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Cholinergic receptor activation on epithelia protects against cytokine-induced barrier dysfunction

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Abstract

Aim: Various types of cholinergic receptors are expressed on intestinal epithelia. Their function is not completely understood. We hypothesize that cholinergic receptor activation on epithelium may serve a protective function in cytokine-induced barrier dysfunction.

Methods: The effect of cholinergic receptor activation on cellular barrier function in epithelial cells was assessed by measuring electrical impedance, and by determining para-cellular transport in transwell experiments. Cell lysates treated with cytokine and/or cholinergic agonists were analysed for cyto- and chemokine production, and tight junction (TJ) protein rearrangement was assessed. Primary colonic epithelial cells were isolated from surgically resected colon tissue of patients with inflammatory bowel disease.

Results: IL-1β induced production of chemokines (CXCL-1, CXCL-10, IL-8, CCL-7) and led to a rearrangement of TJ proteins (occludin and ZO-1). This response was inhibited by pre-treatment with muscarinic, rather than nicotinic, acetylcholine receptor agonists. Treatment with IL-1β enhanced paracellular permeability (4kD dextran) and reduced impedance across the monolayer, which was counteracted by pre-incubation with acetylcholine, or muscarinic receptor agonist bethanechol. The protective effect of acetylcholine was antagonized by atropine, underscoring muscarinic receptor involvement. IL-1β induced transcription of myosin light chain kinase and phosphorylation of myosin light chain, and this cytokine-induced phosphorylation of MLC was inhibited by muscarinic receptor agonists. Furthermore, in epithelial cells from resection material of patients with Crohn's disease and ulcerative colitis, high expression of *CXCL-8* was associated with a reduced *choline acetyl transferase* expression, suggesting an aberrant epithelial production of ACh in inflammatory context.

Conclusion: Acetylcholine acts on muscarinic receptors on epithelial cells to maintain epithelial barrier function under inflammatory conditions.

Keywords cholinergic receptors, epithelial permeability, interleukin-1 beta, myosin light chain, tight junction proteins.

Aberrant barrier function is the basis for the pathogenesis of inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC). Excessive activation and recruitment of proinflammatory immune cells to the mucosa and enhanced flux of luminal antigens into the mucosa are the hallmarks of IBD. Patients with UC exhibit increased intestinal epithelial permeability due to the disruption of TJ proteins (Schmitz *et al.* 1999, Gitter *et al.* 2001). TJs between epithelial cells are the major component of the epithelial barrier and are known to be highly dynamic, opening and closing in response to a number of signalling pathways. This increased gut permeability is reportedly due to a redistribution of TJ proteins such as occludins and claudins, contributing to enhanced permeability. In biopsies from patients with CD, expression of sealing claudin-3, -5 and -8 and occludin was diminished, while pore forming claudin-2 was upregulated (Das *et al.* 2012). It remains debatable whether enhanced intestinal permeability leads to pathogenesis of IBD or is a consequence of inflammation in the gut. Nevertheless, increased permeability was shown to relate to disease severity in patients suffering from Crohn's disease, indicating that intestinal permeability does indeed participate in disease progression (Arnott *et al.* 2000). Furthermore, TJ proteins have also been shown to mediate other cellular processes, such as wound healing and cell proliferation. Recent evidences also indicate a dysbiosis in the intestine of affected individuals with IBD (Tamboli 2004). An altered symbiotic vs. pathobiotic microbe ratio in the intestine, along with a compromised epithelial barrier and impaired wound healing, may elicit inflammatory responses locally or systemically (Round & Mazmanian 2009, Ayres *et al.* 2012). This suggests that developing therapies that restores intestinal TJ integrity may have wide-reaching applications in treatment of patients with IBD.

