British Journal of Pharmacology (2009), 156, 1085–1097

0007-1188/09
www.brjpharmacol.org

RESEARCH PAPER

Loss of Ca^{2+}-mediated ion transport during colitis correlates with reduced ion transport responses to a Ca^{2+}-activated K^+ channel opener

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Background and purpose: Epithelial surface hydration is critical for proper gut function. However, colonic tissues from individuals with inflammatory bowel disease or animals with colitis are hyporesponsive to Cl^- secretagogues. The Cl^- secretory responses to the muscarinic receptor agonist bethanechol are virtually absent in colons of mice with dextran sodium sulphate (DSS)-induced colitis. Our aim was to define the mechanism underlying this cholinergic hyporesponsiveness.
Experimental approach: Colitis was induced by 4% DSS water, given orally. Epithelial ion transport was measured in Ussing chambers. Colonic crypts were isolated and processed for mRNA expression via RT-PCR and protein expression via immunoblotting and immunolocalization.
Key results: Expression of muscarinic M_3 receptors in colonic epithelium was not decreased during colitis. Short-circuit current (I_{SC}) responses to other Ca^{2+}-dependent secretagogues (histamine, thapsigargin, cyclopiazonic acid and calcium ionophore) were either absent or severely attenuated in colonic tissue from DSS-treated mice. mRNA levels of several ion transport molecules (a Ca^{2+}-regulated Cl^- channel, the intermediate-conductance Ca^{2+}-activated K^+ channel, the cystic fibrosis transmembrane conductance regulator, the Na^+/K^+-ATPase pump or the $Na^+/K^+/2Cl^-$ co-transporter) were not reduced in colonic crypts from DSS-treated mice. However, protein expression of Na^+/K^+-ATPase α1 subunits was decreased twofold during colitis. Activation of Ca^{2+}-activated K^+ channels increased I_{SC} significantly less in DSS colons compared with control, as did the protein kinase C activator, phorbol 12-myristate 13-acetate.
Conclusions and implications: Decreased Na^+/K^+-ATPase expression probably contributes to overall epithelial hyporesponsiveness during colitis, while dysfunctional K^+ channels may account, at least partially, for lack of epithelial secretory responses to Ca^{2+}-mediated secretagogues.
British Journal of Pharmacology (2009) **156**, 1085–1097; doi:10.1111/j.1476-5381.2009.00122.x; published online 9 March 2009

Keywords: calcium; calcium-activated K^+ channels; cholinergic; colitis; $K_{Ca}3.1$; ion transport; Kcnn4; muscarinic receptors; protein kinase C; short-circuit current

Abbreviations: BCh, bethanechol; CFTR, cystic fibrosis transmembrane conductance regulator; CLCA6, Ca^{2+}-activated Cl^- channel 6; DCEBIO, 5,6-dichloro-1-ethyl-1,3-dihydro-2*H*-benzimidazol-2-one; DSS, dextran sodium sulphate; FSK, forskolin; $K_{Ca}3.1$, Ca^{2+}-activated K^+ channel; Kcnn4, $K_{Ca}3.1$ gene; NKCC1, $Na^+/K^+/2Cl^-$ co-transporter; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TNBS, trinitrobenzene sulphonic acid; UC, ulcerative colitis

Introduction

Balancing the movement of ions and consequently water across the intestinal epithelium is an active process regulated by multiple mechanisms and a diverse array of mediators. In the colon, ion transport is tipped towards absorption to balance the secretory state required in the small intestine for digestion. Active ion transport is accomplished through the asymmetrical distribution of ion channels, co-transporters and energy-dependent pumps in the plasma membrane of the polarized intestinal epithelial cell. The activity of these ion transport molecules can be up- or down-regulated by increasing or decreasing intracellular messengers, typically calcium and the cyclic nucleotides, cAMP and cGMP. Acetylcholine (ACh) is an important neurotransmitter that directly regulates epithelial ion transport through G-protein-coupled muscarinic receptors, stimulating a fast, transient increase in

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Received 14 May 2008; revised 3 September 2008; accepted 21 November 2008

intracellular Ca^{2+} that leads to a transient increase in chloride (accompanied by water) secretion across the apical membrane. This Ca^{2+}-mediated apical Cl^- efflux is balanced by, and thus requires, Ca^{2+}-mediated potassium efflux through the intermediate-conductance K^+ channel designated as $K_{Ca}3.1$ (also called IK1, IKCa1 or SK4; here, we refer to this channel protein as $K_{Ca}3.1$). Using Kcnn4 null mice, two separate groups have recently demonstrated that this channel is integral to both cholinergic and histamine-mediated modes of Ca^{2+}-activated Cl^- secretion (Flores *et al.*, 2007; Matos *et al.*, 2007). Electrophysiological studies have localized K^+ efflux from this channel to the basolateral membrane of epithelial cells (Bleich *et al.*, 1996; Bowley *et al.*, 2007) as well as the apical membrane (Joiner *et al.*, 2003). Immunostaining and immunoblotting confirmed the expression of $K_{Ca}3.1$ on both the basolateral and apical membrane of epithelial cells (Furness *et al.*, 2003; Joiner *et al.*, 2003), although it is believed that K^+ exit via basolateral $K_{Ca}3.1$ channels is the requirement for Ca^{2+}-mediated Cl^- secretion.

Analyses of tissues from patients with inflammatory bowel disease (ulcerative colitis and Crohn's disease) and from rodent models of colitis, reveal a reduced capacity of the epithelium to respond to pro-secretory stimuli, including ACh, histamine, bradykinin, prostaglandin E_1, substance P and 5-HT (Goldhill *et al.*, 1993b; Kachur *et al.*, 1995; Crowe *et al.*, 1997; Miceli *et al.*, 2002; Perez-Navarro *et al.*, 2005b). Upon challenge of colonic tissues from mice with dextran sodium sulphate (DSS)-induced colitis with the cholinomimetic carbachol, we observed a decrease in short-circuit current (I_{SC}), rather than the transient increase in I_{SC} that occurs in colon from normal mice (Sayer *et al.*, 2002). This drop in I_{SC} was insensitive to the sodium channel blocker, amiloride, but was reduced by removal of Cl^- and HCO_3^- from the solutions bathing the tissues. This drop in I_{SC} evoked by carbachol challenge of colitic colonic tissue was subsequently attributed to the release of nitric oxide from the myenteric plexus of inflamed tissue via the activation of nicotinic, rather than muscarinic, ACh receptors (Green *et al.*, 2004). Thus, using DSS colitis in mice as a model that replicates some aspects of human ulcerative colitis, a hitherto unappreciated role for the cholinergic nicotinic system in the control of epithelial ion transport was revealed. However, the mechanism underlying the inability of carbachol to evoke an increase in I_{SC} (typically a Cl^- efflux across the apical epithelial membrane) in colonic tissue from mice with DSS-induced colitis remained unresolved.

Given the importance of the cholinergic system in the control of enteric water balance and homeostasis, the aim of the current study was to determine the mechanism underlying the lack of secretory responses to the muscarinic agonist bethanechol in colonic tissue from mice with DSS-induced colitis. The data showed that colonic tissues from these mice were profoundly deficient in their ability to respond to not only bethanecol, but also to four other pro-secretory stimuli that function via mobilization of intracellular Ca^{2+}. We hypothesize that this defect is due, at least in part, to a reduced activity of the intermediate-conductance potassium channel, $K_{Ca}3.1$, which is critical in establishing the ion gradients (i.e. driving forces) that allow Cl^- to leave the cell.

Methods

Induction of colitis

All animal care and experimental procedures were approved by the McMaster University or the University of Calgary Animal Care Committees and were performed according to the guidelines of the Canadian Council of Animal Care.

Male Balb/c mice (8–12 weeks old, Harlan Animal Suppliers, Indianapolis, IN) were given a 4% ($w \cdot v^{-1}$) DSS (40 Kd M.W.; ICN Biomedicals Inc., Aurora, OH) drinking water solution for 5 days followed by 3 days of normal drinking water. On day 3 following DSS removal (i.e. 8 days after the start of DSS treatment), mice were killed and segments of colon and, in some experiments, ileum were excised. This treatment regime followed that used in our earlier analyses of colonic ion transport in this model system (Sayer *et al.*, 2002; Green *et al.*, 2004). Colitis was assessed macroscopically, based on weight change, colon length, stool quality, colonic tissue appearance and overall body appearance (Diaz-Granados *et al.*, 2000).

