ORIGINAL ARTICLE

Farnesoid X receptor agonists attenuate colonic epithelial secretory function and prevent experimental diarrhoea in vivo

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ABSTRACT

Objective Bile acids are important regulators of intestinal physiology, and the nuclear bile acid receptor, farnesoid X receptor (FXR), is emerging as a promising therapeutic target for several intestinal disorders. Here, we investigated a role for FXR in regulating intestinal fluid and electrolyte transport and the potential for FXR agonists in treating diarrhoeal diseases.

Design Electrogenic ion transport was measured as changes in short-circuit current across voltage-clamped T_{84} cell monolayers or mouse tissues in Ussing chambers. NHE3 activity was measured as BCECF fluorescence in Caco-2 cells. Protein expression was measured by immunoblotting and cell surface biotinylation. Antidiarrhoeal efficacy of GW4064 was assessed using two in vivo mouse models: the ovalbumin-induced diarrhoea model and cholera toxin (CTX)-induced intestinal fluid accumulation.

Results GW4064 (5 μmol/L; 24 h), a specific FXR agonist, induced nuclear translocation of the receptor in T_{84} cells and attenuated Cl^- secretory responses to both Ca^{2+} and cAMP-dependent agonists. GW4064 also prevented agonist-induced inhibition of NHE3 in Caco-2 cells. In mice, intraperitoneal administration of GW4064 (50 mg/mL) also inhibited Ca^{2+} and cAMP-dependent secretory responses across ex vivo colonic tissues and prevented ovalbumin-induced diarrhoea and CTX-induced intestinal fluid accumulation in vivo. At the molecular level, FXR activation attenuated apical Cl^- currents by inhibiting expression of cystic fibrosis transmembrane conductance regulator channels and inhibited basolateral Na^+/K^+-ATPase activity without altering expression of the protein.

Conclusions These data reveal a novel antisecretory role for the FXR in colonic epithelial cells and suggest that FXR agonists have excellent potential for development as a new class of antidiarrheal drugs.

Significance of this study

What is already known about this subject?
- Although diarrhoeal diseases are common and debilitating conditions, safe and effective drugs for their treatment are still lacking.
- Diarrhoeal diseases primarily occur as a consequence of dysregulated epithelial ion transport, with increased Cl^- secretion being the main driving force for fluid secretion into the intestine.
- The nuclear bile acid receptor, farnesoid X receptor (FXR), is emerging as an important regulator of intestinal function, and is generating a great deal of interest as a new therapeutic target. However, its role in regulating intestinal fluid and electrolyte transport is still unknown.

What are the new findings?
- Agonists of the FXR inhibit Cl^- secretion across colonic epithelial cells in vitro and in vivo.
- FXR agonists prevent intestinal fluid secretion and the occurrence of diarrhoea in mouse models of the disease.
- At the molecular level, FXR agonists exert their antisecretory actions by inhibiting the activity and expression of key transport proteins of the epithelial secretory pathway.

How might it impact on clinical practice in the foreseeable future?
- FXR agonists are already under development for treatment of liver and intestinal diseases, and here we show that they also have excellent potential for development as a new class of antidiarrhoeal drug.
- Because FXR agonists exert their antidiarrhoeal actions at the level of epithelial fluid and electrolyte transport, they may be associated with broader efficacy and fewer side effects than currently available drugs.

INTRODUCTION

Intestinal disorders associated with diarrhoea remain a global problem. According to WHO, there are about 2 billion cases of diarrhoea each year, with their occurrence being most common in developing countries due to the prevalence of infectious disease.[1] Although rarely fatal, epidemics of acute infectious diarrhoea are also common in Western societies,[2] as are intestinal disorders associated with chronic diarrhoea, including inflammatory bowel diseases (IBD), digestive disorders and irritable bowel syndrome (IBS).[3] Such conditions have significant negative impacts on patient quality of life, and represent a huge financial burden to society in terms of healthcare and lost hours of work. However, despite their prevalence, there are still limited options for treating diarrhoeal disease,

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and no drugs are currently available which directly target the epithelial transport processes underlying dysregulated fluid movement.

Intestinal fluid movement is driven by osmotic gradients established by epithelial ion transport. While fluid absorption is primarily driven by cation absorption, secretion is driven by anions, predominantly Cl^-. The molecular pathway for Cl^- secretion has been well-elucidated and is typically stimulated by neuroimmune agonists that elevate intracellular levels of the prosecretory second messengers, Ca^{2+} and cAMP.[4] Cl^- enters epithelial cells, along with Na^+ and K^+, through $Na^+/K^+/2Cl^-$-cotransporters (NKCC1) in the basolateral membrane. The energy to drive this process comes from the activity of Na^+/K^+-ATPase pumps, which pump Na^+ from the cell in exchange for K^+. K^+ does not accumulate intracellularly since it exits through the basolateral channels, KCNN4 and KCNQ1. Thus, the combined activity of these basolateral transport proteins serves to create an electrochemical driving force for Cl^- secretion through channels in the apical membrane, the most characterised of which is the cystic fibrosis transmembrane conductance regulator (CFTR). Molecular mechanisms that regulate the activity and expression of these transport proteins are of great interest since they provide excellent targets for the development of new anti-diarrhoeal therapies.

Bile acids are currently receiving a great deal of interest as targets for the development of new therapeutics for intestinal disease.[5 6] The primary bile acids, chenodeoxycholic acid (CDCA) and cholic acid (CA), are synthesised in the liver and stored in the gallbladder in their glycine- or taurine-conjugated forms. Upon ingestion of a meal, they are released into the proximal small intestine where they perform their classical functions in facilitating lipid digestion and absorption. Most of the released bile acids are reabsorbed in the terminal ileum and returned to the liver via the portal circulation. However, with each cycle of the enterohepatic circulation (EHC), approximately 5% of circulating bile acids enter the colon, where they are metabolised to secondary bile acids, the most common of which is deoxycholic acid (DCA).[7] In the colon, bile acids act as hormones that regulate many aspects of epithelial physiology, including cell growth, mucus production, barrier function and production of inflammatory cytokines.[8–11] A growing body of evidence suggests that a nuclear receptor for bile acids, the farnesoid X receptor (FXR), is a critical mediator of epithelial responses to bile acids. This has led to a great deal of interest in therapeutically targeting the FXR, and indeed, synthetic agonists of the receptor have been shown to exert protective effects in an animal model of colitis.[12]

Bile acids are also important regulators of intestinal fluid and electrolyte transport.[13] In conditions associated with bile acid malabsorption, their delivery to the colon at high concentrations induces fluid and electrolyte secretion,[14] thereby causing diarrhoea.[15 16] However, we have recently shown that, at physiologically relevant concentrations, bile acids chronically downregulate colonic secretory function, an effect that may serve to facilitate normal colonic absorptive function.[17] Here, we set out to examine the role of the FXR in regulating colonic epithelial fluid and electrolyte transport, and the potential for targeting FXR in treatment of diarrhoeal disease.

