, Zeitz-
Instruments GmbH, Munich, Germany) and had a resistance of 3-6Ω when filled with the
pipette solution. The reference electrode was an Ag-AgCl wire in direct contact with the
superfusion bath. Patch-clamp apparatus consisted of a CV-203BU headstage (Axon
Instruments Inc., Union City, CA) connected to an Axopatch 200B series amplifier.
Experiments were performed using the whole-cell patch-clamp configuration and recorded
membrane currents were filtered at 1 kHz and digitized at 5 kHz. Membrane voltage was
clamped from -100mV to +100 mV with ramps of 20 mV from an initial holding potential of
-50 mV. The protocols for patch-clamp and data analysis were established using pClamp 9.2
software (Axon Instruments Inc.) running on a PC, and data were stored for subsequent
analysis. For patch-clamp measurements, the standard bath solution contained (in mM): NaCl
140, KCl 5.4, MgCl₂ 1, CaCl₂ 1.25, Glucose 12.2, and HEPES 10, buffered at pH 7.4 with
NaOH. The patch pipette solution contained (in mM): K-Gluconate 95, KCl 30, Na₂HPO₄ 4.8,
KH₂PO₄ 1.2, EGTA 1, Ca-gluconate 0.73, MgCl₂ 1, Na₂ATP 3, Glucose 5 (pH 7.2). Drug
actions were measured only after steady-state conditions were reached. All patch-clamp experiments were performed at 37°C.

**Immunoprecipitation and Western blotting**

T84 cells were serum-starved for 24 hours prior to treatment. Drugs were added to the basolateral side of the cell monolayers for the required time. After treatment cells were lysed and subjected to standard 10% SDS-PAGE and Western blotting as previously described (Dilly et al., 2004). Immunoblots were developed using specific phospho-antibodies against human PKCα, PKCδ, p42/p44 MAPK and p38 MAPK. Immunoprecipitation assays for KCNQ1 were carried out as previously described (Dilly et al., 2004).

**PKA assay**

PKA activity was detected using the PepTagtrade mark Assay for Non-Radioactive Detection of cAMP-dependent protein kinase. The kit was used according to manufacturer’s instructions using 30μg of protein in each reaction http://www.promega.com/resources/protocols/technical-bulletins/0/peptag-assay-for-nonradioactive-detection-of-pkc-or-campdependent-protein-kinase-protocol/. T84 cells were lysed by hypotonic shock on ice for 45 min (lysis buffer: 20 mM Tris, pH 7.4, 0.5% Nonident P-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, leupeptin 1 μg/μl, 500 mM dithiothreitol, 5 mM phenylmethylsulfonyl fluoride, complete mini EDTA-free protease inhibitor mixture tablets (1 tablet/7 ml of lysis buffer; Roche Applied Science) and phosphatase inhibitors.

**Statistical Analysis**

Densitometric analysis was performed using Genetools software (Syngene, Cambridge UK). Statistical analysis of the data was obtained using paired t-test for analysis between two groups. ANOVA and Tukeys post-hoc test for multiple analyses. P-values of 0.05 and less were considered to be significant. Data are expressed as means ± SEM unless specifically stated to be from a representative experiment.

**Materials**

Phospho-PKCδ/θ (Ser^{643/676}), p44/p42 MAPK, phospho-p42/p44 MAPK (Thr^{202}/Tyr^{204}), and phospho-p38 MAPK (Thr^{180}/Tyr^{182}) antibodies were from Cell Signalling Technologies (Hertfordshire, UK). PKCgralpha and PKAcI antibodies were from BD Transduction Antibodies (NJ, USA). Phospho-PKCα (Ser^{657}) antibody was from Upstate (Dublin, Ireland). KCNQ1 antibody was from Santa Cruz (CA, USA). Chromanol 293B was obtained from Tocris (Avonmouth, UK). Rottlerin, clotrimazole, SB202190 and HBDDE were purchased from Calbiochem (Nottingham, UK). (3R,4S)-(++)-N-[3-hydroxy-2,2-dimethyl-6-(4,4,4-trifluorobutoxy)chroman-4-yl]-N-methylethanesulfonamide (HMR-1556) was kindly provided by Dr. Uwe Gerlach (Aventis Pharma Deutschland, Frankfurt-am-Main, Germany). All other chemicals were purchased from Sigma-Aldrich (Dublin, Ireland). All drugs were dissolved in DMSO or water as stock solutions and diluted appropriately. The final DMSO concentration was less than 0.1%.