It is now well established that excessive production of inflammatory cytokines released by immune cells infiltrating inflamed mucosa (e.g. TNFα, IFNγ, IL-1β and IL-4) induce paracellular permeability in intestinal epithelium in several ways. Recent *in vitro* studies in epithelial cell lines demonstrate that the pro-inflammatory cytokine IL-1β enhances phosphorylation of MLC and paracellular permeability via MEKK-1-mediated activation of canonical NF-κB pathway (Al-Sadi & Ma 2007). It has been shown that via release of ACh, the enteric nervous system also regulates intestinal barrier function (Nzegwu & Levin 1994, Neunlist *et al.* 2007). In homeostatic conditions, stimulation of muscarinic ACh receptors (mAChR) enhances endocytosis (transcellular permeability) of antigens (HRP) in colonic T84 cells and mouse epithelia (Cameron & Perdue 2007). *In vivo* studies suggest that cholinergic agonists reduce conductance across the epithelium (Sheldon *et al.* 1989, Chandan *et al.* 1991, Hayden & Carey 2000), and muscarinic agonists increase paracellular permeability in intestinal epithelium by acting on ACh-stimulated ion channel function (Phillips & Phillips 1987, Greenwood & Mantle 1992, Bijlsma *et al.* 1996). The discrepancies in these studies may be attributed to the use of dissimilar *in vitro* and *in vivo* methods of assessing permeability. However, mice deficient for M3 muscarinic receptor are more susceptible to DSS-induced colitis a phenotype seemingly unrelated to ion channel function (Hirota & McKay 2006a, Hirota & Mckay 2006b). We thus reasoned that in an inflammatory context, when cytokines such as IL-1β and TNFα are abundantly present in the underlying gut mucosa, cholinergic receptor agonists might support barrier function at the level of cytokine receptor signalling, or by ACh-mediated inhibition of NF-kB activity as described earlier (Van Der Zanden *et al.* 2009). A prime source of ACh in the gut is cholinergic neurones, although non-neuronal ACh producing cells are abundant. Notably, epithelial cells express enzymes such as acetylcholine transferase (ChAT), and choline transporters, to allow endogenous ACh production (Klapproth *et al.* 1997, Takahashi *et al.* 2014), allowing for autocrine regulation of mAChR activation.

In the current study, we aimed to investigate the role of AChR signalling in modulating epithelial barrier function under cytokine-induced inflammatory conditions and to highlight the molecular mechanisms underlying this effect. We describe that cholinergic receptor activation on epithelium blocks cytokine-induced phosphorylation of MLC and protects barrier function of the epithelial cell layer. Aberrant cholinergic signalling in the intestine may well affect barrier protective mechanisms in patients suffering from chronic inflammatory diseases of the gut.

Materials and methods

Cell culture

Human epithelial cell line Caco2, HT29 and mouse epithelial cell line CMT93 (purchased from ATCC, Manassas,VA, USA) were grown to confluence in a 24-well plate (5×10^5 cells well^{-1}) using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 1% penicillin and streptomycin and non-essential amino acids. The cells were kept at 37°C in a 5% CO_2 environment. Culture medium was changed every 3 days. Caco2 cells were subcultured after partial digestion with 0.25% trypsin and 0.9 mM EDTA in Ca^{2+} and Mg^{2+} free PBS. For experiments, Caco2 monolayers were cultured for 3–4 weeks after seeding. HT-29 and CMT93 cells were used for experiments 3–5 days post-seeding. On the

day of stimulation, cells were washed twice with PBS and stimulated with ACh (1 nM–1 μM), bethanechol (1 nM–1 μM), neostigmine (50 μM) or atropine (50 μM) followed by incubation with IL-1β (10 ng mL^{-1}) in DMEM for indicated time points.

Cytometric bead array (CBA)

IL-6, IL-10, IL-12p70, CCL-2 and TNF-α were measured in Caco2 homogenates according to the instructions of the manufacturer (Becton Dickinson Biosciences, San Diego, CA, USA). Briefly, 5 μL of sample or the cytokine standard mixture was mixed with 5 μL of the mixed capture beads and 5 μL of the detection antibody-phycoerythrin (PE) reagent and incubated at room temperature for 2 h in the dark. Two-colour flow cytometric analysis was performed using a FACScan® flow cytometer [Becton Dickinson Immunocytometry Systems (BDIS), San Jose, CA, USA]. Data were acquired and analysed using Becton Dickinson CBA software.

Quantitative reverse transcriptase-PCR

Cells were collected and RNA was extracted using TRIpure (Roche, Manheim, Germany); genomic DNA was removed using DNase (Promega, Madison WI, USA), and cDNA was synthesized from 1 μg of total RNA using cDNA synthesizing kit (Thermo Scientific, Vilnius, Lithuania). The SYBR green-based real-time PCR technique was used to detect the expression of transcripts. The cDNA was diluted fourfold for the real-time PCR assay. The PCR mixture consists of 1 μL cDNA, 5 μL SYBR-green master mix (Roche) and 1 mM of each primer in a total volume of 10 μL. Real-time PCR was performed using the Lightcycler 480 (Roche) detection system. Cycling conditions used were 95°C for 15s and 60°C for 1 min, for 40 cycles. Data were analysed using the linreg software, and results were expressed as fold difference relative to the geometric mean expression of the reference genes β-actin and ß2-macroglobulin (B2M).