MATERIALS AND METHODS

Cell culture

T_{84} colonic epithelial cells were cultured as previously described.[18] For electrophysiological measurements, approximately 5×10^5 cells were seeded onto 12 mm Millicel-HA Transwells (Millipore, Bedford, Massachusetts). For western blotting, approximately 10^6 cells were seeded onto 30 mm Millicel-HA Transwells. Cells were cultured on Transwells for 10–28 days and under these conditions, they developed the polarised, electrically resistant phenotype of native colonic epithelia. GW4064 (Tocris Bioscience, Bristol, UK) or obeticholic acid (OCA; gifted from Intercept Pharmaceuticals, New York) were used to activate the FXR.[19] Prior to treatment, T_{84} monolayers were equilibrated in serum-free medium for 2 h, and unless otherwise noted, were treated bilaterally with FXR agonists for 24 h prior to experimentation.

Animal studies

Male C57BL/6 mice (6–9 weeks old) were maintained in an environmentally controlled facility at Beaumont Hospital. GW4064 (50 mg/kg), prepared in a DMSO/corn oil mix (1 : 10, v/v), was injected intraperitoneally. The control group received vehicle alone. Mice were sacrificed 24 h later by cervical dislocation, their distal colon or jejunum was removed, and ion transport was measured in Ussing chambers. All experiments were approved by the RCSI Research Ethics Committee.

The mouse model of allergic diarrhoea was based on a previously published protocol.[20] Male BALB/c mice were sensitised twice, 2 weeks apart, by intraperitoneal injection of 50 mg ovalbumin (OVA) with 1 mg aluminium potassium sulfate adjuvant. Two weeks later, mice were fasted for 4 h and then administered 50 mg OVA, or PBS vehicle, by oral gavage. Mice were challenged with OVA in this way every second day for 2 weeks. Mice were treated with intraperitoneal GW4064 (50 mg/kg), or vehicle, 24 h before each oral OVA challenge. Mice demonstrating profuse liquid stool were recorded as being diarrhoea-positive.

Measurements of cholera toxin (CTX)-induced intestinal fluid accumulation were carried out as previously described.[21] Briefly, male BALB/C mice, weighing 23–26 g, were fasted overnight with free access to water. GW4064 (50 mg/kg) was administered intraperitoneally 24 h and 1 h before CTX. Mice were then treated by oral gavage with CTX (10 mg in 7% $NaHCO_3$, 0.1 ml/mouse). After 6 h mice were euthanased by cervical dislocation, the small intestine (pylorus to the ileocecal junction) was removed, and luminal fluids were collected.

Electrophysiological measurements

For measurements of transepithelial Cl^- secretion, T_{84} cell monolayers or mouse distal colon, stripped of underlying smooth muscle, were mounted in Ussing chambers (aperture=0.6 cm^2 or 0.3 cm^2, respectively) and bathed in Ringer's solution containing (in mmol/L): 40 Na^+, 5.2 K^+, 1.2 Ca^{2+}, 1.2 Mg^{2+}, 119.8 Cl^-, 25 HCO_3^-, 0.4 $H_2PO_4^-$, 2.4 HPO_4^{2-}, and 10 glucose. Tissues were voltage-clamped to zero potential difference and monitored for changes in short circuit current (ΔI_{sc}). Agonist-induced responses were expressed as ΔI_{sc} ($\mu A/cm^2$).

Jejunal Na^+-glucose cotransport was measured as previously described.[22] Intact (unstripped) tissues were mounted in Ussing chambers (aperture=0.5 cm^2) and bathed in modified Ringer's solution, in which 10 mmol/L glucose was substituted basolaterally with 5 mmol/L glucose and apically with 5 mmol/L mannitol. Na^+-glucose cotransport was stimulated by apical addition of 25 mmol/L glucose, with mannitol (25 mmol/L) being added basolaterally to maintain iso-osmolarity.

Na^+/K^+-ATPase activity was measured as previously described.[18] Briefly, T_{84} cell monolayers were mounted in Ussing chambers and bathed bilaterally in low-sodium

(25 mmol/L) Ringer's solution, with NaCl substituted by equimolar N-methyl-D-glucamine (NMDG)-Cl^-. Apical membranes were permeabilised with amphotericin B (50 μmol/L). Under these conditions, changes in I_{sc} reflect electrogenic transport through Na^+/K^+-ATPase pumps, as reflected by their sensitivity to ouabain (10 μmol/L).

Measurements of cAMP-dependent Cl^- currents were carried out using a protocol described by Hallows and colleagues.[23] Basolateral membranes were permeabilised with nystatin (100 μg/mL), and the basolateral Ringer's solution was replaced with a low Cl^- solution, containing 25 mmol/L NaCl and 95 mmol/L Na^+-gluconate, to establish an apical-to-basolateral Cl^- concentration gradient. An apical-to-basolateral gradient is usually employed in these experiments in order to prevent cell swelling. Under these conditions, changes in I_{sc} after stimulation with forskolin (FSK) reflect CFTR-mediated currents I^{Cl}(apical).

Western blotting

Measurements of protein expression were carried out as previously described.[24] Antibodies used in the current studies were: rabbit anti-FXR (1 : 1000 dilution; Santa Cruz, Santa Cruz, California), mouse anti-Na^+/K^+-ATPase α subunit (1 : 1000 dilution or 1 : 500 dilution for biotinylation studies), or mouse anti-β subunit (1 : 1000 dilution; Abcam, Cambridge, Massachusetts), and mouse anti-CFTR, clone M3A7 (1 : 500 dilution and 1 : 300 dilution for biotinylation studies; Millipore, Billerica, Massachusetts). Protein expression was quantified by densitometry.

Surface protein expression

Cell monolayers were washed with PBS and then incubated with Ez-link Sulfo-NHS-Biotin solution (1 mg/mL in PBS; Thermo Fisher Scientific, Waltham, Massachusetts), followed by incubation with 100 mmol/L glycine for 15 min, to quench the reaction. Cells were lysed and, after adjusting for protein content, lysates were incubated overnight on a rotator at 4°C with streptavidin-agarose beads. Samples were centrifuged, the supernatant removed, and the agarose beads were washed with lysis buffer. The beads were then resuspended in 2× loading buffer, and samples were analysed by western blotting.

Extraction of nuclear proteins

Treated cells were scraped into ice-cold buffer A (10 mmol/L Hepes (pH=7.9), 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, Complete Protease Inhibitor Cocktail, 0.5 mmol/L PMSF, and 0.5 mmol/L DTT) and cell suspensions were centrifuged, resuspended in buffer B (buffer A supplemented with 0.1% NP-40), and incubated for 10 min. Samples were centrifuged, and supernatants containing cytosolic proteins were collected. Pellets were washed in buffer A, centrifuged, resuspended in buffer C (20 mmol/L Hepes (pH=7.9), 420 nmol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 25% glycerol, Complete Protease Inhibitor Cocktail, 0.5 mmol/L PMSF, and 0.5 mmol/L DTT), and incubated on ice for 10 min. Samples were centrifuged, and the supernatant containing nuclear proteins was collected. FXR levels in cytosolic and nuclear fractions were then analysed by western blotting.