**Results**

**Berberine inhibits cAMP-stimulated Cl− secretion in colonic epithelial cells**

Short-circuit current measurements across T84 monolayers under basal conditions revealed a small $I_{Sc}$ reflecting a low level of Cl− secretion. When the epithelium was treated with forskolin (10 μM), the $I_{Sc}$ rapidly increased from 4 ± 2 to 48 ± 6 μA·cm$^{-2}$ and remained
significantly elevated for over 30 min. Berberine when added to the basolateral solution at the peak of the forskolin response decreased the $I_{SC}$ (control $46 \pm 5 \mu A/cm^2$, berberine $5 \pm 3 \mu A/cm^2; n = 8, P < 0.01$) (Figure 1A). The inhibitory effect of berberine on forskolin-induced Cl$^-$ secretion was concentration-dependent between 10 and 300 $\mu M$ with an IC$_{50}$ of $80 \pm 3 \mu M$ (Figure 1B). No significant effect on $I_{SC}$ was observed when berberine was added in the absence of forskolin, however, subsequent treatment with forskolin failed to increase $I_{SC}$ (not shown). In approximately 75% of the experiments berberine induced a small and transient increase in $I_{SC}$ (Figure 1A). This response was prevented by addition of BaCl$_2$ (2 mM) to the apical bath suggesting berberine also inhibits an apical K$^+$ conductance responsible for K$^+$ secretion (data not shown). Berberine treatment for as long as 2 hours did not decrease the transepithelial resistance $R_t$ compared to vehicle controls, suggesting that the effects of berberine, at the concentrations used in this study, were not due to cytotoxicity (Table 1).

**Berberine effects on apical membrane Cl$^-$ conductance and basolateral Na$^+-$K$^+$-ATPase activity and K$^+$ conductance**

Nystatin, added to the basolateral bathing solution of T84 monolayers increased the apical membrane Cl$^-$ specific current $I_{Cl}$ from $3 \pm 1$ to $14 \pm 2 \mu A/cm^2$ ($n = 6$). This likely reflects a constitutively active apical membrane Cl$^-$ conductance. Subsequent addition of forskolin (10 $\mu M$) further increased the $I_{Cl}$ to $88 \pm 12 \mu A/cm^2$. Treatment of T84 cell monolayers with berberine (300 $\mu M$) on either apical or basolateral bath for 15 min had no effect on either basal or forskolin-activated apical membrane $I_{Cl}$ (Table 2).

The addition of amphotericin B to the apical bathing solution containing 25 mM Na$^+$ increased the basolateral membrane Na$^+$ specific pump current $I_{Na}$ from $3 \pm 1$ to $16 \pm 3 \mu A/cm^2$ ($n = 6$). Under these conditions, the amphotericin-induced $I_{Na}$ was completely inhibited by 100 $\mu M$ ouabain. When T84 monolayers were treated with berberine (300 $\mu M$) for 10 min, the amphotericin-induced $I_{Na}$ was not different from that of vehicle controls (Table 2).

When amphotericin B was added to the apical bath in the presence of an apical to basolateral K$^+$ gradient (80:5 mM) and basolateral ouabain (100 $\mu M$), the $I_K$ immediately began to increase and reached maximal levels within 15 min (from $5 \pm 3$ to $53 \pm 4 \mu A/cm^2$; $n = 6$). In these conditions, $I_K$ was almost completely inhibited by addition of BaCl$_2$ (2 mM), chromanol 293B (10 $\mu M$) or HMR-1556 (500 nM) to the basolateral bath (Figure 2A). When monolayers were treated with berberine (300 $\mu M$), $I_K$ was rapidly inhibited (53 $\pm 4$ to $9 \pm 2 \mu A/cm^2$; $P < 0.01, n = 6$) (Figure 2B) with half-maximal inhibition produced at a concentration of 150 $\mu M$ (Figure 2C).