Measurement of epithelial barrier integrity

Barrier function across Caco2 monolayers was assessed using apical 4kD fluorescein isothiocyanate (FITC)-conjugated dextran (Sigma, St Louis, MO, USA) with cells cultured on 0.9 cm^2 filter inserts. After incubation for 72 h with cytokine and cholinergic agonists, the basal and apical medium was analysed. The medium in the lower chamber was refreshed with medium, and the apical medium was replaced with 250 μM FITC-dextran solution. Samples (100 μL) were taken at 0, 30, 60, 90 and 120 min from the basolateral side and replaced with an equal volume of fresh medium for each monolayer examined. Fluorescence was measured in opaque 96-well Nunc™ plates (Fisher Scientific UK, Loughborough, UK) using an FLX-800 Fluorometer (Bio-Tek Instruments, Winooski, VT, USA) as described (Prasad *et al.* 2005). FITC-dextran flux was calculated as pmol h^{-1} $cm^{2-}1$, normalized to IL-1β (100% flux) and expressed as the percentage change. Impedance measurements were performed using ECIS technology (Applied Biophysics, Troy, NY, USA). Stimulations of the epithelial cells were performed in quadruplicates and were carried out as described previously (Hiemstra *et al.* 2014).

Scratch assay

Cells were seeded in 6-well plates until confluence and incubated overnight in serum-free medium. The confluent cell layer was scratched using a 1000-μL pipette tip, and the compounds were added and layered with mineral oil (Sigma, nr. M3516) to prevent evaporation of the culture medium. Cell cultures were imaged using a Leica IR-BE (Leica Microsystems GmbH, Germany) inverted wide field microscope at 37°C in an atmosphere containing 5% CO_2. Phase contrast images were acquired at 10 min time intervals for 72 h using a 10× objective. Images were processed and analysed using custom-made software and IMAGE PRO PLUS (Mediacybernetics, Carlsbad, CA, USA).

Fluorescence microscopy

Monolayers were fixed in 2% paraformaldehyde in PBS and stained as described previously (Zolotarevsky *et al.*, 2002). Rabbit polyclonal antibodies against ZO-1, occludin and P65 were from Zymed (South San Francisco, CA, USA). Alexa Fluor 488- and Alexa Fluor 594-conjugated goat anti-rabbit and goat anti-mouse antibodies, Alexa Fluor 488-conjugated phalloidin and Hoechst 33342 were from Invitrogen. Stained monolayers were mounted in vinol and images collected using 63× N.A. 1.32 or 100× N.A. 1.35 PLAN APO objectives mounted on a Leica DMLB microscope equipped with an 88000 filter set (Chroma Technology, Brattleboro, VT, USA).

Epithelial fraction isolation

Resected colonic tissue (10 cm^2) from UC, CD and unaffected controls was dissected from the submucosa, and processed as described earlier (Prasad *et al.* 2005). The tissue pieces were separated with a strainer and taken up in fresh isolation buffer. Loosened cells were confirmed epithelial cell type by light micros-

copy, centrifuged, resuspended in TRIpure and stored at −80 °C for RNA isolation. Patient material was gathered along guidelines described in Good Publication Practice in Physiology, and the study is conform with Persson PB. Good Publication Practice in Physiology 2013 Guidelines for Acta Physiol (Oxf). 2013 Dec;209(4):250–3.

SDS–PAGE and western blotting

Western immunoblotting was carried out according to (Prasad *et al.* 2005). Membranes were probed sequentially with rabbit anti-human claudin 3, rabbit anti-human occludin, rabbit anti-human phospho-IKKα/β, rabbit anti-human IKKα/β, mouse anti-human MLC and rabbit anti-human phospho-MLC. All blots were probed with mouse anti-human Na/K ATPase (Dako) as a loading control for epithelial proteins. The secondary antibodies were HRP-rabbit anti-mouse and HRP-goat anti-rabbit (Dako) and were detected with the ECL Plus kit (Amersham Biosciences, Piscataway, IL, USA).

Measurement of cell viability

Cell viability was assessed by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay based on the reduction of MTT by mitochondrial dehydrogenases of viable cells to a purple formazan product (Mosmann, 1983). Caco2 cells were plated in transwells for 21 days. After differentiation, cells were treated with IL-1β or cholinergic agonists for 72 h. Subsequently, the cells were washed with 200 μL of PBS and incubated with 100 μL of 500 μg mL^{-1} MTT in PBS at 37 °C for 3 h. The MTT-formazan product dissolved in 200 μL of DMSO was estimated by measuring the absorbance at 570 nm in a Bio-Rad multi-well plate reader, and the IC_{50} was calculated. The cell viability of Caco2 cell line was expressed as the per cent viability of treated cells compared with the untreated control.

Cytokine Elisa

After 24-h incubation, the supernatants were collected and subjected to ELISAs for several cytokines, according to the instructions provided by the manufacturer (TNFα and IFNγ, BD Bioscience; IL-1β, R&D Systems, Minneapolis, MN, USA).

Statistical analysis

Data are presented as mean ± SE. All experiments were performed with triplicate or greater samples, and data shown are representative of three or more independent studies. P values were determined by ANOVA and were considered to be significant if $P < 0.05$.