Na^+/H^+ exchange activity

Na^+/H^+ exchange activity in polarised Caco-2/bbe cells expressing Adeno-HA-NHE3 was determined using the intracellular pH-sensitive dye BCECF, as previously described.[25] Cells were treated with GW4064 (5 μmol/L) for 24 h prior to conducting the assay. Initial rates of Na^+-dependent intracellular alkalinisation (efflux of H^+, in micromoles per second) in response to carbachol (10 μM) were calculated for a given pH over ~1 min during the initial linear period of intracellular alkalinisation. Data were expressed as ΔpH/ΔT.

mRNA expression

RNA was extracted using RNeasy Mini-Kits (Roche Diagnostics), according to the manufacturer's protocol. cDNA was synthesised by reverse transcription using the Improm-II Reverse Transcription kit (Promega, Madison, Wisconsin), and was then used for PCR reactions using GoTaq green master mix (Promega). The sequences of primers used were: FGF19 (forward: 5′- GAGGACCA-AAACGAACGAAATT-3′, reverse 5′-ACGTCCTTGATGGCAAT-CG-3′, 18S rRNA forward 5′- GTCCCCCAACTTCTTAGAG-3′ reverse 5′-CACCTACGGAAACCTTGTTAC-3′ (Invitrogen, Carlsbad, California).

ATP levels

Cell monolayers were lysed in hypotonic lysis buffer (100 mmol/L Tris base, 2 mmol/L EDTA, pH adjusted to 7.5 with acetic acid), and ATP levels were measured using a commercially available kit (ENLITEN ATP Assay System, Promega). ATP levels were normalised to total protein concentration.

Statistical analyses

Data are expressed as mean±SE for a series of *n* experiments. Paired t tests, or one-way analysis of variance (ANOVA) with the Student Neuman–Keuls post-test were employed as appropriate. p Values ≤0.05 were considered to be statistically significant.

RESULTS

FXR activation inhibits Cl^- secretion in T_{84} cells

The FXR agonist, GW4064 (5 μmol/L), significantly increased nuclear localisation of FXR to 369±70% of that in control cells after 6 h (n=4; p<0.05) (figure 1A and B). Confocal analysis of GW4064-treated cells confirmed increased FXR staining in the nucleus (figure 1C). Furthermore, treatment of T_{84} cell monolayers with GW4064 (5 μmol/L) robustly increased expression of the FXR target gene, FGF19 (figure 1D), demonstrating that the agonist effectively activates FXR in these cells.

To determine the effects of FXR activation on colonic epithelial secretory function, T_{84} cell monolayers were treated with GW4064 (5 μmol/L; 24 h), mounted in Ussing chambers, and Cl^- secretion was stimulated with the prototypical, Ca^{2+} and cAMP-dependent agonists, CCh (100 μmol/L) and FSK (10 μmol/L), respectively. GW4064 significantly reduced CCh- and FSK-stimulated currents to 38±6% (n=9; p<0.01) and 52±5% (n=9; p<0.001) of control responses, respectively (figure 2A and B). Antisecretory effects of GW4064 were observed at concentrations as low as 0.1 μmol/L (figure 2B) and were apparent after 12 h of treatment (figure 2C). In similar experiments, FXR activation also decreased Cl^- secretion induced by the naturally occurring secretagogues, DCA (500 μmol/L) and cholera toxin (CTX; 0.1 μg/mL) to 64±10% (n=8; p<0.05) and 81±6% (n=7; p<0.05) of controls, respectively. The effects of GW4064 were not due to cellular toxicity as it did not alter transepithelial resistance (TER), nor did it cause lactate dehydrogenase (LDH) release, which in GW4064 (5 μmol/L)-treated cells was 107±10% of that in controls (n=4). Furthermore, antisecretory actions were not specific to GW4064, since a structurally distinct FXR agonist, OCA (6-ethyl-CDCA), also attenuated secretory responses to CCh and FSK (figure 2D).