**Berberine effects on whole-cell potassium currents in T84 cells**

Berberine produced a strong inhibitory effect on forskolin-activated basolateral membrane $I_K$ in T84 monolayers (Table 2). The effect of berberine on cAMP-stimulated whole-cell currents in T84 cells was examined (Figure 3A). Forskolin (10 $\mu M$) stimulated a mean maximal increase in whole-cell current of $359 \pm 71 \mu A$ (at $V_p = +100 mV; n = 7, P < 0.001$). Berberine (100 $\mu M$) addition to isolated T84 cells reduced the forskolin-activated current by 68% (corresponding to a reduction of $245 \pm 44 \mu A$ (at $V_p = +100 mV, n = 7, P < 0.01$). Consistent with the increase in whole-cell current, forskolin stimulated an increase in the chord conductance at $+100 \text{ mV} (\gamma_{+100})$ from $1.7 \pm 0.3 \text{ nS}$ to $5.2 \pm 0.4 \text{ nS} (P < 0.001, n = 7)$, which was reduced to $2.8 \pm 0.2 \text{ nS} (P < 0.01, n = 7)$ following treatment with berberine.

The identity of the K$^+$ channels underlying the current stimulated by forskolin and inhibited by berberine was investigated using a variety of K$^+$ channel blockers. Chromanol 293B (10 $\mu M$) completely inhibited the forskolin-stimulated current below the level of the
control current measured prior to the addition of forskolin (at Vp +100 mV; forskolin = 665 ± 193 pA; forskolin + chromanol 293B = 86 ± 51 pA; P < 0.05, n = 3) (Fig. 3B). Superfusion with chromanol 293B also reduced the forskolin-induced chord conductance at +100 mV from 7.2 ± 1.4 nS to 1.2 ± 0.5 nS (P < 0.05, n = 3). Other specific K⁺ channel inhibitors (of large and small conductance, Ca²⁺-dependent K⁺ channels) such as iberiotoxin (BK, 200 nM), apamin (SK, 500 nM) and TRAM-34 (KCNN4, 500 nM) did not affect the forskolin-induced whole cell K⁺ currents.

Berberine effect on protein kinases activity in T84 cells

PKCα activation was assessed by probing with a specific antibody to phospho-Ser⁶⁵⁷. Berberine treatment increased PKCα phosphorylation levels at 5 and 10 min with fold increases of 6.4 ± 0.8 and 7.4 ± 1.8 respectively (n = 3, P < 0.01) compared to vehicle controls (Figure 4A). PKCδ activity was measured using an antibody specific to phospho-Ser⁶⁴³. Berberine treatment had no effect on PKCδ phosphorylation levels (5 min 1.1 ± 0.1; 10 min 0.9 ± 0.1; n = 3) compared to vehicle controls within the time period assayed (Figure 4B). These results show that berberine selectively activates the classical PKCα with no effect on the novel PKCδ. Berberine pre-treatment activated PKA at 10 min with a fold increase of 2.2 ± 0.1 compared to vehicle controls (n = 3, P < 0.001) (Figure 4C). Pre-treatment for 10 min with 100 μM HBDDE, a selective PKCa inhibitor at this concentration (Kashiwada et al., 1994), prevented berberine-induced PKA activation (1.0 ± 0.1; n = 3) (Figure 4C). This result indicates that PKA is activated in response to berberine and is downstream to PKCα activation.

Activation of p38 MAPK was assessed by probing with a specific antibody to phospho-Thr¹⁸⁰/Tyr¹⁸². Berberine treatment induced p38 MAPK phosphorylation at 5 min with a fold increase of 1.8 ± 0.02 (n = 3, P < 0.05) (Figure 5A). The activity of p42/p44 MAPK was measured using an antibody specific to phospho-Thr²⁰²/Tyr²⁰⁴. Treatment with berberine had no effect on basal p42/p44 MAPK phosphorylation levels compared to vehicle controls (0.8 ± 0.3, P < 0.04, n = 4) within the time points assayed (Figure 5B). However, pre-treatment of the cells with berberine for 5 min inhibited activation of p42/p44 MAPK by epidermal growth factor (EGF 100 ng·ml⁻¹) with similar potency to that of PD98059 (20 μM), a specific MEK1 inhibitor that prevent receptor mediated activation of p42/p44 MAPK, (EGF 10.3 ± 1.1; berberine + EGF 1.6 ± 0.7; PD98059 + EGF 0.6 ± 0.1; n = 4, P < 0.01) (Figure 5B). These results confirm that berberine selectively activates the p38 MAPK isoform but does not activate p42/p44 MAPK isoforms. Moreover, berberine prevents the EGF-induced activation of p42/p44 MAPK.