Results

Fully differentiated Caco2 cells respond to the proinflammatory cytokines IL-1β and TNFα

First, we used differentiated human colonic adenocarcinoma cells lines (Caco2) as an *in vitro* model, to assess cytokine and chemokine production by CBA array (Fig. 1a). We established that differentiated Caco2 cells did not respond to various TLR/NOD ligands (LPS, MDP, PGN and LTA), whereas cytokines (IL-1β and TNFα) elicited a significant IL8 release (Fig. 1b). Besides IL-8, IL-1β and TNFα significantly induced transcription of GRO, IP10, CCL-2 and CCL-7 (Fig. 1a), while other cytokines were below detection limits (data not shown). This increased release of IL-8 was not associated with cytotoxicity or cell death (Fig. 1c), as no changes in cell viability were observed in presence of IL-1β or ACh. The data indicate that IL-1β-induced IL-8 was a relevant readout for immunological activation in differentiated Caco2 cells.

Muscarinic cholinergic receptors counteract IL-1β-induced epithelial barrier dysfunction

Next, we examined changes in paracellular permeability and its association with cholinergic receptor activation. Previous studies have reported an enhanced flux of HRP across the epithelium in presence of cholinergic agonist carbachol (Cameron & Perdue 2007). In resting cells, no increase in paracellular permeability in the presence of ACh was observed (Fig. 2a). However, consistent with previous findings, co-incubation with IL-1β (10 ng mL^{-1}) significantly induced paracellular flux of FITC-dextran (4 kD) across Caco2 monolayers in a progressive time-dependent manner (Fig. 2b). To assess whether cholinergic receptor activation affected IL-1β-induced TJ permeability, we pre-incubated fully differentiated Caco2 cells with ACh (1 nM–1 μM) for 72 h. Pre-incubation with ACh significantly attenuated IL-1β-induced barrier loss in a dose-dependent manner (Fig. 2b).

A variety of both mAChR and nicotinic AChRs (nAChRs) are expressed on intestinal epithelia (Richardson *et al.* 2003, Takahashi *et al.* 2014). To assess whether nAChR or mAChR were involved, we next investigated whether pre-incubation with nAChR agonist nicotine or mAChR agonist bethanechol ameliorated IL-1β -induced paracellular permeability.