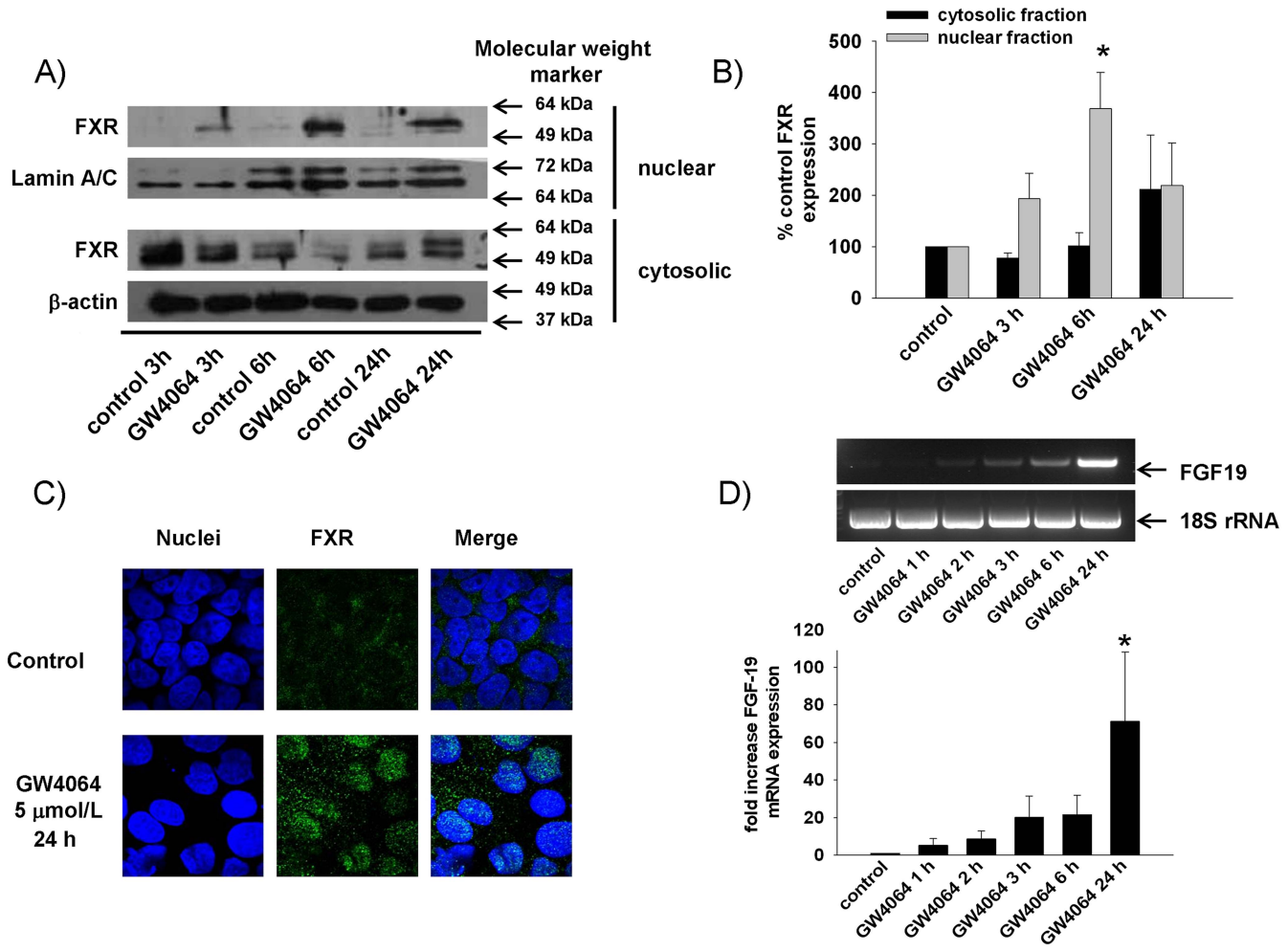

Figure 1 GW4064 activates FXR in colonic epithelial cells. (A) Monolayers of T_{84} cells were treated with GW4064 (5 μmol/L) and FXR expression in nuclear and cytosolic fractions was measured after 3, 6 or 24 h. FXR expression in the nuclear fraction was normalised to lamin A/C expression and FXR expression in cytosolic fraction was normalised to β-actin (n=4). Panel (B) shows densitometric analysis of 4 similar experiments. (C) Confocal analysis confirmed that GW4064 caused translocation of FXR from the cytosol to the nucleus after 24 h of treatment (image is representative of 3 similar experiments). (D) RT-PCR analysis revealed that the FXR target gene, FGF19, was significantly increased after GW4064 treatment (n=5). *p<0.05.

We also examined effects of FXR activation on electrogenic Na^+ absorption through ENaC, which represents the major route for Na^+ absorption in human distal colon. Since there are no cellular models available that are suitable for measurements of intestinal epithelial ENaC activity, we used a previously described model, in which treatment of T_{84} cells with 4-phelylbutyrate (5 mmol/L; 24 h) reliably induces expression of the channel.[26] We also found 4-phelylbutyrate to induce expression of the ENaCα subunit (data not shown) in T_{84} cells and amiloride-sensitive currents in T_{84} cells. However, these currents were unaffected by cotreatment of the cells with GW4064 (n=4; figure 2E).

Sodium–hydrogen exchange (NHE) is also an important mechanism of fluid absorption throughout the intestine and, in humans, is mediated predominantly by apical NHE3. Using a well established Caco-2 cell model,[25] we found that treatment with GW4064 did not significantly alter basal NHE3 activity, but prevented inhibition of the transporter by CCh (figure 2F).

FXR activation inhibits fluid and electrolyte secretion in vivo

To test effects of FXR activation on epithelial transport in a more physiological setting, mice were treated by intraperitoneal injection with GW4064 (50 mg/kg) and after 24 h, colonic tissues were mounted in Ussing chambers. Transmucosal resistance was similar in colonic tissues from control and GW4064-treated mice (data not shown). However, Cl^- secretory responses to CCh and FSK in tissues from treated mice were significantly reduced to 79±6 (p<0.001) and 77±7% (p<0.01) of those in control tissues, respectively (n=11; figure 3A). By contrast, Na-glucose cotransport, stimulated by apical addition of glucose (25 mmol/L) to jejunal tissues from these mice, was unaltered by GW4064 treatment (figure 3B).

Next, were examined the effects of GW4064 in two different mouse models of diarrhoeal disease. In the first model of allergic disease, oral challenge with ovalbumin-induced severe diarrhoea in 100% of presensitised mice, as evidenced by the lack of solid stool in the colons of these mice compared with controls (figure 3C). By contrast, in mice pretreated with GW4064 (50 mg/kg), diarrhoea occurrence was markedly attenuated and solid stool pellets could be clearly observed in the colons from these animals (n=10). In the second model, we found that GW4064 also prevented the prosecretory effects of CTX in an in vivo model of intestinal fluid accumulation (figure 3D). In mice treated with CTX alone, fluid accumulation in the small