Berberine inhibition of cAMP-stimulated Cl⁻ secretion is mediated by protein kinases

To assess the role of berberine-induced protein kinase activation on its antisecretory effect, T84 monolayers mounted in Ussing chambers were pre-treated with specific kinase inhibitors. Treatment of T84 monolayers with the PKCα inhibitor HBDDE and the specific p38 MAPK inhibitor SB202190 (10 μM) had no effect on basal or forskolin-stimulated Iₛ. However, HBDDE significantly reduced the inhibitory effect of berberine on Iₛ by 65 ± 7 % (Figure 6A). SB202190 also reduced the effect of berberine but only by 14 ± 5 % (Figure 6A). Co-incubation with both inhibitors inhibited the effect of berberine by 82 ± 4 % suggesting parallel and synergistic signalling pathways. In contrast, inhibitors of PKCδ (rottlerin 10 μM) and p42/p44 MAPK (PD98059 20 μM) failed to prevent the antisecretory effect of berberine. Figure 6B shows percentages of inhibition of the berberine antisecretory action on forskolin-stimulated Iₛ by several kinase inhibitor treatments.
Berberine pre-treatment increased the association of PKCα with the KCNQ1 channel at 5 min with a fold increase of 2.4 ± 0.1 (n = 3, P < 0.05) (Figure 7A). Upon activation, PKA catalytic subunits (PKAcI) dissociate from their regulatory subunits and interact with their substrates. Berberine treatment increased PKAcI association with the KCNQ1 channel with a fold increase of 2.2 ± 0.4 (n = 3, P < 0.05) (Figure 7B). These experiments demonstrate the association of two kinases, PKCα and PKAcI, with the KCNQ1 channel upon treatment with berberine.

**Discussion**

The results from this study show that the plant alkaloid berberine inhibits cAMP-dependent Cl⁻ secretion through a kinase-dependent inhibition of the KCNQ1 potassium channel located at the basolateral membrane of human colonic T84 cells. These data identify the ion channel target of berberine to produce inhibition of transepithelial Cl⁻ secretion which we described in early studies in human colonic epithelia (Taylor et al., 1999). Taken together our results are consistent with the hypothesis that blockade of Cl⁻ secretion by berberine in colonic epithelia is secondary to blockade of basolateral membrane K⁺ channels involved in cAMP-regulated Cl⁻ secretory pathways.

At least two types of K⁺ channels are present in T84 cells (Kunzelmann and Mall, 2002; Barrett and Keely, 2000). One channel is activated by agents that elevate cytosolic Ca²⁺ and has been identified as the KCNN4 channel (Warth et al., 1999). A separate K⁺ channel is activated by cAMP-dependent agonists. Electrophysiological and pharmacological studies have demonstrated this K⁺ conductance to correspond to the KCNQ1 channel (Schroeder et al., 2000) and this channel has been shown to be rate-limiting for secretion in colon (Preston et al. 2010). In our study, berberine significantly reduced the basolateral membrane K⁺ conductance. This conductance was stimulated by the cAMP-dependent agonist forskolin and was sensitive to basolateral addition of Ba²⁺, a non-specific K⁺ channel blocker, and chromanol 293B and HMR-1556, two specific KCNQ1 channel blockers, suggesting the main component of the basolateral K⁺ conductance is composed of current flow through KCNQ1 channels. Similarly, berberine inhibited a cAMP-stimulated and chromanol 293B sensitive whole-cell conductance, suggesting berberine inhibits KCNQ1 channels. Berberine had no effect on the apical Cl⁻ conductance nor did it affect the basolateral Na⁺/K⁺-ATPase pump activity determined as ouabain-sensitive basolateral membrane Na⁺ current. Therefore, this data suggests berberine exerts its antisecretory effect primarily by inhibiting KCNQ1 channels, thus, decreasing K⁺ recycling at the basolateral membrane.