Measurement of ion transport

Non-ulcerated whole-thickness segments of distal colon (the distal 50–60% of colon was used, up to two segments per mouse, following removal of the rectal portion) were opened along the mesenteric border and mounted in Ussing chambers (0.6 cm^2 opening). Briefly, tissues were bathed in oxygenated Krebs buffer at 37°C (composition in $mmol \cdot L^{-1}$: NaCl 115, KH_2PO_4 2, $MgCl_2$ 1.1, $NaHCO_3$ 25, KCl 8, $CaCl_2$ 1.25) containing either 10 $mmol \cdot L^{-1}$ glucose (serosal side) or 10 $mmol \cdot L^{-1}$ mannitol (luminal side). Net active ion transport across the epithelium was measured via the injection of a short-circuit current (I_{SC}, expressed as $\mu A \cdot cm^{-2}$) through the tissue under clamped voltage conditions. After ~30 min equilibration period, the baseline I_{SC} and potential difference (PD, expressed as mV) were recorded and ion conductance calculated using Ohm's law ($G = I \cdot V^{-1}$, expressed as $mS \cdot cm^{-2}$). The maximum change in I_{SC} (ΔI_{SC}) that occurred within 30 min of addition of various pharmacological agents was recorded. Bethanechol was used at 100 $\mu mol \cdot L^{-1}$ as previous studies (Sayer *et al.*, 2002) revealed that mouse colonic tissues with the attendant external musculature have minimal responses to cholinergic stimuli below this concentration, presumably because of muscarinic receptors in the muscle layers. At the end of every experiment, each tissue was challenged with forskolin (FSK; 10^{-5} or 10^{-6} $mol \cdot L^{-1}$) to ensure viability and secretory responsiveness, and I_{SC} and PD were again recorded and G calculated to ensure that tissue had maintained viable electrical properties.

Immunodetection of the muscarinic M3 receptor

Segments of colonic tissue were fixed in Zamboni's fixative for 24–72 h, embedded in Tissue-Tek® OCT (Sakura Finetek USA Inc., Torrance, CA), frozen in liquid nitrogen and stored at –20°C. Cryostat sections (8 μm) were collected on poly-D-lysine-coated slides, air-dried, and stored at –20°C until ready for use, at which time slides were thawed to room temperature, rinsed in phosphate buffered saline (PBS) and then

treated with 5% goat serum in PBS with 0.1% Triton x-100 for 30 min at room temperature. Following three washes with PBS, the sections were incubated with rabbit anti-human M_3 muscarinic receptor antibody (1:200; US Biological, Swampscott, MA) for 24 h at 4°C (as a specificity control, some sections were not incubated with the anti-M_3 antibody). This was followed by three washes in PBS and a 1 h incubation at room temperature with anti-rabbit IgG antibody conjugated to Alexa Fluor 488 (6 μg·mL^{-1}; Molecular Probes, Burlington, ON); sections were then rinsed in PBS and distilled H_2O and coverslips were applied. Sections were viewed and images recorded on a Zeiss LSM 510 laser scanning confocal microscope on an inverted stage (Carl Zeiss Canada Ltd., North York, ON).

Intestinal crypt isolation

Mouse intestinal tissue was removed and rinsed three times in 4°C PBS or flushed once with 4°C Hank's Balanced Salt Solution (HBSS; both methods provided a good crypt yield). The tissue was laid flat on Whatman filter paper and cut into small segments (~0.5 × 0.5 cm) that were immersed in BD™ Cell Recovery Solution (BD Biosciences, Mississauga, ON) and incubated for 2–4 h at 4°C (method adapted from Perreault and Beaulieu, 1998). Segments were then shaken individually with fine forceps to release crypts into the solution. In some experiments, crypts in solution were first passed through a cell strainer (100 μm pore size) to remove large debris. Crypts were then washed 3 times in PBS or HBSS (at 4°C) for further processing.

Western blotting

Intestinal crypts were lysed in either RIPA buffer or T-PER® Tissue Protein Extraction Reagent (Pierce Biotechnology, Inc., Rockford, IL) containing protease inhibitors (complete protease inhibitor cocktail; Roche, Indianapolis, IN). Samples were centrifuged (17 900 × *g* for 5 min) and stored at –70°C. Total protein content in the supernatant was determined by the Bradford microplate assay (Bio-Rad Laboratories, Hercules, CA; protein samples were diluted at least 1:8 to reduce effects of buffer components on Bradford assay) and, depending on the experiment, 30 or 40 μg of protein was mixed with loading buffer, boiled for 3–5 min and equal amounts of protein were loaded into 4–10% or 4–12% acrylamide/bisacrylamide (29:1) SDS gels. Proteins were separated by electrophoresis followed by electrical transfer onto either PVDF or nitrocellulose membranes, which were then blocked in 4–5% non-fat powdered milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for either 1 h at room temperature or overnight at 4°C. The membranes were then incubated with the primary antibody in blocking buffer for 1–4 h at room temperature or overnight at 4°C [goat anti-M_3 muscarinic receptor (C-20), 1:500, Santa Cruz Biotechnology, Santa Cruz, CA; goat anti-NKCC1 (N-16), 1:300, Santa Cruz Biotechnology; rabbit anti-CFTR (H-182), 1:500, Santa Cruz Biotechnology; goat anti-Na^+/K^+-ATPase α1, 1:1000, Cell Signaling Technology, Danvers, MA; rabbit anti-$K_{Ca}3.1$, 1:250, Alomone Labs, Jerusalem, Israel; goat anti-cytokeratin 19 (N-13), 1:500, Santa Cruz Biotechnology; rabbit anti-calmodulin (FL-149), 1:300, Santa Cruz]. The membranes were washed 3 times in TBST followed by incubation for 40–60 min at room temperature with a horseradish peroxidase-conjugated secondary antibody (anti-goat IgG, 1:3000–1:10 000, Santa Cruz Biotechnology or Jackson ImmunoResearch Laboratories, West Grove, PA; anti-rabbit IgG, 1:5000–1:10 000, Santa Cruz Biotechnology or Jackson ImmunoResearch Laboratories). The membranes were washed several times in TBST and then finally incubated in an enhanced chemiluminescence reagent (Amersham Pharmacia, Piscataway, NJ, Millipore, Billerica, MA, or Perkin-Elmer, Boston, MA) and either exposed to Kodak Blue XB-1 film or directly imaged on a Molecular Imager ChemiDoc XRS System (Bio-Rad Laboratories) to visualize immunoreactive proteins.

RT-PCR

RNA was extracted from intestinal crypts using either the TRIzol® extraction method (Invitrogen, Burlington, ON) or the Aurum™ Total RNA Mini Kit (Bio-Rad). Total RNA extracted was further purified with 2.5 mol·L^{-1} $LiCl_2$. cDNA was reverse transcribed from the purified RNA (as a negative control samples were also prepared without reverse transcriptase) and then 1 μg of cDNA was incubated in a reaction mixture that included 1.25 Units Platinum® Taq DNA Polymerase (Invitrogen), 1.5 mmol·L^{-1} $MgCl_2$ and two sets of nucleotide primers for the following mouse genes (Table 1): M_3 muscarinic ACh receptor (M3), β-actin, cytokeratin 19 (CK19), the α1 subunit isoform of the Na^+/K^+-ATPase (ATPaseα1), the β1 subunit isoform of the Na^+/K^+-ATPase

Table 1 Primer information

*Gene**	*GenBank™ accession no.*	*Product size (bp)*	*Forward primer (5′ to 3′)*	*Reverse primer (5′ to 3′)*	*Primer concentration*
M3	NM_033269	502	ctg ctg gta ctg ctg ttt gc	tca ggc cca gaa gag tat gg	300 nmol·L^{-1}
β-actin	NM_007393	437	cca gag caa gag agg tat cc	ctg tgg tgg tga agc tgt ag	100 or 200 nmol·L^{-1}
CK19	NM_008471	519	ttg aga cag aac acg cct tg	tca cgc tct gga tct gtg ac	100 nmol·L^{-1}
ATPaseα1	NM_144900	380	ctc ctt tga caa gac gtc agc	cct tca gct ctt cat cca gtg	300 nmol·L^{-1}
ATPaseβ1	NM_009721	301	aga ttc ccc aga tcc aga aga	ttg ata atg atg cag ggc ttc	300 nmol·L^{-1}
NKCC1	NM_009194	347	tct gtg ggt tcg tgt gtt gt	cca cga agc ggt tca tta tt	400 nmol·L^{-1}
Kcnn4	BC010274	215	aag cac act cga agg aag ga	ccg tcg att ctc ttc tcc ag	400 nmol·L^{-1}
CFTR	NM_021050	336	cat tct tca cgc ccc tat gt	gct cga agt gtc cag agt cc	400 nmol·L^{-1}
CLCA6	NM_207208	~540	tat gtg cct agt tct tga tg	ctt ctg ggt gat atc agc att ttc tga agc cag	200 nmol·L^{-1}

*see text for full name.