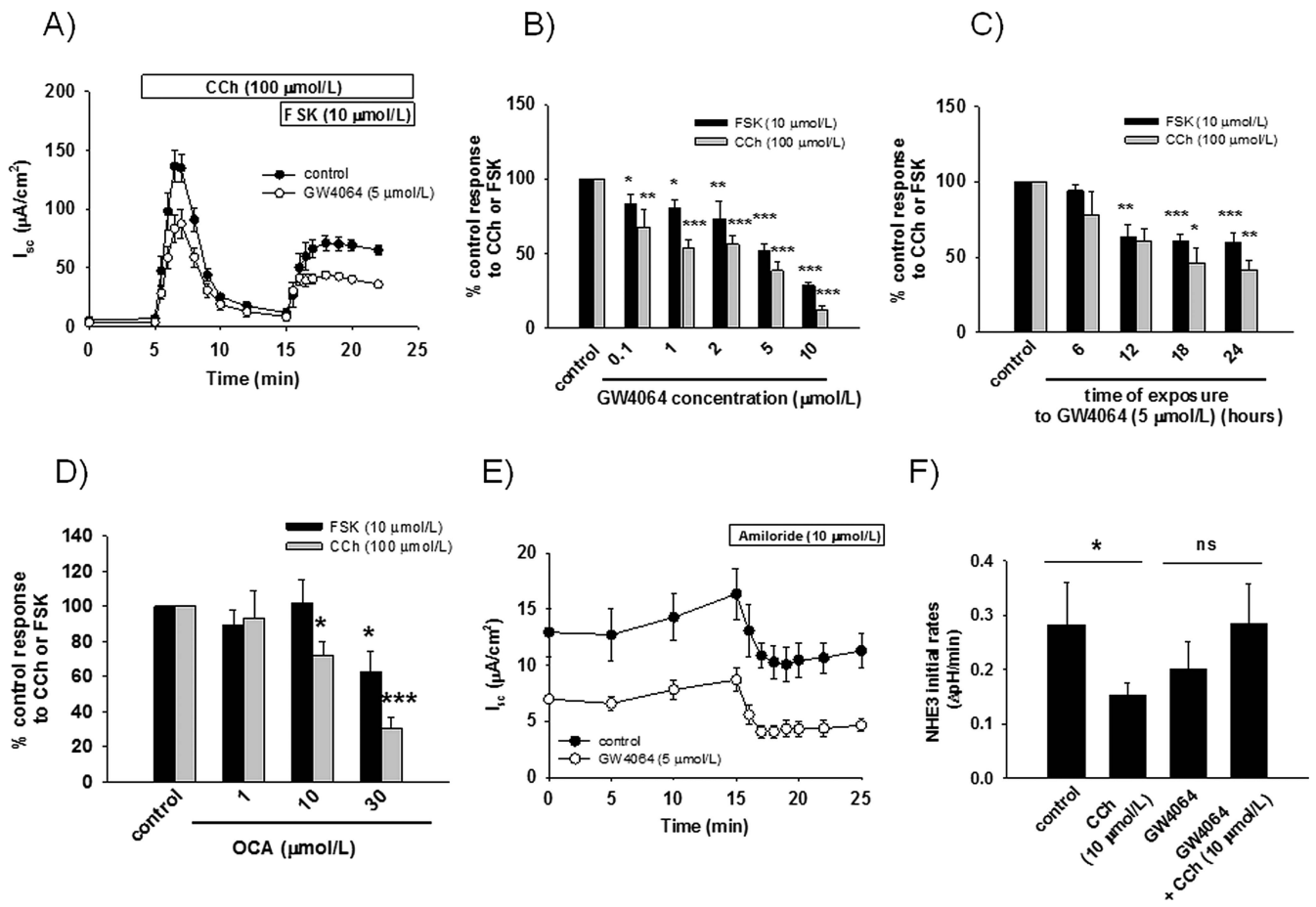

Figure 2 FXR activation inhibits colonic epithelial Cl^- secretion in vitro. (A) Monolayers of T_{84} cells were treated with GW4064 (5 µmol/L) for 24 h before Cl^- secretory responses to CCh (100 µM; basolateral) and FSK (10 µM, apical) were measured in Ussing chambers (n=8). (B) T_{84} cells were exposed to GW4064 at various concentrations for 24 h before Cl^- secretory responses to CCh and FSK were measured (n=3–6). (C) T_{84} cells were exposed to GW4064 (5 µmol/L) for varying periods of time before subsequent I_{sc} responses to CCh and FSK were measured (n=3–7). (D) T_{84} cells were exposed to OCA at various concentrations for 24 h before Cl^- secretory responses to CCh (n=7) and FSK (n=6) were measured. (E) T_{84} cells were treated with GW4064 in presence of sodium 4-phenylbutyrate (5 mmol/L) for 24 h. Cells were then mounted in Ussing chambers and amiloride (10 µM) was added apically to inhibit ENaC (n=4). (F) Monolayers of Caco-2 cells were treated with GW4064 (5 µmol/L; 24 h) and basal and CCh-induced inhibition of NHE3 activity were measured as changes in BCECF fluorescence in response to $NHCl_2$ (50 mmol/L) (n=4). *p<0.05, **p<0.01, ***p<0.001, ns; no significant difference.

intestine was 0.41±0.04 mL, compared to 0.25±0.04 mL in mice pretreated with GW4064 (n=9; p<0.05). Fluid accumulation in untreated control mice was 0.03±0.01 mL (n=3).

FXR activation inhibits Na^+/K^+-ATPase activity and CFTR Cl^- currents in colonic epithelial cells

Having found FXR activation to inhibit colonic fluid and electrolyte secretion in vivo, we returned to our in vitro model to begin to elucidate molecular mechanisms involved. Using a well-established protocol for measuring Na^+/K^+-ATPase transport function,[18] we found that GW4064 inhibited transport activity of the pump to 61±10% of that in control cells (n=6; p<0.05) (figure 4A). Interestingly, this inhibitory effect was not associated with altered expression of either the catalytic α, or regulatory β, subunits of the protein (figure 4B). Furthermore, using a biotinylation approach to label cell surface proteins in the basolateral membrane, we found that membrane expression of the α subunit was not altered in GW4064-treated cells (figure 4C). No β-actin was detected in the biotinylated protein fraction, confirming specific isolation of surface proteins (n=3). Furthermore, cellular ATP, which is required to drive Na^+/K^+-ATPase transport activity, was found to be unaltered by FXR activation, with levels of ATP in GW4064-treated cells being 96 ±13% of those in controls (n=6).

Finally, we examined the effects of FXR activation on apical CFTR channels, which constitute the primary exit pathway for Cl^- in colonic epithelial cells.[23] In these experiments, we found that FSK-induced CFTR currents were attenuated by pretreatment of the cells with GW4064 (5 µmol/L, 24 h) (figure 5A). Interestingly, GW4064 inhibition of CFTR-mediated Cl^- currents was associated with a significant decrease in CFTR protein expression to 33±9% of that in controls (n=4; p<0.001) (figure 5B), while apical surface expression of the protein was reduced to 35±6% of controls (n=4; p<0.001) (figure 5C).

DISCUSSION

There is currently a great deal of research interest in the therapeutic potential of the FXR. Agonists of this nuclear receptor are already in advanced clinical trials for treatment of liver diseases,[27] and they have also been proposed as potential targets for therapy of several other conditions, including obesity, type II diabetes, atherosclerosis and IBD.[12 28] The current studies suggest that, due to their potent antisecretory actions on intestinal epithelial cells, FXR agonists may also be beneficial in