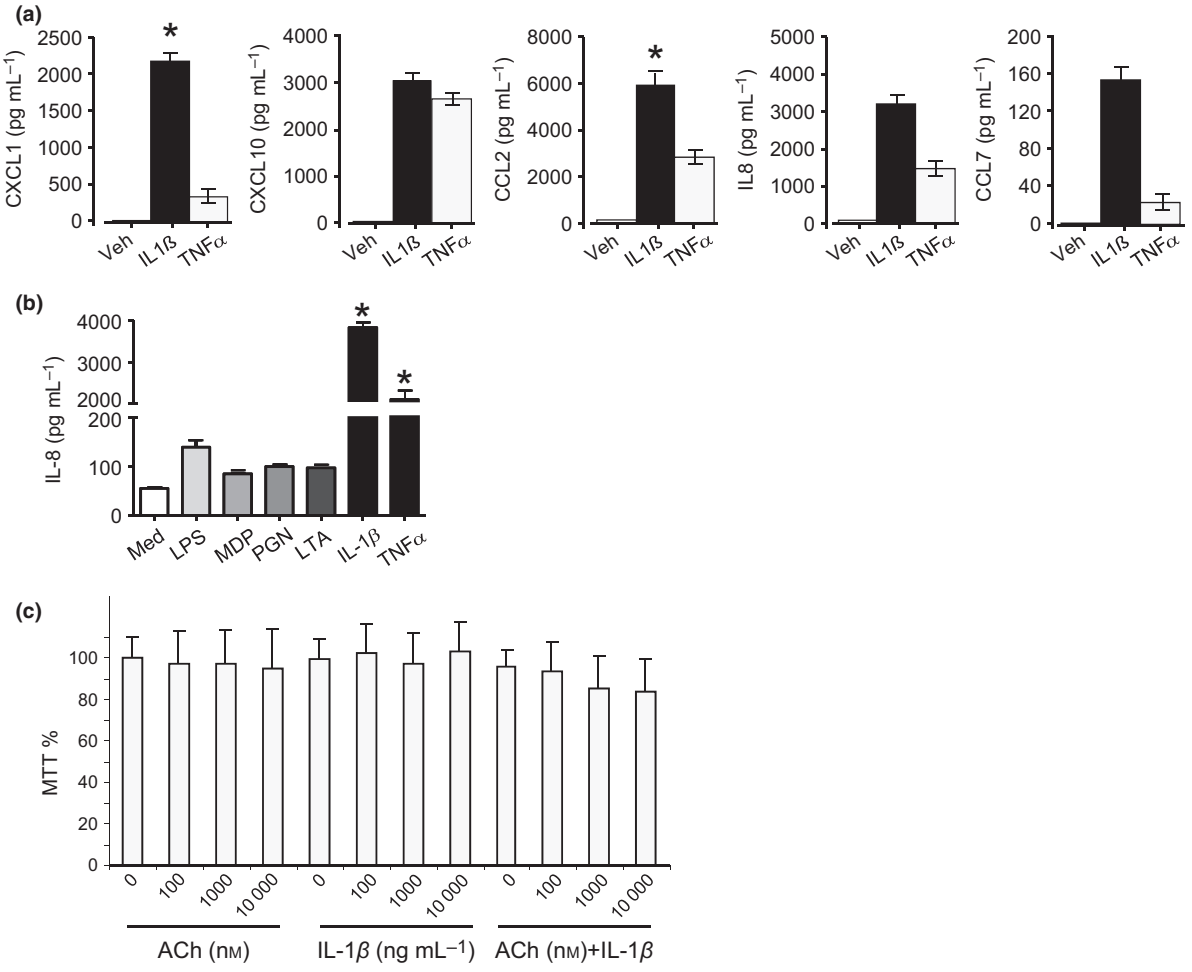

Figure 1 Differentiated Caco2 cells respond to IL-1β and TNFα. (a) Polarized Caco2 monolayers were stimulated with IL-1β (10 ng mL^{-1}) and TNFα (10 ng mL^{-1}). After 24 h, release of GRO, CXCL10, CCL2, IL-8 and CCL7 was measured in the supernatant by cytokine bead array. *$P < 0.05$. Values shown represent $n = 3 \pm$ SEM. (b) Caco2 cells were treated with indicated cytokines or TLR/NOD ligands (Med indicates Medium). IL-8 levels were determined by ELISA 24 h after stimulation. (c) Differentiated Caco2 cells were treated with IL-1β (10 ng mL^{-1}), ACh (1 μM), IL-1β +ACh 72 h after which cell viability was measured by MTT assay.

Pre-incubation with bethanechol (1 nM–1 μM) significantly antagonized IL-1β -induced dextran flux (Fig. 2c). In contrast, nicotine (1 nM–1 μM) failed to affect IL-1β-induced paracellular permeability (Fig. 2d). Furthermore, atropine (50 μM) blocked the protective effects of ACh (Fig. 2e), resulting in an enhanced flux of FITC-dextran upon IL-1β incubation, which strongly suggests that muscarinic receptors and not nicotinic receptors mediate paracellular permeability in Caco2 cells. Furthermore, to confirm that the observed effects of cholinomimetics were exclusive to paracellular transport of macromolecules and not transcellular (endocytic) transport, we performed a real-time assessment of low-voltage (500v) electrical impedance changes (measured by ECIS) across a differentiated monolayer of Caco2 cells. In correspondence to our earlier observed protective effects of muscarinic receptor activation on IL1 receptor signalling, similar protective effects of cholinergic receptor activation on electrical impedance in Caco2 cells were noted (Fig. 2f and a sample impedance tracing shown in g). Hence, our ECIS data confirmed blocking of IL-1β-mediated decrease in cellular impedance. Moreover, as observed with permeability studies, cholinergic receptor-mediated effects were significantly abolished by pre-incubation with atropine (50 μM), confirming that protective effects are mediated via mAChRs. However, no protective effects of mAChRs were observed in high-voltage (>5000V) ECIS studies (data not shown), suggesting that cholinergic receptors modulate cell–cell TJ rearrangement, rather than processes involved in endocytosis or transcytosis.