(ATPaseβ1), the Na^+-K^+-$2Cl^-$ co-transporter (NKCC1), the intermediate-conductance calcium-activated potassium channel (Kcnn4), the cystic fibrosis transmembrane conductance regulator (CFTR), and a calcium-activated chloride channel (CLCA6). All primers, except those for CLCA6, were designed with the Primer3 program. CLCA6 primer sequences were reported by Evans *et al.* (2004). Gene product size, primer sequences and final primer concentrations are listed in Table 1. cDNA was amplified for 32–35 cycles on a Techne PHC-3 thermal cycler (Mandel Scientific Co., Guelph, ON) with denaturing, annealing and extending temperatures of 94–95°C, 57–58°C and 72°C respectively. The final PCR product was run on a 2% agarose gel and the amplified bands viewed via DNA binding to ethidium bromide under ultraviolet light.

Image analysis

Densitometry was performed using the ImageJ software (downloaded from: http://rsb.info.nih.gov/ij/). Regions of interest were selected and mean pixel density was measured and multiplied by total area if area differed between sample bands from the same experiment.

Statistical analysis

Data are expressed as mean ± SEM. Comparisons between two groups were analysed using Student's unpaired two-tailed *t*-test; comparisons of events within a single animal were analysed using Student's paired two-tailed *t*-test. Multiple group comparisons were made using a one-way ANOVA followed by a *post hoc* test as indicated. In all comparisons $P \leq 0.05$ was accepted as a statistically significant difference.

Drugs

The following drugs were used at the indicated concentrations: bethanechol (dissolved in water, added serosally to tissue mounted in the Ussing chamber), GF 103209X (1 μmol·L^{-1}, dissolved in water, added serosally and mucosally), calcium ionophore A23187 (10 μmol·L^{-1}, dissolved in DMSO, added mucosally), clotrimazole (30 μmol·L^{-1}, dissolved in DMSO, added serosally), cyclopiazonic acid (CPA, 10 μmol·L^{-1}, dissolved in DMSO, added mucosally), DCEBIO (100 μmol·L^{-1}, dissolved in DMSO, added serosally, Tocris, Ellisville, MO), FSK (1 or 10 μmol·L^{-1} as indicated, dissolved in DMSO or EtOH, added serosally), histamine (100 μmol·L^{-1}, dissolved in water, added serosally), phorbol 12-myristate 13-acetate (PMA, 1 μmol·L^{-1}, dissolved in DMSO, added serosally and mucosally), thapsigargin (10 μmol·L^{-1}, dissolved in DMSO, added mucosally), W-13 (50 μmol·L^{-1}, dissolved in water, added serosally and mucosally, Tocris). Unless indicated otherwise, drugs were purchased from Sigma-Aldrich (Oakville, ON).

Nomenclature

Naming of receptors, transporters and ion channels follows the recommendations of Alexander *et al.* (2008).

Results

DSS-induced colitis results in loss of muscarinic receptor-mediated ion transport that is not rescued by priming with cAMP

Mice that received 4% (w·v^{-1}) DSS in their drinking water developed clinical signs of colitis as previously reported (Green *et al.*, 2004). Histological evidence of colitis has also been observed in the DSS model by our laboratory (Diaz-Granados *et al.*, 2000; Sayer *et al.*, 2002; Green *et al.*, 2004) and was not repeated for this study. At the time of killing, DSS-treated mice had lost 13.7 ± 1.2% of their initial body weight ($n = 17$) compared with control mice, whose weight increased by 1.4 ± 0.6% ($n = 12$; $P < 0.0001$); DSS-treated mice had shorter colons than control mice (6.8 ± 0.1 cm, $n = 17$, compared with 9.6 ± 0.2 cm for control mice, $n = 17$; $P < 0.0001$) and had poorly formed, soft stool, with occasional signs of faecal blood. The distal colon of DSS-treated mice was often devoid of contents and the tissue was paler and more rigid than colons from control mice.

Challenge of mid-distal colonic segments from control mice mounted in Ussing chambers with bethanechol (10^{-4} mol·L^{-1}) resulted in a transient increase in I_{SC} (Figure 1), which is due to increased epithelial Cl^- secretion and decreased epithelial Na^+/Cl^- absorption (Bajnath *et al.*, 1992; Sayer *et al.*, 2002). Colonic tissues from DSS-treated mice, however, were non-responsive to bethanechol (Figure 1), complementing our earlier observations with the cholinomimetic carbachol (Green *et al.*, 2004). Despite this lack of response to bethanechol, tissues from DSS-treated mice were viable, displaying slightly increased but normal passive ion conductance values (DSS tissues = 21.9 ± 1.3 and control tissues = 18.8 ± 0.9 mS·cm^{-2}, $n = 71$, $P = 0.054$) and a reduced baseline I_{SC} (DSS tissues = 44.1 ± 2.2 μA·cm^{-2} and control tissues = 55.5 ± 3.0 μA·cm^{-2}, $n = 71$, $P = 0.003$). Also, while significantly less effective, FSK (10^{-5} mol·L^{-1}) was still able to elicit an increase in I_{SC} in colonic tissue from DSS-treated mice (DSS tissues = 64.9 ± 9.1 and control tissues = 247.7 ± 9.8 μA·cm^{-2}, $n = 32$; $P < 0.001$).

cAMP- and Ca^{2+}-driven Cl^- secretion are closely linked and it has been shown that basal amounts of cAMP are required for Ca^{2+}-mediated ion transport responses (Mall *et al.*, 1998). Hence, we postulated that mobilizing a cAMP-driven event would facilitate a greater response to bethanechol in tissues from DSS-treated mice. Segments of colon were pretreated with FSK for approximately 20 min in order to allow I_{SC} responses to plateau. Treatment with FSK activates adenylate cyclase to produce cAMP, resulting in increases in I_{SC} in both control and DSS-colitic tissue, although the ΔI_{SC} was smaller in colon from DSS-treated mice (see above). Upon subsequent bethanechol challenge, the resulting ΔI_{SC} was reduced in control tissues compared with tissues that had not received FSK and this may be a consequence of the large magnitude of the response of the epithelium to 10^{-5} mol·L^{-1} FSK. Moreover, FSK (10^{-7}–10^{-5} mol·L^{-1}) pretreatment did not enhance/induce a Cl^- efflux in response to bethanechol in colon from mice treated with DSS (Figure 1A).