Recent studies have examined the effects of berberine on protein kinase activity in several tissues. These reports have shown that the antihyperglycemic activity of berberine is mediated by activation of p38 MAPK and AMP-activated protein kinase (Cheng et al., 2006; Lee et al., 2006). The antiproliferative activity of berberine has been reported to be mediated by inhibition of cyclin-dependent kinases and the MAPK pathway in several tissues (Mantena et al., 2006; Liang et al 2006). In T84 cells, no effect on basal p42/44 MAPK activity was observed, however, berberine did prevent EGF-induced activation of p42/44 MAPK. Several studies have described the ability of EGF receptor-mediated signalling in regulating Cl⁻ secretion though MAPK (Bertelsen et al., 2004). Therefore, it is possible that berberine may also reduce Cl⁻ secretion by interfering with the EGF signalling. Also, the inhibitory effect of berberine on EGF signalling may be relevant in the antiproliferative action of berberine.

Berberine activated p38 MAPK, PKA and PKCα.Activation of PKA was observed to be dependent on PKCα activity, and this is the first study to demonstrate berberine-induced activation of PKA and PKCα. Upon activation by berberine, both kinases were shown to transiently associate with the KCNQ1 channel even though both kinases remained activated.
for a longer period. Several studies have demonstrated regulation of K+ channels by protein kinases. We have previously shown that estrogen causes a female-gender specific inhibition of KCNQ1 channels in rat colonic crypts, producing an anti-secretory response which is also dependent on PKA activation and association with the KCNQ1 channel (O’Mahony et al. 2009). Therefore, we investigated the role of berberine-induced kinase activity on the antisecretory effect of berberine. Using specific kinase inhibitors we demonstrated that the antisecretory effect of berberine could be halved by the PKCα inhibitor HBDDE. Pre-treatment with SB202190, a specific inhibitor of p38 MAPK, also significantly decreased the effect of berberine on secretion, although to a much lesser degree. In agreement with these findings, several studies have shown that the phorbol ester PMA inhibits the basolateral K+ conductance of T84 cells and that this effect reduces transepithelial Cl− secretion (Reenstra, 1993; Matthews et al., 1993). The molecular mechanism by which berberine-induced activation of PKCα and p38 MAPK inhibits basolateral KCNQ1 channels in T84 is an important goal of future studies. One possibility to be considered is a change in the phosphorylation state of the channel protein, which is an important determinant of K+ channel activity (Kurokawa et al., 2003; Li et al., 2004; Kathöfer et al., 2003) and which we have shown to mediate the estrogen-induced inhibition of KCNQ1 in rat colonic crypts (O’Mahony et al. 2007).

The present study has shown activation of PKA by berberine. It is well documented that activation of these enzyme lead to an increase in CFTR activity in colonic crypts (Kunzelmann and Mall, 2000; Barrett and Keely, 2000; Schultheiss and Diener, 1998). However, in this study berberine did not affect the apical Cl− current but had an overall inhibitory effect on Cl− secretion. This paradox of activation of PKA without activation of CFTR can be explained if we consider that the cAMP-PKA pathway is tightly regulated at several levels to maintain specificity in the multitude of signal inputs (Tasken and Aandahl, 2004; Cooper, 2005). Ligand-induced changes in cAMP concentration vary in duration, amplitude, and localization into the cell, and cAMP microdomains are shaped by adenylyl cyclases that form cAMP as well as phosphodiesterases that degrade cAMP. Different PKA isozymes with distinct biochemical properties and cell-specific expression contribute to cell and organ specificity. Activated kinase anchoring proteins target PKA to specific substrates and distinct subcellular compartments providing spatial and temporal specificity for mediation of biological effects channelled through the cAMP-PKA pathway (McConnachie et al., 2006).