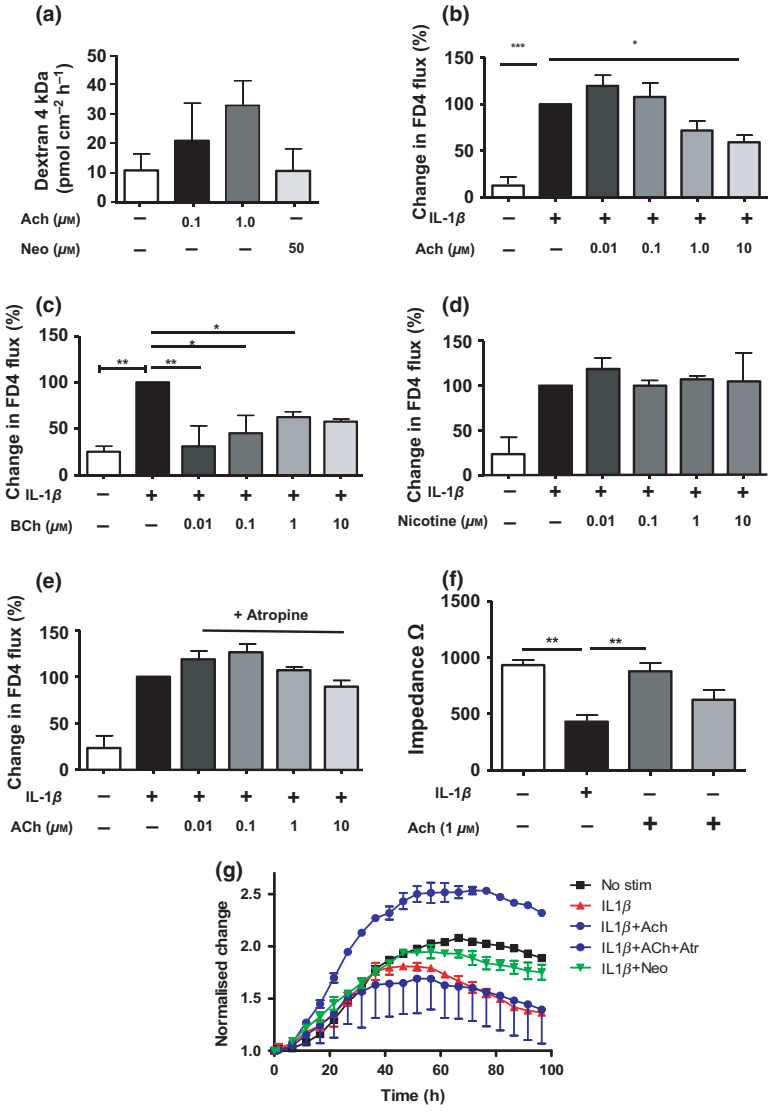

Figure 2 IL-1β increases Caco2 monolayer permeability. Caco2 cells were cultured in transwell plates for 21 days, and IL-1β (10 ng mL^{-1}) was added to the medium basolaterally to the monolayer for 72 h. Cholinergic agonists (0.01–10 μM) were applied basally 10 min before IL-1β treatment. (a) Flux of FITC-dextran measured after incubation of Caco2 cells with ACh (0.1–1 μM) (b, c, d, e) paracellular flux of FITC-dextran measured at 72 h after co-incubation of Caco2 cells with ACh (0.01–1 μM), bethanechol (0.01–1 μM), nicotine (0.01–1 μM) and IL-1β (10 ng mL^{-1}). (Means ± SE, n = 5) (f and g) changes in cell impedance over time with compounds (Atr = atropine 50 μM, Neo = neostigmine 50 μM) added as indicated, measured by electrical impedance (ECIS). In panel F, ECIS data are quantified at time point 72 h of measurement. (Means ± SEM, n = 4).

Cholinergic receptors influence epithelial barrier function by modulating TJ protein expression in Caco2 cells

Given the evidence that ACh antagonizes cytokine-induced paracellular permeability in Caco2 cells, and that the effect is mediated via activation of muscarinic receptors, we next investigated the underlying molecular mechanisms. Previous studies have shown that activation of IL-1β receptor leads to activation of the NF-kB pathway, resulting in transcription of MLCK, leading to a reduced expression of TJ proteins such as occludin and ZO-1. Immunoblot and immunostaining analysis of TJ proteins revealed IL-1β -induced reduction of occludin expression (Fig. 3a), and rearrangement of ZO-1 cellular localization (Fig. 3b). Furthermore, occludin expression was elevated upon pre-incubation with bethanechol (10 nM–1 μM) and ACh (Figs 4a and 3a). This effect was not observed upon pre-incubation with nicotine (data not shown). Surprisingly, we observed a strong induction of claudin 3 expression in presence of bethanechol, while incubation with IL-1β had no effect (Fig. 4a). Consistent with this data, a significant reduction in IL-1β-induced phosphorylation of MLC was observed on pre-treatment with ACh and muscarinic agonist bethanechol (Fig. 4b), indicating that cholinergic receptor activation acts on MLCK activity. As these effects might be the result of a stimulation of cellular proliferation or migration, as described earlier for cholinergic receptors (Heeschen *et al.* 2002, Ng *et al.* 2007), we tested whether this increase might be the result of proliferative changes of the epithelial cells. To this end, we performed a real-time scratch assay test, to validate whether cholinergic agonists might affect the migratory capacity to heal the scratch. As a positive control, we first tested the capacity of epidermal growth factor