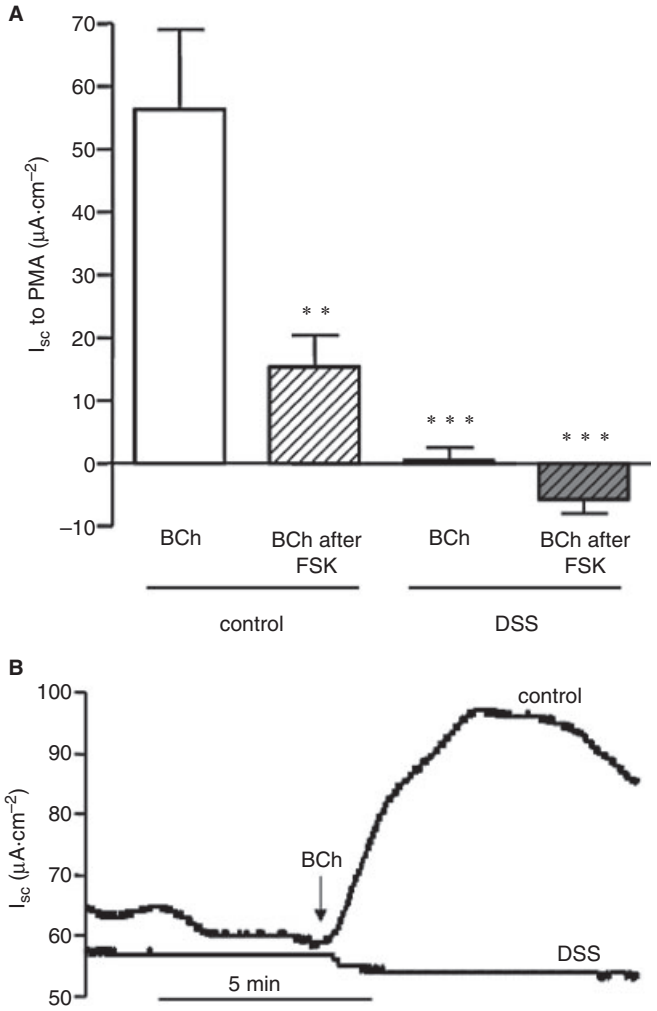

Figure 1 Colonic epithelial ion transport responses to the muscarinic agonist bethanechol (BCh, 100 μmol·L^{-1}, serosal addition) were absent in mice treated with 4% (wt vol^{-1}) dextran sodium sulphate (DSS) for 5 days followed by 3 days of normal tap water. (A) Average peak ion transport response in colonic tissue from control mice compared with tissues from mice with DSS-induced colitis. Prior treatment with forskolin (FSK; 10 μmmol·L^{-1}, added serosally) to initiate cAMP-driven Cl^- secretion did not restore secretory responsiveness to BCh in colonic tissue from DSS-treated mice (mean ± SEM; $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control BCh response; $n = 6$–7). (B) Representative I_{SC} tracings following BCh challenge (arrow) of colonic tissue from a control mouse and a DSS-treated mouse.

Loss of muscarinic receptors does not account for the hyporesponsiveness to bethanechol

The M_3 muscarinic receptor is a major contributor to direct cholinergic effects on epithelial ion transport. Mucosal tissue was isolated from colons of control and DSS-treated mice and RT-PCR revealed no difference in the ratio of M_3 receptor/β-actin mRNA between control and DSS-treated mice (Figure 2A, control = 0.875 ± 0.278 and DSS = 1.007 ± 0.043 mean pixel density; $n = 3$). Immunoblotting of whole cell protein lysates of colonic crypts also revealed no significant difference between M_3 receptor expression in tissues from control or DSS-treated mice (Figure 2B), an observation that was supported by densitometry analysis (control = 118.9 ± 8.9 and DSS = 100.8 ± 8.2 mean pixel density; $n = 4$). Protein gels were stained with Coomassie blue to ensure similar protein loading across all samples (not shown). In addition, immunofluorescence localization studies that focused on the epithelium did not show any notable differences in the cellular or tissue distribution of the M_3 receptor on cryostat sections of colon from control and DSS-treated mice (Figure 2C).

DSS colitis results in a global diminution of Ca^{2+}-driven active epithelial ion transport

Since muscarinic cholinergic control of epithelial ion transport is dependent on intracellular Ca^{2+}, the ability of colonic tissue to respond to other Ca^{2+}-mediated secretagogues was assessed. Responses to four agents (histamine, thapsigargin, CPA and the Ca^{2+} ionophore A23187) that increase intracellular Ca^{2+} via three different mechanisms from intracellular or extracellular sources were profoundly reduced or absent in colonic tissue from DSS-treated mice as compared with controls (Table 2). In the case of thapsigargin, CPA and A23187, statistical analysis (two-tailed *t*-test assuming unequal variance) revealed that the I_{SC} events in tissue from DSS-treated mice were not significantly different from zero. Positive increases in I_{SC} evoked by histamine [100 and 500 μmol·L^{-1} (latter not shown)] were observed in DSS tissues, but these were significantly reduced compared with control. It is possible that along with activation of histamine H_1 receptors on the epithelium, this increase in I_{SC} could also reflect activation of additional histamine receptors on other mucosal cells.

DSS colitis is not accompanied by qualitative decreases in mRNA of epithelial ion transport molecules

A lack of I_{SC} response to secretagogues that utilize Ca^{2+} signalling pathways could reflect a decrease in the expression of specific ion transport molecules. To begin to address this possibility at the level of transcription, total RNA was isolated from colonic and ileal crypts from control and DSS-treated mice and mRNA for the $Na^+/K^+/2Cl^-$ co-transporter (NKCC1), the α1 and β1 subunits of the Na^+/K^+-ATPase (ATPaseA1 and ATPaseB1 respectively), the basolaterally located Ca^{2+}-activated K^+ channel (Kcnn4), and the apical CFTR Cl^- channel were assessed and compared with levels of cytokeratin 19 (CK19) mRNA (Owens and Lane, 2004), which served as the house-keeping gene. As estimated by RT-PCR, no significant differences in mRNA expression of any of these three key ion transport molecules could be detected when control and DSS samples were compared (Figure 3). Expression of mRNA for the Ca^{2+}-activated Cl^- channel, CLCA6, was also examined. In comparison to the level of expression in colonic crypts from control mice, and counter-intuitive to the diminished I_{SC} response to bethanechol observed in tissues from DSS-treated mice, there was an increase in CLCA6 mRNA expression in colonic crypts from DSS-treated mice (Figure 3). Furthermore, a second band was apparent in extracts of colonic crypts from DSS-treated mice that may represent a splice variant of the CLCA6 gene, as previously reported by Evans *et al.* (2004). Collectively, these findings provide no explanation for the hyporesponsiveness of colonic tissues

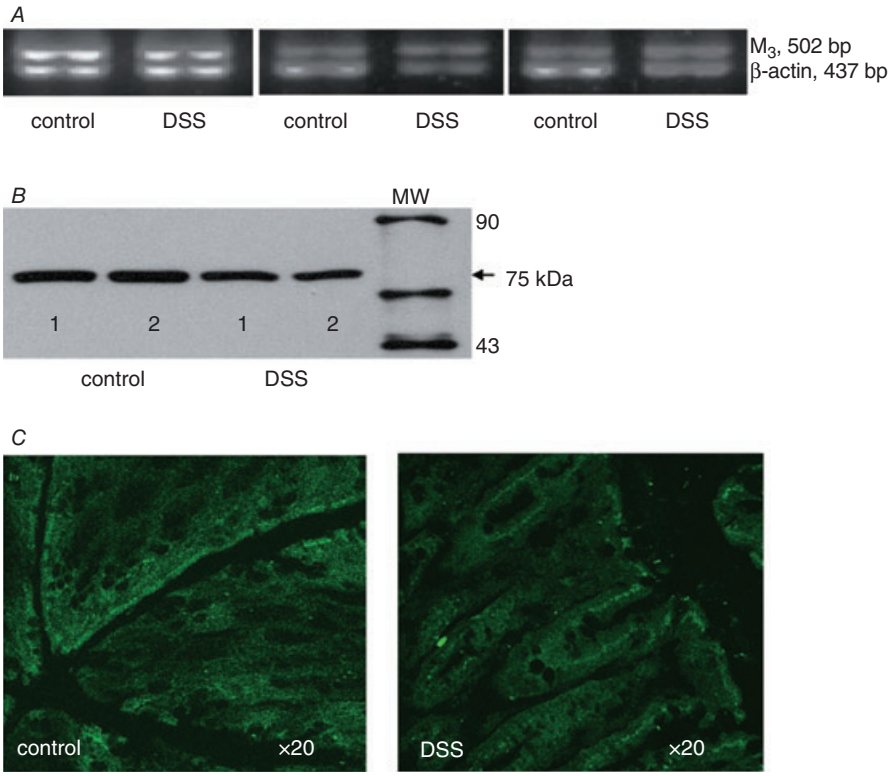

Figure 2 Expression of muscarinic M_3 receptor mRNA and protein was not reduced in DSS-colitic tissue compared to control. (A) RT-PCR bands amplified from colonic mucosal tissue from control and DSS-treated mice showing no difference in the ratio of M_3 receptor/β-actin gene product between the two groups (each column is from a separate mouse). (B) representative immunoblot of protein extracted from colonic epithelial crypts showing no significant difference in total M_3 receptor protein between control and DSS-treated mice (molecular weight of M_3 receptor protein is 75 kDa); blots were stripped and re-probed for β-actin and no significant differences were noted in the expression of this house-keeping protein when extracts from control and DSS-treated mice were compared. (C) Representative images showing the distribution of M_3 receptor immunoreactivity in the colon.