Therefore, the activation of PKA by berberine does not necessarily imply phosphorylation and activation of CFTR located in the apical membrane. Berberine is freely permeable across human intestinal epithelia and its absorption is significantly increased by P-glycoprotein inhibitors (Pan et al. 2002). It is possible that berberine interacts with a known receptor to effect its anti-secretory action. Recently berberine has been shown to affect the activity of sex steroid receptors including the androgen receptor (agonist) in prostate cancer cells (Li et al. 2011) and the pregnane X receptor (agonist) in HepG2 cells (Yu et al. 2011). Berberine has been shown to enhance the anticancer effect of estrogen receptor antagonists on human breast cancer cells (Liu et al. 2009). The only known receptor-mediated inhibition of KCNQ1 channels in intestine is the estrogen receptor (O’Mahony et al. 2007). 17-B estradiol (E2) inhibits Cl− secretion via PKC and PKA dependent phosphorylation of the KCNQ1:KCNE3 K+ channels in colonic crypts and berberine displays a remarkably similar mechanism of anti-secretory action to E2 (both inhibit KCNQ1 channels by protein kinase phosphorylation and neither molecule inhibits CFTR). It is possible that berberine modulates estrogen receptor signaling in intestinal cells to inhibit KCNQ1 channels similar to E2. The chemical structure of berberine is similar to the novel GPR30 receptor agonist G-1 and this orphan estrogen receptor may also be a target of
berberine. A hypothetical schema of the anti-secretory action of berberine in intestinal epithelium is presented in figure 8.

The ability of berberine to inhibit salt and water secretion from native intestinal tissue, as evidenced by blockade of stimulated $I_{SC}$ in rabbit and rat colon in vitro, raises the distinct possibility that berberine and related compounds may display utility in the clinical treatment of secretory diarrhoeas. High doses of berberine have already been administered orally to humans (Rabbanii et al., 1987; Tang and Eisenbrand, 1992), and can be therapeutically safe over the long term. In support of this view, we found that berberine had no detectable effect on the transepithelial resistance of T84 monolayers over a 2-hour treatment period. Thus, berberine may inhibit basal and cAMP-induced fluid secretion from intestine without affecting the net transport and absorption of nutrient substrates or absorptive capacity. In fact, berberine has been shown to stimulate Na$^+$ absorption (Tai et al., 1981). Unlike most antidiarrheals, berberine exhibits several pharmacological actions useful to the treatment of secretory diarrhoea. Firstly, it reduces the cause of infection via its antimicrobial activity. Secondly, berberine increases stool transit time through the intestine via its antimotility action. Finally, as shown here, berberine decreases basolateral membrane K$^+$ recycling, thus reducing the electrical driving force for whole secretory process in the enterocytes.

Other laboratories have also explored the use of K$^+$ channel blockers to inhibit epithelial Cl$^-$ secretion. The antifungal antibiotic, clotrimazole, has been shown to prevent fluid and electrolyte secretion in rabbit and mouse intestine triggered by Ca$^{2+}$- and cAMP-mediated agonists via specific inhibition of basolateral K$^+$ conductances (Rufo et al., 1997). Furthermore, levamisole (and other phenylimidazothiazoles) inhibits Ca$^{2+}$- and cAMP-activated Cl$^-$ secretion in T84 cell monolayers and isolated human distal colon, apparently by blocking basolateral K$^+$ channels (Mun et al., 1998). Greger and colleagues noted that a group of chromanol compounds selected from agents screened for the ability to block $I_{SC}$ in isolated rabbit colon turned out to be ineffective as Cl$^-$ channel blockers, but effective as blockers of K$^+$ channel activity elicited by cAMP-dependent agonists. The most potent of these was chromanol 293B (Lohrmann et al., 1995), which here was shown to block KCNQ1 channels in T84 cells. Chromanol 293B has been described to block CFTR currents when expressed in Xenopus oocytes (Bachmann et al. 2001) but this has not been observed in native epithelia. In our study, the KCNQ1 inhibitors, chromanol 293B and HMR-1556 were added from the basolateral side without affecting apical Cl$^-$ currents. The inhibitory effects of these molecules on transepithelial Cl$^-$ secretion is indirect via block of KCNQ1 channels. Like chromanol 293B, berberine also reduced the basolateral K$^+$ current activated by cAMP-dependent agonists. Our finding that berberine has a marked inhibitory effect on basolateral KCNQ1 channel current in T84 cells highlights this component of the intestinal Cl$^-$ secretory process as a target for new antidiarrhoeal strategies. Identification of the mechanism of kinase regulation of basolateral K$^+$ channels in colonic epithelia and a better understanding of the signalling processes involved may provide a basis for the development of new antisecretory drugs with greater potency and specificity.