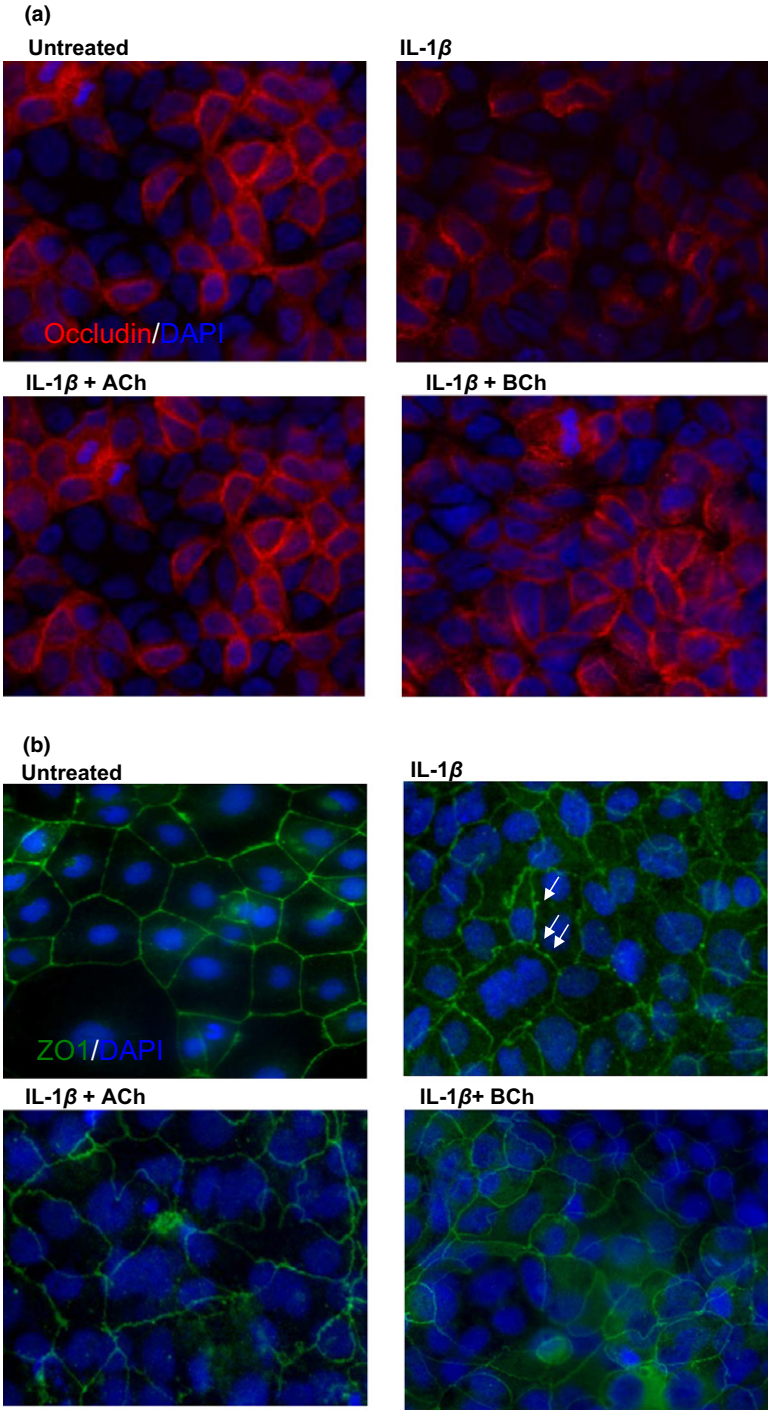

Figure 3 IL-1β disrupts rearrangement of TJ proteins, occludin and ZO-1. Caco2 cells were incubated with IL-1β, IL-1β + ACh or IL-1β + bethanechol (for 72 h), followed by TJ protein immunostaining and visualization of occludin (a) and ZO-1 (b) using confocal microscopy. Stainings are representative of 3 independent cell preparations.

(EGF) to enhance scratch healing. Real-time analysis revealed that both HT-29 and CMT93 cell lines repair mechanically induced wound significantly better in presence of EGF, whereas no significant differences were observed upon incubation with ACh or bethanechol compared to saline controls (Fig. 4c,d). This observation suggests that mAChR activation-induced TJ expression in epithelial cells affects barrier function properties exclusively, and this effect is unlikely to rest on proliferative or migratory effects of mAChR on epithelial cells.

Cholinergic agonists inhibit IL-1β-induced phosphorylation of myosin light chain via an NF-kB independent mechanism

As previous studies have reported, cytokine induced degradation of TJ proteins signals via activation of the