Table 2 I_{SC} responses to Ca^{2+}-dependent secretagogues in colonic tissue from control and DSS-treated mice

Pro-secretory agent	*Concentration. (μmol·L⁻¹)*	*Activity*	*Maximum I_{SC} response (μA·cm⁻²)*	
			Control	*DSS*
Histamine	100	H_1 receptor agonist	25.6 ± 6.4 ($n = 4$)	4.8 ± 0.5* ($n = 4$)
Thapsigargin	10	SERCA pump inhibitor	22.8 ± 6.6 ($n = 5$)	1.6 ± 0.9*** ($n = 18$)
Cyclopiazonic acid	10	SERCA pump inhibitor	75.4 ± 13.6 ($n = 11$)	2.0 ± 1.3** ($n = 6$)
A23187	3	Ca^{2+} ionophore	17.2 ± 2.2 ($n = 14$)	2.6 ± 1.3*** ($n = 12$)

Mean ± SEM; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with corresponding control group.

from DSS-treated mice to muscarinic stimulation or to the other Cl^- secretagogues that operate through Ca^{2+} signalling.

Reduced expression of Na^+/K^+-ATPase α1 likely contributes to the overall hyposecretory state associated with DSS-induced colitis

Because mRNA may not necessarily translate into expressed protein and since RT-PCR is a qualitative measure of mRNA we assessed protein levels of NKCC1, CFTR, the α1 subunit of Na^+/K^+-ATPase (and the $K_{Ca}3.1$ channel – see below) and compared these against CK19 protein expression. In agreement with RT-PCR results, neither NKCC1 nor CFTR protein expression appeared to differ between control and DSS-treated mice (mean NKCC1/CK19 density = 0.43 ± 0.02 in control crypts vs. 0.46 ± 0.03 in DSS crypts, $P = 0.58$; CFTR/CK19 density = 0.18 ± 0.02 in control crypts vs. 0.18 ± 0.04 in DSS crypts, $P = 0.90$; $n = 3$ mice per group). However, while RT-PCR did not detect any significant differences in mRNA for the Na^+/K^+-ATPase α1 or β1 subunits between the two groups, protein levels of the Na^+/K^+-ATPase α1 subunit (the catalytic subunit) were about twofold lower in colonic crypts from DSS-treated mice (Figure 4).

Reduced activity of epithelial K^+ channels may contribute to reduced Cl^- secretory responses in colon from DSS-treated mice

Epithelial Cl^- secretion can only occur if K^+ is transported out of the epithelium. During Ca^{2+}-mediated Cl^- secretion,

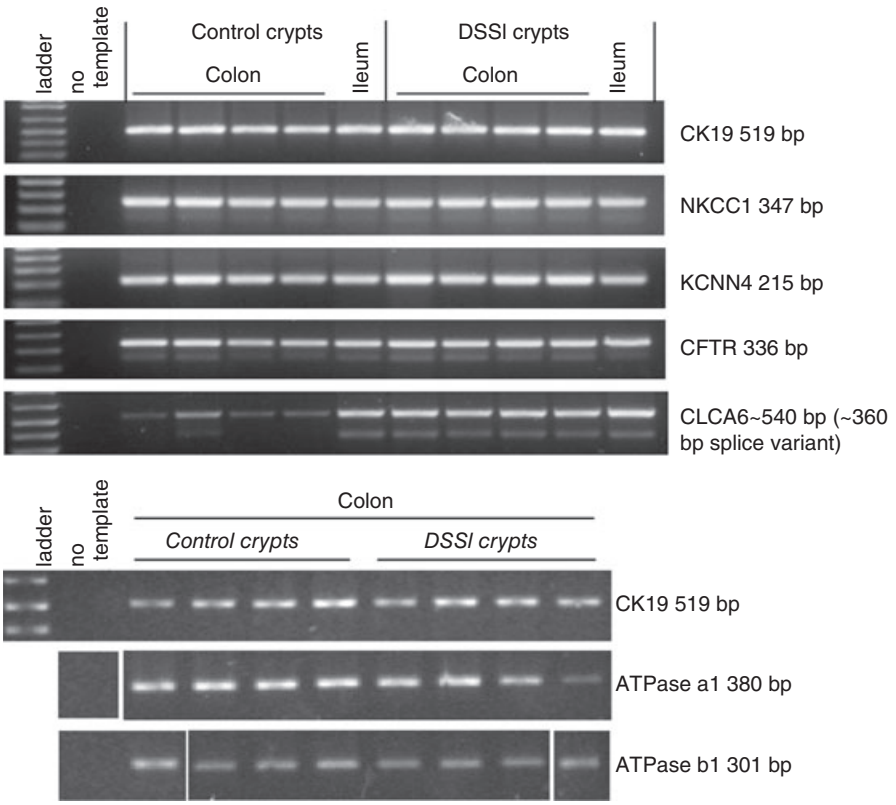

Figure 3 Decreased transcription of mRNA for epithelial ion transport molecules did not account for the lack of Ca^{2+}-mediated ion transport responses observed in colonic tissue from mice with DSS-induced colitis. Intestinal epithelial crypts were isolated from ileum (samples pooled) or colon of control and DSS-treated mice. RT-PCR was performed using specific primers to the epithelial-specific marker cytokeratin 19 (CK19, used as a housekeeping gene), the $Na^+/K^+/2Cl^-$ transporter (NKCC1), a Ca^{2+}-activated K^+ channel (Kcnn4), the cystic fibrosis transmembrane conductance regulator (CFTR), a Ca^{2+}-activated Cl^- channel (CLCA6), and the Na^+/K^+-ATPase (both the α1 and the β1 subunits, ATPaseα1 and ATPaseβ1). CLCA6 mRNA was paradoxically increased in colonic crypts from DSS-treated mice, while message levels for the other genes remained unchanged. Negative controls in which reverse transcriptase was omitted are not shown; each band represents colonic crypts extracted from separate mice.

the K^+ channels involved in balancing Cl^- secretion are intermediate-conductance Ca^{2+}-activated K^+ channels, specifically those transcribed from the Kcnn4 gene ($K_{Ca}3.1$ channels) (Hardcastle and Hardcastle, 1986; Joiner *et al.*, 2003). The Ca^{2+} sensitivity of this channel is conferred by calmodulin (CaM), which binds tightly to the C-terminal end of the channel (Fanger *et al.*, 1999; Jensen *et al.*, 2001). Activation of these channels with the benzimidazolone compound, DCEBIO, directly stimulates Cl^- secretion (von Hahn *et al.*, 2001). Furthermore, in colonic tissue from control mice, inhibition of CaM activity with 50 $\mu mol \cdot L^{-1}$ W-13 inhibits subsequent ion transport responses to bethanechol (ΔI_{SC} to bethanechol, $22.7 \pm 4.3\ \mu A \cdot cm^{-2}$; ΔI_{SC} to bethanechol + W13, $6.5 \pm 2.4\ \mu A \cdot cm^{-2}$; $n = 6$; $P < 0.05$), an effect that is likely due to its regulation of $K_{Ca}3.1$ channels.

Analysis of the $K_{Ca}3.1$ channel protein expression revealed no significant difference between colonic crypts from control mice and those from DSS-treated mice (Figure 5A; $n = 3$ mice per group). However, CaM expression was increased in crypts from DSS-treated mice (Figure 5A; $n = 3$ mice per group) as revealed by densitometry analysis normalized to expression of the house-keeping protein cytokeratin 19 (mean CaM/CK19 density = 0.81 ± 0.22 in control crypts vs. 2.00 ± 0.17 in DSS crypts, $P < 0.05$; $n = 3$ mice per group). Thus, it is possible that while $K_{Ca}3.1$ expression remains unchanged, its activity could be aberrant in the colon of DSS-treated mice.