In summary, based on the results of our previous studies in rat colon in vitro and the present studies in T84 cells monolayers, we propose that the effect of berberine on chloride ion secretion in intestine is mediated by a protein kinase dependent reduction in basolateral KCNQ1 channel activity.
Acknowledgements
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References


**Table 1.** Effect of berberine on transepithelial resistance.
Transepithelial resistance ($R_t$) was calculated using Ohms law by measuring the $I_{SC}$ resulting from 5 seconds square voltage pulses (2 or 4 mV) imposed across the monolayer.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Vehicle</th>
<th>Berberine 100 μM</th>
<th>Berberine 300 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$1302 ± 212$</td>
<td>$1235 ± 205$</td>
<td>$1301 ± 182$</td>
</tr>
<tr>
<td>1</td>
<td>$1287 ± 234$</td>
<td>$1198 ± 192$</td>
<td>$1246 ± 235$</td>
</tr>
<tr>
<td>2</td>
<td>$1176 ± 187$</td>
<td>$1099 ± 210$</td>
<td>$1155 ± 211$</td>
</tr>
</tbody>
</table>

Values are mean ± SEM; $n = 8$ for each group.

**Table 2.** Berberine effect on membrane conductances.
Forskolin (10 μM) was added to stimulate CFTR and cAMP-dependent $K^+$ conductance. Berberine (300 μM) was added to the basolateral bath. The berberine effect was measured 15 minutes after the drug addition.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$I_{Cl}$ (μA/cm²)</th>
<th>$I_{Na}$ (μA/cm²)</th>
<th>$I_{K}$ (μA/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>14 ± 2</td>
<td>16 ± 3</td>
<td>53 ± 4</td>
</tr>
<tr>
<td>Basal + berberine</td>
<td>12 ± 3</td>
<td>13 ± 3</td>
<td>9 ± 2 **</td>
</tr>
<tr>
<td>Forskolin</td>
<td>88 ± 12</td>
<td>_</td>
<td>94 ± 2</td>
</tr>
<tr>
<td>Forskolin + berberine</td>
<td>84 ± 10</td>
<td>_</td>
<td>15 ± 4 **</td>
</tr>
</tbody>
</table>

$n = 6$ for each group, **$P < 0.05$
Figure Legends

Figure 1. Effect of berberine on forskolin-induced Cl⁻ secretion. (A) Representative short-circuit current recording of the effect of berberine (300 µM) on forskolin-stimulated $I_{SC}$ in T84 cell monolayers. Drugs were added at arrow and remained in the bathing solutions throughout the experiment (B) Concentration-response curve for the antisecretory effect of berberine on forskolin-stimulated $I_{SC}$ in T84 cell monolayers. Change in $I_{SC}$ measured 15 min post berberine application at different concentrations. Values are mean ± S.E.M., n = 8 for each point.

Figure 2. Effect of berberine on basolateral K⁺ current ($I_K$) across apically permeabilized T84 cell monolayers. (A) Effect of KCNQ1 channel inhibitors on $I_K$ in T84 cell monolayers. Amphotericin B (10 µM) was added to apical bath after ouabain (100 µM) was added to basolateral bath. BaCl₂ (2 mM), chromanol 293B (10 µM) or HMR-1556 (500 nM) were added to the basolateral bath. Values were measured at the point of maximal inhibition of $I_K$ by each inhibitor. (B) Short-circuit currents recording of the effect of berberine (300 µM, filled circles) on $I_K$ across T84 cells monolayers compared to a vehicle control (DMSO 0.05%, open circles). Drugs were added at arrow and remained in the bathing solutions throughout the experiment. Values are means ± S.E.M., **P < 0.01 compared to controls, n = 6 for each group. (C) The concentration dependence of the inhibitory effect of berberine on basolateral membrane K⁺ current showed a half-maximal inhibition at 150 µM.

Figure 3. (A) Current/voltage relationships illustrating the effect of berberine (100 µM) on the increase in whole-cell current stimulated by forskolin (10 µM) in T84 cells. Traces are control (○), forskolin (■), and forskolin + berberine (▲). Data are mean ± SEM (*P < 0.01 between the forskolin-stimulated current and berberine, n = 7). (B) Current/voltage relationships illustrating the effect of chromanol 293B (10 µM) on the increase in whole-cell current stimulated by forskolin (10 µM) in T84 cells. Traces are control (○), forskolin (■), and forskolin + chromanol 293B (▲). Data are mean ± S.E.M. (*P < 0.01 between the forskolin-stimulated current and chromanol 293B, n = 3).