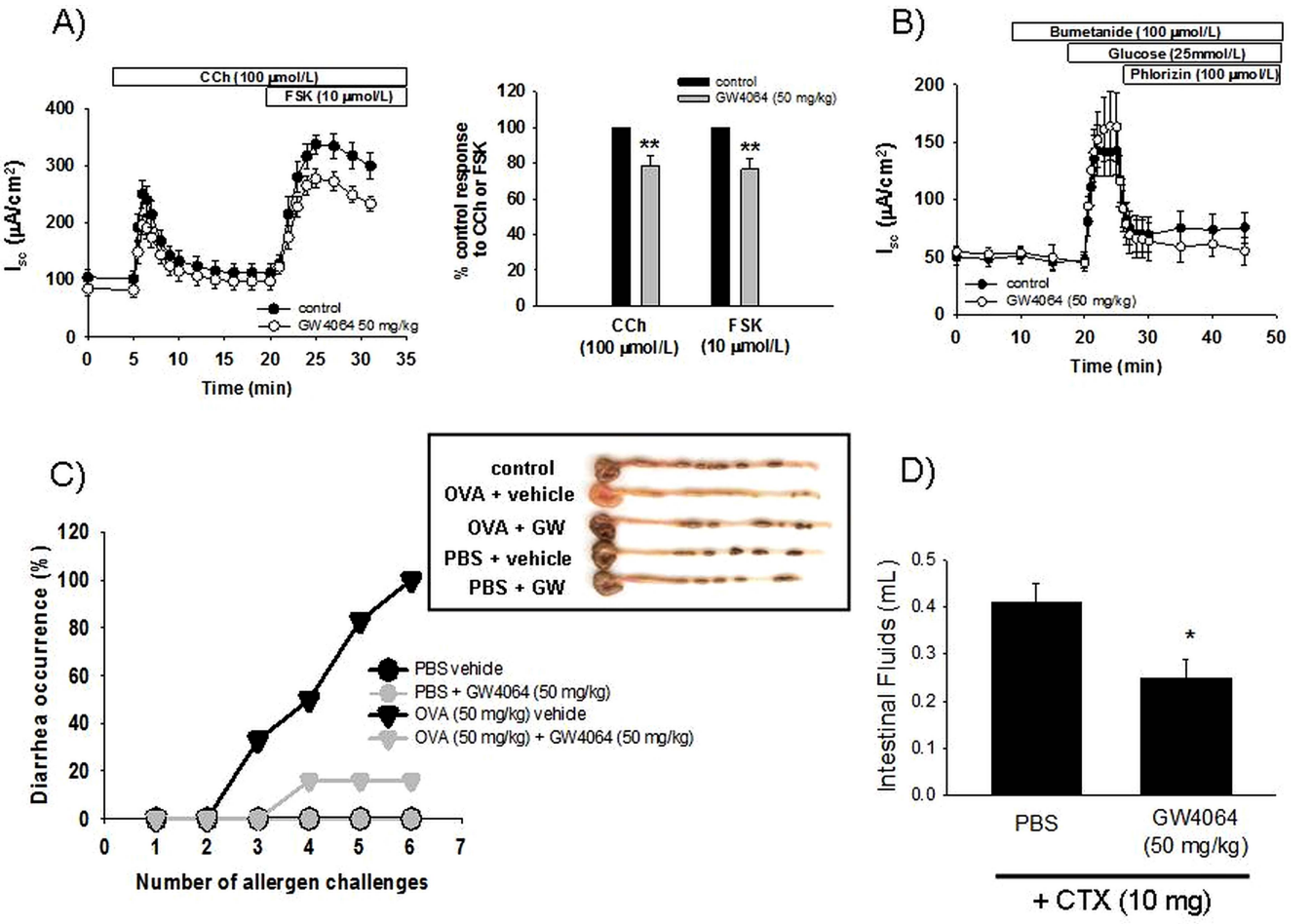

Figure 3 FXR activation inhibits colonic epithelial fluid and electrolyte secretion in vivo. (A) Male C57BL/6 mice were treated with GW4064 (intraperitoneal, 50 mg/kg), or vehicle, for 24 h after which muscle-stripped sections of distal colon were mounted in Ussing chambers for measurements of CCh (100 µmol/L) and FSK (10 µmol/L)-stimulated Cl^- secretion. Amiloride (10 µmol/L) was present throughout to exclude any contribution of ENaC to observed I_{sc} responses. The left panel shows a mean trace of n=11 experiments and the right panel shows maximal responses to CCh and FSK in GW4064-treated tissues as a percent of controls (n=11). (B) Intact sections of mouse jejunum from control or GW4064-treated mice were mounted in Ussing chambers and Na^+-glucose cotransport was stimulated by apical addition of glucose (5 mmol/L). Bumetanide (Bum) (100 µmol/L) was present throughout to exclude the contribution of Cl^- secretion to observed responses. Phlorizin, was added at the end of each experiment to confirm that responses were due to Na^+/glucose exchange through SGLT-1 (n=8). (C) Ovalbumin-sensitised male Balb/C mice were treated with GW4064 (intraperitoneal, 50 mg/kg) or vehicle, and after 24 h were challenged by oral gavage with ovalbumin (50 mg). Mice were treated in this way every 2 days, and those demonstrating profuse liquid stool within 1 h after OVA challenge were recorded as being diarrhoea-positive. The graph shows diarrhoea occurrence as a percent of the number of mice in each group (n=10), while the inset shows a representative image of colons from each group. Note the lack of fecal pellets in ovalbumin-treated mice, indicating diarrhoea, compared to those pretreated with GW4064. D) Male Balb/C mice were treated with GW4064 (intraperitoneal, 50 mg/kg) or vehicle, and 24 h and 1 h prior to oral challenge with cholera toxin (CTX). After 6 h mice were sacrificed, the small intestine (pylorus—ilieocaecal junction) was removed, and luminal fluid was collected and measured (n=10). *p<0.05; **p<0.01.

treatment of intestinal disorders associated with secretory diarrhoea. We found that two structurally unrelated FXR agonists, GW4064 and OCA, attenuated Ca^{2+}- and cAMP-dependent Cl^- secretory responses across cultured colonic epithelia. Furthermore, ex vivo colonic tissues from GW4064-treated mice also displayed attenuated responsiveness to secretagogues. Since Cl^- secretion is the primary driving force for fluid secretion in the intestine, on the basis of our data, one would expect that FXR agonists should reduce fluid movement into the lumen. This hypothesis was borne out by our experiments in a murine model of allergic diarrhoea. Intestinal fluid loss in this model has been previously shown to be due to secretory diarrhoea,[29] and was reversed by pretreatment of the mice with GW4064. Further support for this hypothesis comes from our studies showing that GW4064 prevents small intestinal fluid accumulation in an in vivo model of CTX-induced secretion. Thus, FXR agonists exert antisecretory effects in vitro, ex vivo and in vivo, suggesting that they could be useful in the treatment of diarrhoeal diseases. It should be noted that although the G protein-coupled bile acid receptor, TGR5, is also expressed in colonic epithelia,[30] it is unlikely to contribute to the antisecretory actions of FXR agonists described here. First, FXR and TGR5 have distinct ligand binding characteristics[31] with FXR agonists, such as OCA, being poor agonists of TGR5, and second, we have recently published data which show that TGR5 activation with INT-777, a poor agonist of FXR, exerts actions on colonic Cl^- secretion that are distinct to those we describe here for FXR agonists.[30]

The antisecretory effects of FXR agonists appear to be due to inhibition of multiple components of the epithelial Cl^- secretory