Application of DCEBIO to the serosal side of colonic tissue from control mice produced a large increase in I_{SC} that remained elevated for up to 1 h (Figure 5B, top panel). The ability of DCEBIO to open Ca^{2+}-activated K^+ channels was confirmed by the addition of clotrimazole, a Ca^{2+}-activated K^+ channel inhibitor, which reduced the DCEBIO response by $69.0 \pm 5.8\%$ ($n = 6$; $P < 0.001$; bottom panel Figure 5B). Pretreating control colonic tissues with DCEBIO prior to bethanechol addition led to smaller bethanechol responses (Figure 5C, first two columns), while pretreatment of DSS tissue with DCEBIO did not restore bethanechol responses in this tissue (not shown). These results suggest that these drugs both converge on $K_{Ca}3.1$ to produce changes in ion transport and that this concentration of DCEBIO produces near maximal current responses through $K_{Ca}3.1$. We also confirmed that bethanechol-evoked increases in I_{SC} were dependent on Ca^{2+}-activated K^+ channel activity in control tissue by pretreatment with clotrimazole (Figure 5C, last two columns). These results show that basolateral K^+ channel activity is essential for muscarinic-receptor driven Cl^- secretory responses in normal mouse colon. As a control for the specificity of clotrimazole in our system, we examined the effects of this

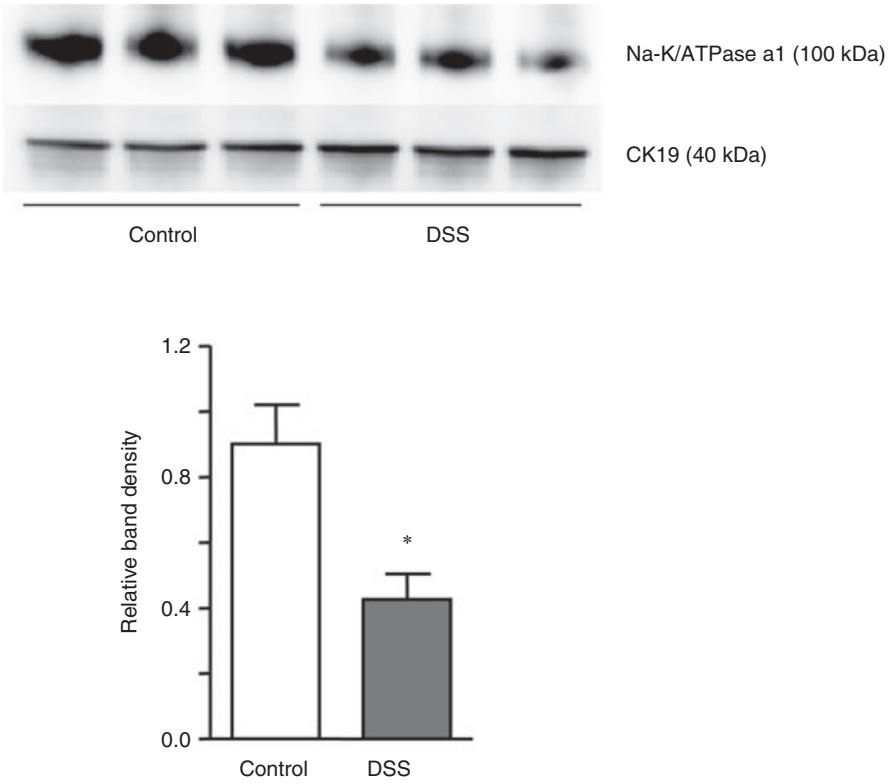

Figure 4 Decreased protein expression of epithelial Na^+/K^+-ATPase α1 was associated with DSS-induced colitis. The upper panel shows immunoblots of whole cell lysates from isolated colonic crypts probed with specific antibodies against Na^+/K^+-ATPase α1 (upper blot) and for the epithelial marker protein CK19 (lower blot). Each lane represents colonic crypts from an individual mouse sample. The lower panel shows relative densitometry of Na^+/K^+-ATPase α1:CK19 bands from the blots above (*$P < 0.05$; $n = 3$ mice per group).

inhibitor on I_{SC} responses to FSK (added at the end of each experiment). Clotrimazole (30 μmol·L^{-1}) had no effect on subsequent responses to 1 μmol·L^{-1} FSK (FSK: 84.5 ± 9.8 μA·cm^{-2}, $n = 14$; clotrimazole + FSK: 92.6 ± 8.8 μA·cm^{-2}, $n = 12$, mean ± SEM). When DCEBIO was used to evoke an increase in I_{SC} in colon from DSS-treated mice, the response was reduced by ~60% compared with controls (Figure 5D). Together, these data suggest that a dysfunctional basolateral K^+ channel is a significant contributing factor to the reduced responsiveness of DSS tissue to muscarinic- as well as Ca^{2+}-driven Cl^- secretion in general.

Ion transport responses to direct activation of PKC with PMA are absent in colonic tissue from DSS-treated mice

Protein kinase C (PKC) has been implicated in cholinergically mediated intestinal ion transport and Ca^{2+}-mediated ion transport in general (see Hirota and McKay, 2006). It is possible that the function of one or more PKC isoforms is altered during DSS colitis. Activation of PKC with the phorbol ester, PMA, can directly evoke a Cl^- secretory response in some studies (Lindeman and Chase, 1992; Vaandrager *et al.*, 1992; Kanwar *et al.*, 1995), although others have also reported inhibitory effects of PMA on both cAMP- and, in at least one study, Ca^{2+}-mediated Cl^- secretion (Dechecchi *et al.*, 1992; Matthews *et al.*, 1993; Reenstra, 1993; Shen *et al.*, 1993). Treatment with PMA resulted in increased I_{SC} in tissue from control mice but had negligible effects in colonic segments from mice with DSS-induced colitis (Figure 6). In addition, inhibition of classical and novel PKC isoforms with the pan-PKC inhibitor GF 103209X (1 μmol·L^{-1}) significantly inhibited bethanechol-induced I_{SC} responses in colonic tissue from control mice: bethanechol, 37.1 ± 10.2 versus GF 103209X + Bethanechol, 8.4 ± 2.5 μA·cm^{-2} ($n = 5$; $P < 0.05$). The effects of GF 103209X in these experiments did not significantly affect I_{SC} responses to FSK (10 μmol·L^{-1}: data not shown). Similar experiments could not be done with tissues from mice with DSS-induced colitis since they failed to respond to bethanechol exposure.

Discussion

Efficient regulation of water movement is critical in gut homeostasis, facilitating hydration at the epithelial surface, which allows for digestion and absorption and serves as an element of epithelial barrier function. An increase in electrogenic ion transport into the gut lumen or mucosa will lead to an increase in a similarly directed water movement: excessive water loss can result in life-threatening diarrhoea, while constipation and intestinal stasis can contribute to bacterial overgrowth with equally dire consequences. Diarrhoea is a hallmark symptom of inflammatory bowel disease, with decreased absorption of both Na^+ and Cl^- as a major contributing factor rather than increased Cl^- secretion (see Sandle, 2005). Furthermore, many studies report a