Figure 4. Berberine effect on PKC isoforms and PKA activity in T84 cells. (A) Representative blot of PKCα phosphorylation levels at Ser⁶⁵⁷ in cellular extracts from T84 cells after berberine treatment (300 grmuM) for 5 and 10 min compared to vehicle controls. (B) Representative blot of PKCδ phosphorylation levels at Ser⁶⁴³ in cellular extracts from T84 after berberine treatment (300 grmuM) cells for 5 and 10 min compared to vehicle controls. grbeta-Actin was used as an internal control for protein loading. The graphs represent densitometric analysis of PKC blots or PKA activity assays. Values are given as fold changes in PKC phosphorylation (activation) or PKA activity respect to an untreated control. Values are displayed as mean ± S.E.M. (n = 3 for each group, **P < 0.01).

Figure 5. Berberine effect on MAPK signalling in T84 cells. (A) Representative blot of p38 MAPK phosphorylation levels at Thr¹⁸⁰/Tyr¹⁸² in cellular extracts from T84 cells after berberine treatment (300 µM) for 5 and 10 min compared to vehicle controls. (B) Representative blot of p42/p44 MAPK phosphorylation levels at Thr²⁰²/Tyr²⁰⁴ in cellular extracts from T84 cells after berberine treatment (300 grmuM) for 5 and 10 min compared to vehicle controls. T84 cells were treated with EGF (100 ng·ml⁻¹) for 5 min to stimulate p42/p44 MAPK phosphorylation. PD98059 (20 grmuM) was added 15 min prior EGF-treatment. B-Actin was used as an internal control for protein loading. The graphs represent...
densitometric analysis of blots. Values are given as fold changes in MAPK phosphorylation (activation) respect to an untreated control. Values are displayed as mean ± S.E.M. (n = 3 for each group, *P < 0.05, ***P < 0.001).

**Figure 6.** Role of protein kinases on the antisecretory effect of berberine. (A) Short-circuit current recordings in T84 cell monolayers of the effect of protein kinases inhibitors on the antisecretory effect of berberine (300 μM). Cells were pre-incubated for 15 min with HBDDE 100μM (▲), SB202190 10μM (○) or vehicle DMSO 0.05% (●). (B) Mean ± SEM (n = 6) change in total $I_{SC}$ caused by preincubation with several kinase inhibitors on the antisecretory effect of berberine on forskolin-stimulated $I_{SC}$. Values are mean ± S.E.M., n = 6, *P < 0.05, **P < 0.01 compared to controls.

**Figure 7.** Berberine effect on protein kinase association with KCNQ1 channel. (A) Representative blot of PKCα association with the KCNQ1 channel in response to berberine (300 grmuM) in T84 cells. (B). Representative blot of PKACI association with the KCNQ1 ion channel in response to berberine (300 grmuM) in T84 cells. Total KCNQ1 pools were immunoprecipitated from total cellular lysates using an antibody specific to KCNQ1. Associated PKCα and PKACI were quantified by Western blot analysis. The graphs represent densitometric analysis at specific time points of berberine treatment compared to vehicle controls. Values are given as fold changes in kinase association with KCNQ1 respect to an untreated control. Values are displayed as mean ± S.E.M. (n = 3 for each group, *P < 0.05, ***P < 0.001).

**Figure 8.** Hypothetical schema of the anti-secretory action of berberine in intestinal epithelium. Berberine activates p38 MAPK, PKCα and PKA to phosphorylate the KCNQ1:KCNE3 K$^+$ channel causing the channel to become inactive either through closure or removal from the plasmamembrane. Inhibition of basolateral K$^+$ channel reduces the electrical driving force for Cl$^-$ exit across the apical membrane through CFTR and Calcium-activated Cl$^-$ channels. The receptor transducing the response to berberine may be the estrogen receptor or GPR30/GPCR as previously described for similar anti-secretory and rapid protein kinase responses to estrogen in the intestine.
Figure 5: (A) Bar graph showing p38 MAPK activation A.U. over time with Berberine and Vehicle treatments. (B) Bar graph showing p42/44 MAPK activation A.U. with various treatments over time.