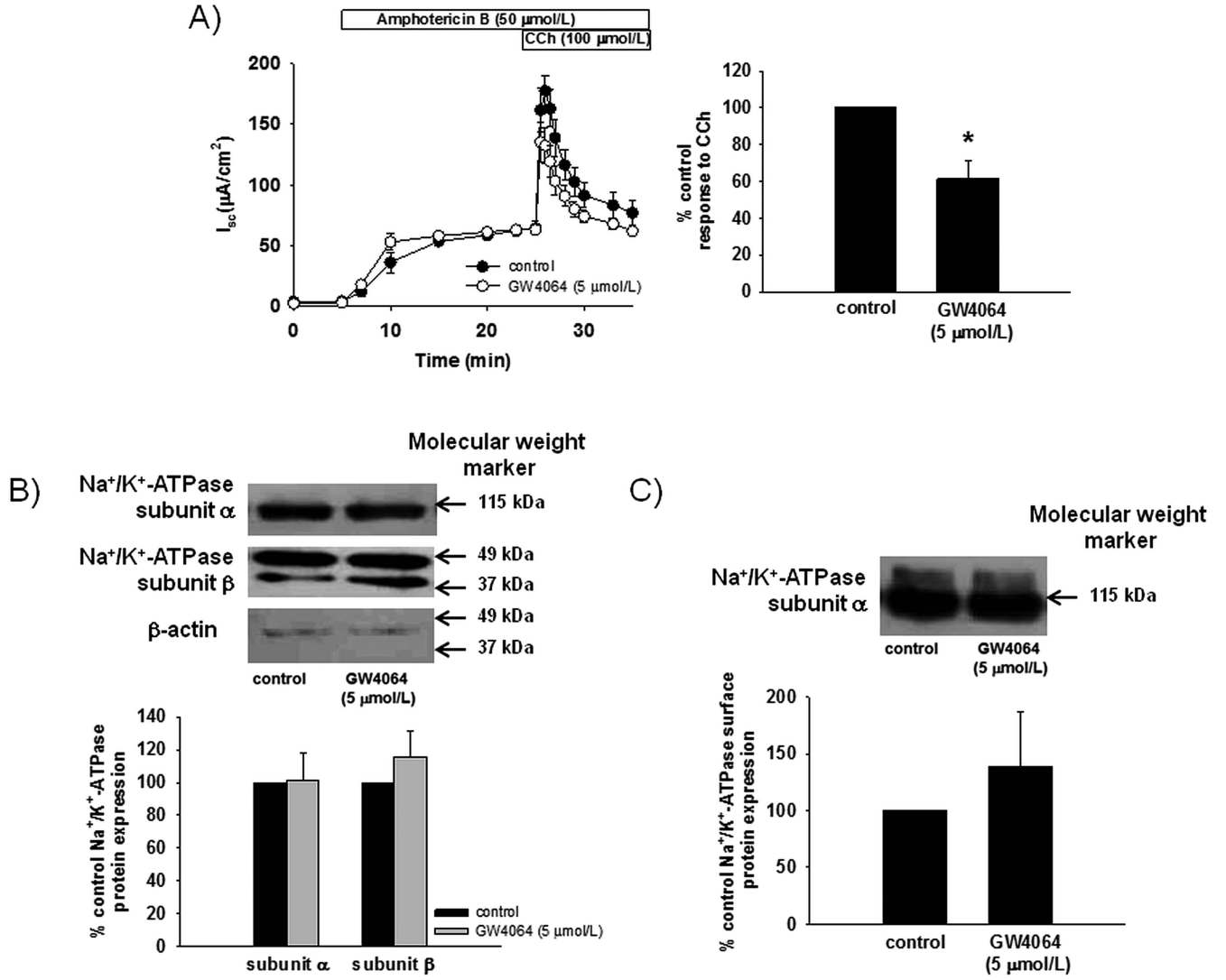

Figure 4 FXR activation decreases Na^+/K^+-ATPase activity in colonic epithelial cells. (A) GW4064 (5 µmol/L; 24 h)-treated T_{84} cell monolayers were mounted in Ussing chambers for measurements of Na^+/K^+-ATPase activity, as described in Methods (n=6). The right panel shows maximal changes in CCh-induced I_{sc} in GW4064-treated cells as a percent of controls. (B) Cells were treated with GW4064 (5 µmol/L; 24 h) after which Na^+/K^+-ATPase α or β subunit expression were measured by western blotting. Na^+/K^+-ATPase expression was normalised to β-actin expression. The lower panel shows densitometric analysis of three similar experiments. (C) After treatment with GW4064 (5 µmol/L; 24 h) Na^+/K^+-ATPase α subunit was detected by cell surface biotinylation of the basolateral membrane. The lower panel shows densitometric analysis of surface Na^+/K^+-ATPase α expression from six similar experiments. *p<0.05.

pathway, with both basolateral Na^+/K^+ ATPase activity, and apical CFTR Cl^- channel currents being attenuated upon treatment. Thus, activation of the FXR prevents fluid secretion by inhibiting both the driving force for Cl^- uptake across the basolateral membrane, and its exit across the apical membrane via CFTR. Furthermore, the effects of FXR activation on inhibition of transport protein function appear to be specific, since basolateral K^+ channel currents, which are necessary to drive Cl^- secretion, were found to be unaltered by GW4064 (data not shown). Thus, the effects of GW4064 do not appear to be due to a loss of cell viability, and this is further supported by observations that, even at the highest concentrations tested, GW4064 did not alter TER or LDH release from cultured cells, nor did it have any apparent toxicity in vivo. Furthermore, at concentrations similar to those used in the current study, previous studies have shown that GW4064 is devoid of liver and kidney toxicity.[32 33]

Interestingly, mechanisms underlying FXR-induced inhibition of CFTR currents and Na^+/K^+ ATPase activity appear to be different. Inhibition of CFTR Cl^- currents was associated with attenuated cellular expression of the protein, accompanied by a decrease in its cell surface expression. Notably, the degree to which FXR activation inhibited CFTR surface expression was somewhat greater than its effects on apical Cl^- currents, which correlates with previous studies demonstrating that there is an excess of CFTR expressed in intestinal epithelia, and that more than 60% inhibition of its expression is necessary before altered Cl^- secretory responses are observed. How FXR activation might bring about alterations in CFTR expression remains to be determined. However, our preliminary, unpublished, data show that GW4064 also downregulates CFTR mRNA expression, suggesting its effects are likely mediated either at the transcriptional or post-transcriptional level. By contrast, FXR-induced inhibition of Na^+/K^+ ATPase activity was not associated with altered expression of either the catalytic α or regulatory β subunits of the protein. Furthermore, activation of the FXR did not alter cellular levels of ATP which is required to drive Na^+/K^+