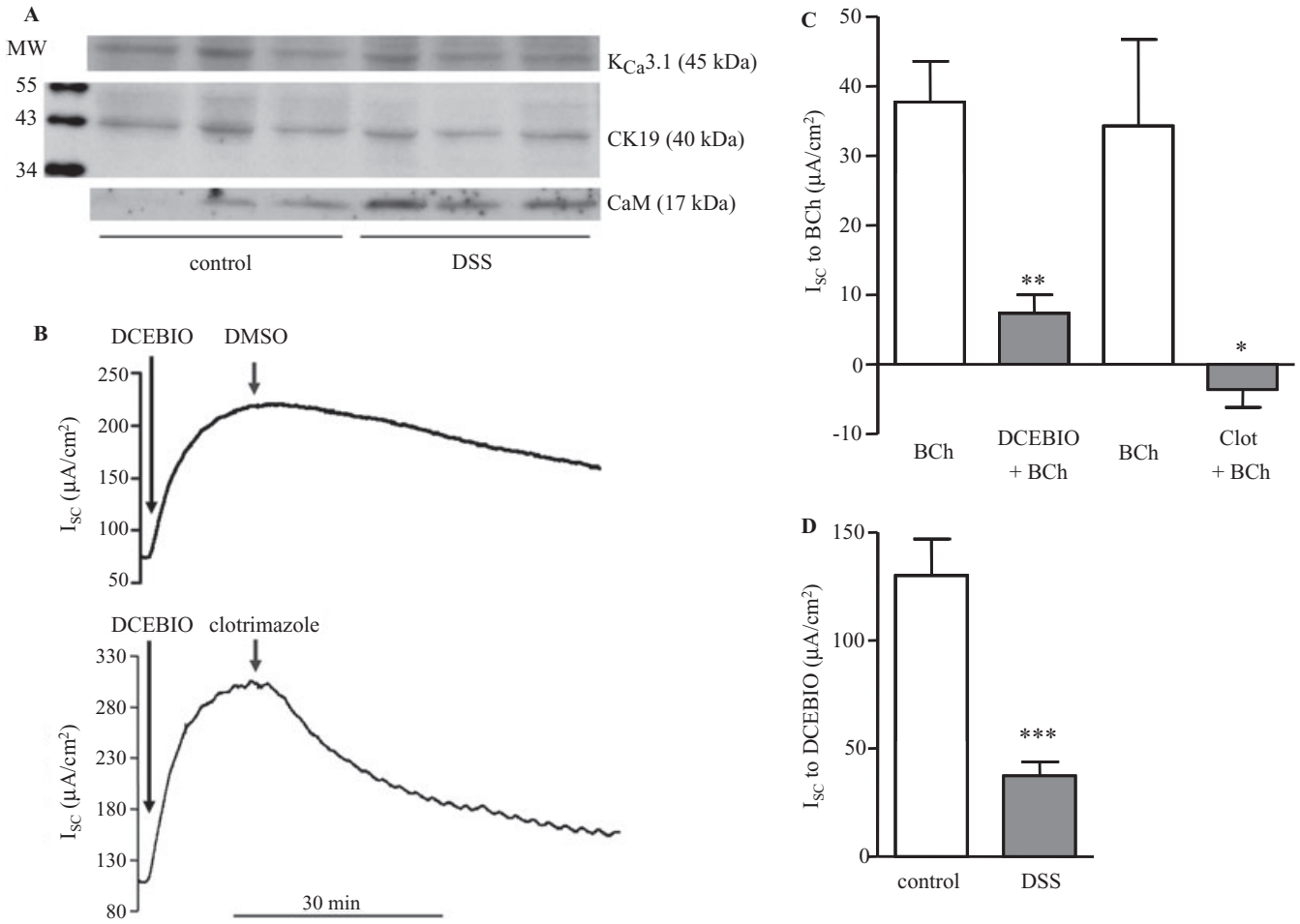

Figure 5 Activity of the $K_{Ca}3.1$ Ca^{2+}-activated K^+ channel was reduced in colonic epithelium from DSS-treated mice. (A) expression of the Ca^{2+}-activated K^+ channel $K_{Ca}3.1$ protein was not reduced in colonic crypts from DSS-treated mice compared to control (membrane was stripped and re-probed with an antibody against cytokeratin 19 as a loading control). Calmodulin expression was increased in DSS samples (lower panel, same blot as upper two panels stripped and re-probed for calmodulin). (B) representative current tracing showing the Ca^{2+}-activated K^+ channel activator DCEBIO (100 μmol·L^{-1})-induced increase in I_{SC} in tissue from a control mouse, and inhibition of the response by the Ca^{2+}-activated K^+ channel inhibitor clotrimazole (30 μmol·L^{-1}, added serosally; lack of a DMSO-vehicle effect is shown in the upper panel). (C) in colonic tissue from control mice, addition of bethanechol (BCh) at the peak of the I_{SC} response to DCEBIO further increased the ion transport response only minimally compared to the ΔI_{SC} observed with BCh challenge only (**$P < 0.01$; $n = 8$ mice) and inhibition of Ca^{2+}-activated K^+ channels with clotrimazole (Clot; 30 μmol·L^{-1}, added serosally) completely blocks subsequent ion transport responses to BCh (100 μmol·L^{-1}, added serosally) in tissue from control mice (*$P < 0.05$; $n = 6$ mice). (D) ΔI_{SC} stimulated by DCEBIO is significantly reduced in colonic tissues from DSS-treated mice compared to the control mice (***$P < 0.001$; $n = 7$–8 mice).

diminished capacity of tissues from patients with colitis and animal models of colitis to respond to pro-secretory stimuli (Kachur *et al.*, 1995; Crowe *et al.*, 1997; Diaz-Granados *et al.*, 2000; Perez-Navarro *et al.*, 2005a). Whether this represents a protective response to dampen water loss or is of pathophysiological significance (e.g. increasing bacterial contact with the epithelium) is unclear. The mechanisms underlying the reduced responsiveness of ion transport or secretory systems are not fully elucidated and vary depending on the secretory response examined and the nature of the colitis. For example: nitric oxide contributes to epithelial hyporesponsiveness in colitis induced by tri-nitrobenzene sulphonic acid (TNBS) or DSS (Asfaha *et al.*, 1999; Green *et al.*, 2004); decreased epithelial cAMP production has been implicated in the depressed secretory responses observed in TNBS colitis in rats (Sanchez de Medina *et al.*, 2002), but not rabbits (Goldhill *et al.*, 1993a), and alterations in the enteric nervous system can also contribute to the hypo-secretion observed in colitis (Goldhill *et al.*, 1993b; Sanchez de Medina *et al.*, 2002; Green *et al.*, 2004; Perez-Navarro *et al.*, 2005b).

Using the mouse model of DSS-induced colitis, we found that I_{SC} responses to FSK (mobilizes cAMP) and carbachol (mobilizes Ca^{2+}) were both significantly reduced compared with controls and, moreover, the response to carbachol was a drop in I_{SC} and not the expected transient increase in I_{SC} (Sayer *et al.*, 2002). Inhibition of nitric oxide signalling prevented the drop in I_{SC} evoked by carbachol challenge, but failed to restore the increase in I_{SC}, which is typically mediated by Cl^- efflux (Green *et al.*, 2004). In the present study, we used the cholinergic muscarinic receptor agonist, bethanechol, and confirmed that colonic segments from mice with DSS-induced colitis were completely unresponsive to muscarinic stimulation.

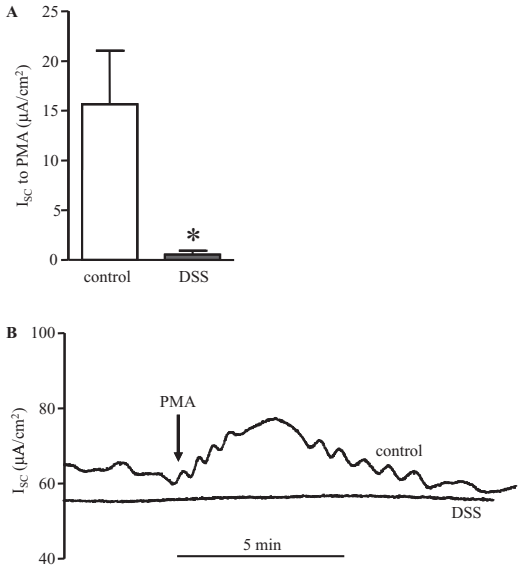

Figure 6 (A) Colonic tissue from DSS-treated mice did not respond to activation of PKC by the phorbol ester PMA (1 μmol·L^{-1}, added serosally and mucosally) with an increase in I_{SC} (*$P < 0.05$; $n = 4–5$ mice). (B) I_{SC} tracings showing typical responses of mid-distal colonic segments from control and DSS-treated mice to PMA.

There is evidence of cross-talk between Ca^{2+}- and cAMP-driven ion transport responses (Dharmsathaphorn and Pandol, 1986; Mall *et al.*, 1998). Furthermore, a synergistic relationship exists between agents that activate serosal-to-mucosal Cl^- conductances at the apical membrane (i.e. FSK) and those that increase basolateral mucosal-to-serosal K^+ conductance (e.g. carbachol) in eliciting a net current response (Devor *et al.*, 1996). Thus, we postulated that stimulation with FSK prior to bethanechol could engage the ion transport machinery and restore, at least partially, subsequent responses to bethanechol. Contrary to this prediction, pretreatment with FSK had no significant effect on subsequent bethanechol responses in DSS tissue, indicating, in our view, a very profound perturbation of Ca^{2+}-regulated Cl^- secretion in the colitic epithelium.

Assessing the mechanism of the epithelial hyporesponsiveness to bethanechol, RT-PCR and immunodetection analyses revealed neither loss nor reduced expression of the muscarinic M_3 receptor, suggesting that the defect lay downstream of this muscarinic receptor. We, and others (van Bergeijk *et al.*, 1998; Depoortere *et al.*, 1999; Gonzalez and Sarna, 2001), observed decreased contractile responses in longitudinal muscle from mice with DSS-induced colitis, which occurred in the absence of any shift in the EC_{50} for bethanechol (unpubl. data), further pointing towards a problem downstream of the muscarinic receptor.

An immediate consequence of activation of epithelial M_3 receptors is a rise in intracellular Ca^{2+} that leads to increased ion channel activity, with Cl^- efflux across the apical cell membrane as a primary consequence (Hirota and McKay, 2006). Use of four different agents that evoke increases in I_{SC} via Ca^{2+}-activated Cl^- efflux revealed diminished responses in tissue from colitic mice compared with those in control tissue: responses to histamine [acting through H_1 receptors (Wang *et al.*, 1990; Keely *et al.*, 1995)], thapsigargin and CPA (both SERCA-pump inhibitors that prevent Ca^{2+} re-entry into intracellular stores leading to cytosolic accumulation), and the calcium ionophore A23187 were reduced in colonic tissues from DSS-treated mice. Thus, one consequence of the inflammatory milieu in DSS colitis is the inability of enterocytes to appropriately mobilize and/or direct Ca^{2+} signals from intracellular or extracellular sources to drive electrogenic Cl^- secretion.