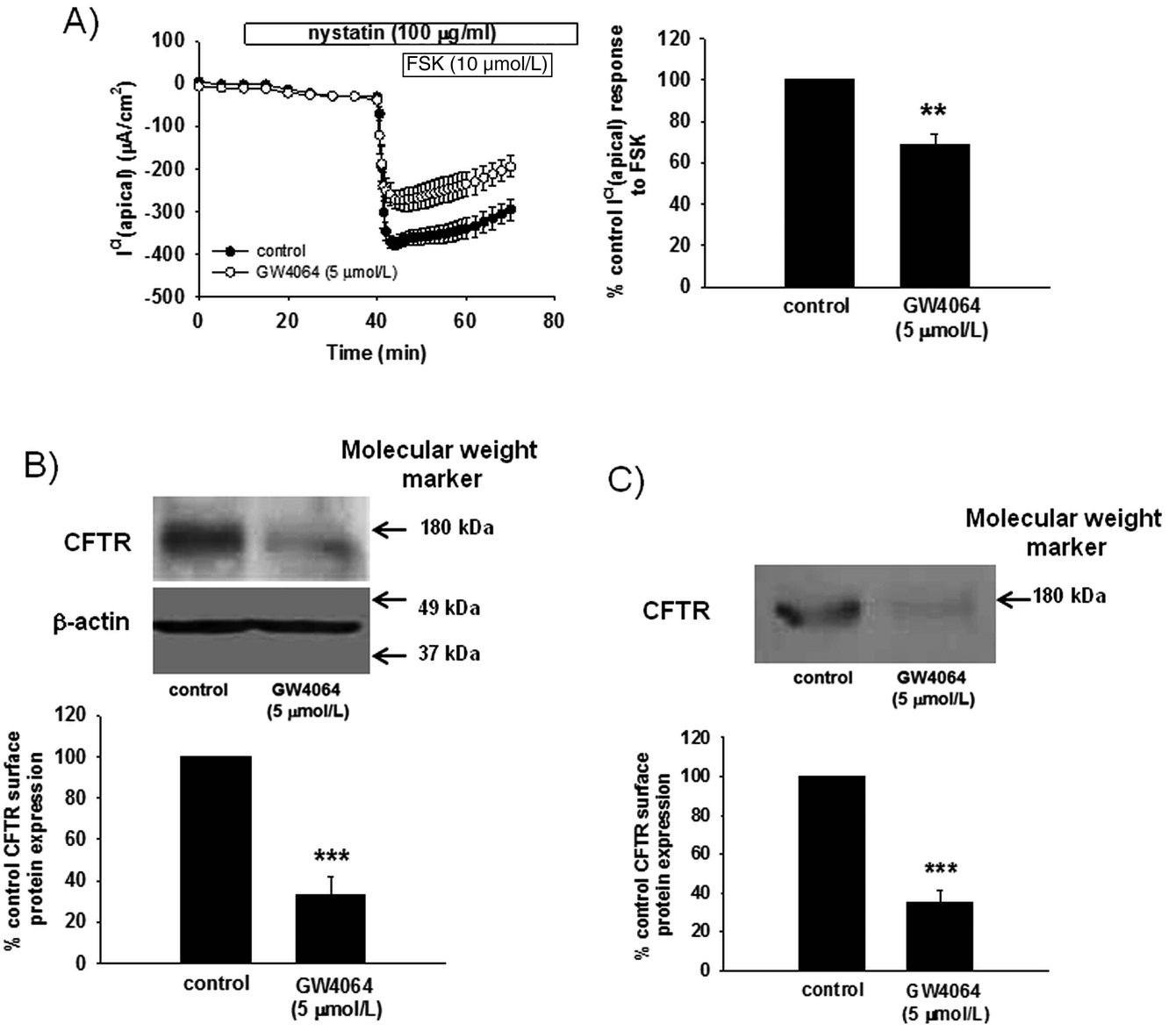

Figure 5 FXR activation inhibits CFTR currents and protein expression. (A) GW4064 (5 µmol/L; 24 h)-treated T_{84} cell monolayers were mounted in Ussing chambers for measurements of apical CFTR currents (I_{Cl}^-), as described in Methods (n=6). The right panel shows maximal changes in FSK-induced I_{Cl}^- in GW4064-treated cells as a percent of controls (n=9). (B) T_{84} cells were treated with GW4064 (5 µmol/L; 24 h) after which CFTR expression was measured by western blotting. CFTR expression was normalised to β-actin expression. The lower panel shows densitometric analysis of seven experiments. (C) After treatment with GW4064 (5 µmol/L; 24 h) CFTR was detected by biotinylation of the apical membrane. The lower panel shows densitometric analysis of surface CFTR expression from four similar experiments. **p<0.01; ***p<0.001.

ATPase activity. Thus, it seems likely that FXR actions on Na^+/K^+ ATPase transport activity involves intermediary proteins that regulate pump activity. A number of such proteins exist, including a family of FXYD proteins,[34] and modulator of Na^+/K^+ ATPase (MONaKA).[35] Such proteins are expressed in a cell-specific manner, thereby enabling differential regulation of Na^+/K^+ ATPase activity in different cell types, and our preliminary studies show that both FXYD proteins and MONaKA are expressed in colonic epithelial cells (data not shown).

In the current studies, we also examined the effects of FXR activation on absorptive processes. In the intestine, electrogenic Na^+ absorption through ENaC, electroneutral absorption through NHE3, and Na^+-glucose cotransport through SGLT1 are the primary mechanisms driving fluid absorption. Using a number of different models to measure their activity, we found that FXR activation was without effect on the activity of any of these transport proteins under basal conditions. However, we found that treatment with GW4064 reversed agonist-induced inhibition of NHE3 activity. This is important since many prosecretory agonists, typified by CCh, are known to simultaneously inhibit absorption through NHE3,[36] an effect which would serve to enhance fluid accumulation in the lumen. Thus, in vivo, FXR agonists would be expected to prevent the onset of diarrhoea, not only through inhibition of secretory responses, but also through promotion of absorption. How FXR activation prevents secretagogue-induced inhibition of NHE3 remains to be determined. However, previous work has shown that inhibition of NHE3 activity by Ca^{2+}-dependent agonists, such as CCh, is mediated by endocytosis of the transporter from the cell surface.[37] Thus, since FXR activation does not alter basal NHE3 activity, it is unlikely that FXR agonists act by increasing expression of the protein, but rather by interfering with the

inhibitory pathway induced by CCh. Future studies will aim to more thoroughly investigate this hypothesis.

Collectively, our data suggest that FXR agonists prevent fluid accumulation in the intestine through pathways that ultimately inhibit Na^+/K^+ ATPase activity and CFTR expression, and which also prevent secretagogue-induced inhibition of Na^+ absorption through NHE3. An intriguing question that arises from these studies is how activation of the FXR can have such diverse effects on different transport processes. The answer to this question likely lies in the ability of activated FXR to influence multiple aspects of gene expression regulation, both at transcriptional and post-transcriptional levels. For example, activated FXR may regulate epithelial function by binding to FXR response elements on genes encoding either transport proteins or the regulatory proteins that control their activity. Alternatively, FXR activation could downregulate the expression of transporters, or their regulatory proteins, through a number of mechanisms, including binding to negative response elements within the promoter regions of their genes, induction of DNA methylation, or stimulation of the expression of miRNAs that specifically target mRNA for these proteins. Each of these mechanisms has been shown to mediate FXR actions in other cell types,[38 39 40] and in our future studies we will aim to elucidate these complex FXR-dependent pathways in intestinal epithelial cells and their involvement in regulation of fluid and electrolyte transport.

While most currently available antidiarrhoeals exert their effects by indirect mechanisms, FXR agonists inhibit Cl^- secretion through direct actions on epithelial cells, and therefore may be useful in treating a range of secretory diarrhoeas. For example, diarrhoea induced by CTX is driven by excessive Cl^- secretion through CFTR,[41] and since CFTR expression is downregulated by FXR activation, targeting the receptor as a therapy for cholera-induced diarrhoea may be a useful approach. Recent studies suggest that FXR agonists may also have a role to play in treating a subset of patients diagnosed as having IBS, but who actually suffer from bile acid diarrhoea (BAD). Approximately 30% of all diagnosed IBS patients have the diarrhoea-predominant form, IBS-D,[42] and it has been recently shown that of these patients, approximately 30% have increased bile acid loss (as measured by SeHCAT retention) and increased bile acid synthesis.[43 44] Interestingly, dysregulation of bile acid biosynthesis in these patients appears to be due to defective production of the FXR target protein, FGF-19, an enterohepatic messenger that downregulates bile acid biosynthesis. Normally, FGF-19 production is induced by bile acids acting on FXR in ileal epithelial cells, and loss of this signal leads to enhanced bile acid biosynthesis and increased delivery into the colon. Since bile acids exert prosecretory effects at such pathophysiological concentrations, this induces the onset of diarrhoea. Thus, one would expect that this particular subset of BAD patients, which constitutes approximately 1% of the population of Western countries,[42] should benefit doubly by the use of FXR agonists through restoration of FGF-19 production and exertion of anti-secretory actions on the colonic epithelium.

In summary, these studies reveal an important role for the FXR in regulating intestinal fluid and electrolyte secretion. The effects of FXR activation are mediated by direct actions at the level of epithelial cells and occur though inhibition of multiple components of the Cl^- secretory mechanism. While its precise role in regulating epithelial transport function under normal physiological circumstances remain to be elucidated, our studies suggest that the FXR represents an excellent target for the development of a new class of antidiarrhoeal drugs.

Correction notice This article has been corrected since it was published Online First. Three author names have been updated with initials.

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