The appropriate expression and placement of ion transport channels, pumps and co-transporters in the cell membrane is critical to electrogenic ion fluxes. In our model, assessment of the mRNA levels of NKCC1, the CFTR Cl^- channel, the intermediate-conductance Ca^{2+}-activated K^+ channel Kcnn4, the Ca^{2+}-activated Cl^- channel CLCA6 (Evans *et al.*, 2004) and subunits of the Na^+/K^+-ATPase pump revealed no obvious decreases in colonic crypts from DSS-treated mice. Indeed, CLCA6 mRNA expression, both full-length transcript and shorter splice variant (of unknown function), was increased in colonic crypts from DSS-treated mice, which may represent an attempt to compensate for the hyporesponsiveness to Ca^{2+}-dependent secretagogues during colitis. Because RT-PCR is not particularly quantitative and does not address potential post-transcriptional alterations associated with DSS colitis, we also examined protein expression of ion transport molecules. Corroborating the RT-PCR results, protein levels of NKCC1 and CFTR appeared unaltered by DSS-induced colitis. However, the α1 subunit of the Na^+/K^+-ATPase pump was decreased twofold in samples from DSS-treated mice. Since Na^+ and K^+ transport as well as ATPase function are attributed to this subunit [the structural β subunit allows assembly and membrane insertion of the pump; see Aperia (2007)] and because activity of the Na^+/K^+-ATPase pump is required for all forms of active epithelial ion transport, decreased α1 expression almost certainly contributes to the overall hyposecretory state observed during DSS-induced colitis. This is in keeping with findings in which human small intestinal xenografts (Bertelsen *et al.*, 2004) and T84 cells (Sugi *et al.*, 2001) treated with IFNγ, a pro-inflammatory cytokine which is increased in DSS colitis (Egger *et al.*, 2000; Ito *et al.*, 2006), displayed decreased expression of the α subunit of the Na^+/K^+-ATPase pump, which resulted in a decreased Cl^- secretory response to carbachol.

At its worst, ulcerative colitis can be associated with hypokalaemia, attributable to severe diarrhoea-induced loss of electrolytes (Fedorak and Thomson, 2000). Decreased expression and/or activity of basolateral epithelial K^+ channels during colitis could contribute to this problem indirectly by greatly diminishing a major epithelial K^+ recycling pathway. Indeed, Sandle *et al.* (2004; 2005) report that whole cell K^+ conductance is decreased in patients with ulcerative colitis, which was mainly attributed to impairment of basolateral Ca^{2+}-activated K^+ channel activity [recent data from this group implicate a K^+ channel in the apical cell membrane in this event (Sandle *et al.*, 2007)]. As DSS colitis in mice recapitulates some aspects of human ulcerative colitis and because epithelial Cl^- secretion is critically dependent on basolateral K^+ recycling, further assessment of the epithelial ion transport machinery was focused on the Ca^{2+}-activated K^+ channel, $K_{Ca}3.1$ (i.e. the Kcnn4 gene product). $K_{Ca}3.1$ protein

expression was unaltered by DSS-induced colitis, while expression of calmodulin, which tightly binds to $K_{Ca}3.1$ and confers its Ca^{2+} sensitivity, was increased in the same extracts. While the full significance of increased calmodulin expression needs to be determined, it may, like the increased expression of the Ca^{2+}-activated Cl^- channel, be an attempt to compensate for reduced Ca^{2+}-mediated ion transport. Activation of $K_{Ca}3.1$ with DCEBIO in control tissue evoked a large increase in I_{SC} and bethanechol responses require activation of this channel, as I_{SC} responses to bethanechol were reduced by the $K_{Ca}3.1$ channel inhibitor clotrimazole and by pretreatment with DCEBIO. This latter point may also reflect that treatment of colonic tissue with DCEBIO brings the epithelium close to its maximum secretory capacity (ΔI_{SC} responses to DCEBIO were greater than 200 $\mu A \cdot cm^{-2}$ compared with typical bethanechol responses of <70 $\mu A \cdot cm^{-2}$). However, normal expression of $K_{Ca}3.1$ protein need not necessarily translate into normal channel activity. Indeed, this was the case with colon from DSS-treated mice, in which I_{SC} responses to DCEBIO were significantly reduced. Interestingly, the parent compound of DCEBIO, EBIO, has been shown to increase intracellular cAMP levels in colonic crypts (MacVinish *et al.*, 2001), which may explain the residual ion transport responses to DCEBIO in tissue from DSS-treated mice and why clotrimazole could not completely block these responses. Collectively, these data suggest that DSS-induced colitis is associated with significantly reduced $K_{Ca}3.1$ channel activity, which is not due to reduced calmodulin expression, and that reduced $K_{Ca}3.1$ activity contributes to the tissues' non-responsiveness to Ca^{2+}-driven Cl^- secretagogues.

The $K_{Ca}3.1$ channel contains two putative PKC phosphorylation sites (Jensen *et al.*, 2001) and reduced PKC expression and activity can occur as a consequence of colitis (Sakanoue *et al.*, 1992). Thus, altered PKC signalling could underlie the reduced $K_{Ca}3.1$ activity in the colon of mice treated with DSS (Brown *et al.*, 1999; Chang *et al.*, 2000). Use of the pan-PKC inhibitor GF 103209X indicated a role for PKC in bethanechol-induced increases in I_{SC} in control tissue. A similar approach could not be applied to tissues from DSS-treated mice as they did not respond to bethanechol and hence there was no response to block pharmacologically. The absence of a secretory response in DSS tissue to the PKC activator, PMA, could point to dysfunctional PKC signalling, but could equally reflect inactive $K_{Ca}3.1$ channels. Thus, a putative role for PKC in regulating $Ca^{2+}/K_{Ca}3.1$-mediated epithelial Cl^- secretion will require further research.

Speculating on the significance on the hyporesponsive nature of colonic epithelium from mice with DSS-induced colitis, we see this as an element of the integrated tissue response to inflammation. For instance, enhanced cholinergic activity can be immunosuppressive, but if this results in excessive secretory diarrhoea (i.e. ACh-driven epithelial Cl^- secretion), then the net outcome of increased ACh release would be detrimental and not beneficial. Thus, a temporary loss of the epithelium's responsiveness to ACh would, at least theoretically, allow the benefits of the immunosuppression to dominate.

In conclusion, DSS-induced colitis in mice results in a profound hyporesponsiveness of the colonic epithelium to prosecretory agents and a complete loss of response to muscarinic receptor activation. The general hyposecretory phenotype, as exemplified by reduced ion transport responses to FSK, is very likely attributable in large part to reduced expression of the $\alpha 1$ subunit of the Na^+/K^+-ATPase pump, which is absolutely required for all forms of active ion transport in the intestine. The complete loss of muscarinic receptor-driven ion transport did not arise from the loss of M_3 receptor expression or from a reduced expression of ion transport molecules selectively associated with Ca^{2+}-driven ion transport (i.e. CLCA6, $K_{Ca}3.1$); rather, the current pharmacological analysis likely reflects an inability to activate appropriately $K_{Ca}3.1$ channels, although other events such as altered Ca^{2+} handling remain a possibility. Currently, the identity of the inflammatory mediator(s) that evokes this change is unknown and the precise relationship between Ca^{2+}, calmodulin and $K_{Ca}3.1$, as well as perhaps PKC isoforms, remains to be defined. However, the current study has revealed that in designing therapeutic agents to regulate intestinal epithelial water balance, consideration of Na^+ and Cl^- channels needs to be complemented by an awareness of the activation and putative perturbations in Ca^{2+} signalling and $K_{Ca}3.1$ channel activity.

Acknowledgements

This work was supported by funding from the Canadian Institutes of Health Research (MT-13421) and the Crohn's and Colitis Foundation of Canada. D.M.M. is a recipient of a Canada Research Chair (Tier 1) and an Alberta Heritage Foundation for Medical Research (AHFMR) Scientist Award. C.L.H. was funded by an Ontario Graduate studentship.

Statement of conflicts of interest

None.

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