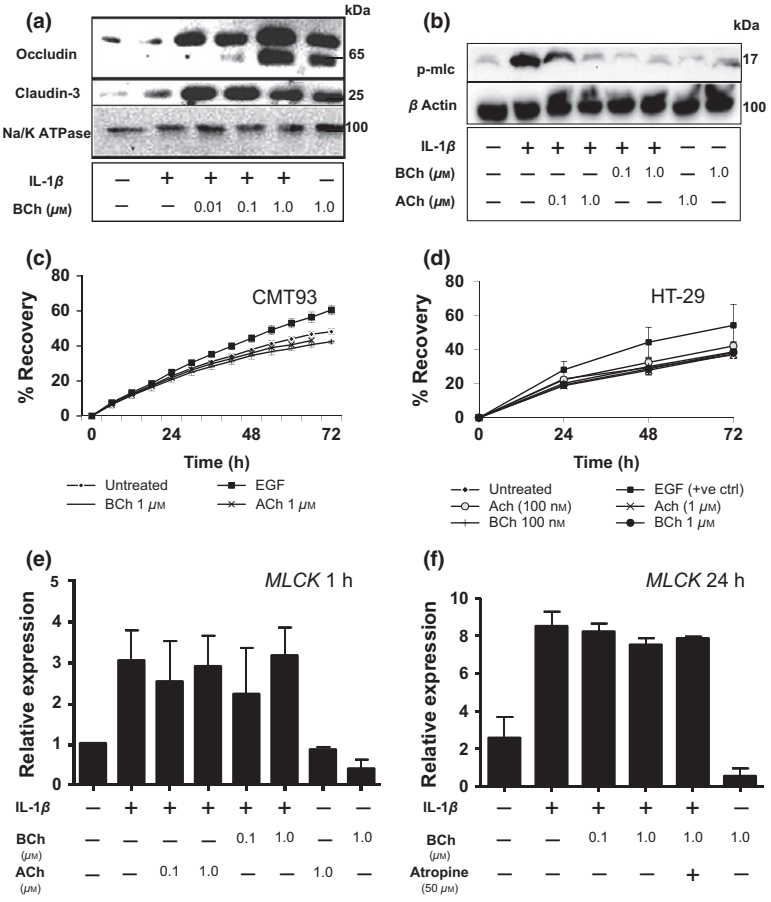

Figure 4 Cholinergic agonists inhibit phosphorylation of MLC (a) Caco2 cells were incubated without (−) or with (+) IL-1β (10 ng mL^{-1}) or bethanechol (0.01–1 μM) for 72 h, and then whole cell lysates were analysed by Western blot for expression of the indicated junctional proteins. (b) MLC phosphorylation determined by Western blotting in Caco2 cells after treatment with IL-1β (10 ng mL^{-1}) or bethanechol (1 μM) or acetylcholine (1 μM) for 48 h. (c, d) Quantification of scratch wound healing assay was carried out by measuring the uncovered area at given time points. HT29 and CMT93 cells were cultured to confluence, mechanically wounded by scratching, and then incubated in medium containing the indicated reagents; EGF (10 ng mL^{-1}) was used as a positive control. (Means ± SE, n = 3). (e, f) Relative *MLCK* mRNA levels were measured by qPCR (1 h and 24 h) in Caco2 cells treated with IL-1β (10 ng mL^{-1}) or bethanechol (0.1–1 μM) or acetylcholine (0.1–1 μM), (Means ± SE, n = 4). *P < 0.005.

canonical NF-kB pathway (Turner 2009). This is initiated by nuclear translocation of p65 subunit, and transcription of NF-kB-dependent genes, such as *MLCK* and *IL-8*. We observed a significant increase in IL-8 protein levels in supernatants of Caco2 cells treated with IL-1β, which were not antagonized by pre-treatment with either ACh or bethanechol (Fig. 5a–c). Furthermore, we observed an increased transcription of *MLCK*, both 1 h and 24 h after stimulation with IL-1β, neither of which were affected by cholinergic agonists (Fig. 4e,f). Nevertheless, these observations are not directly indicative of p65 translocation and NF-kB activation, and to this end we used NF-kB-luciferase Caco2 cells lines (kindly provided by Prof. J.Plat University of Maastricht, the Netherlands) and visualized p65 protein translocation by immunofluorescence microscopy. Consistent with previous findings, IL-1β -induced p65 translocation remained unaffected by cholinergic receptor activation in Caco2 cells (Fig. 5d,f). To determine whether cholinergic signalling interferes with IL-1R signalling upstream nuclear translocation of p65, we examined the phosphorylation of IKKα by immunoblotting. As expected, we observed a strong phosphorylation of IKKα upon incubation of Caco2 cells with IL-1β. In line with previous results, we observed no significant reduction in phosphorylation of IKKα protein on pre-treatment with cholinergic agonists, ACh and bethanechol (Fig. 5e). In conjunction, these results clearly establish the protective role of muscarinic receptor activation in IL-1R-mediated TJ disruption acts via inhibition of MLCK activity, via a mechanism independent of NF-kB pathway (summarized in Fig. 7).

Reduced ChAT expression in inflamed UC intestine

As epithelial cells have an endogenous ACh metabolic capacity (Proskocil *et al.* 2004), we wondered whether in colitis patients this system would be impaired, contributing to the inflammatory TJ disruption in colitic epithelia. We compared the expression levels of *ChAT* and *CXCL8* within inflamed IBD and non-inflamed control. A significant difference was observed in the expression levels of *CXCL8*, in unaffected vs. UC and unaffected vs. CD patients. This indicates that the epithelium isolated from patients with UC and CD was exposed to inflammatory stimuli, which was also confirmed by the pathology scores (not shown). MRNA

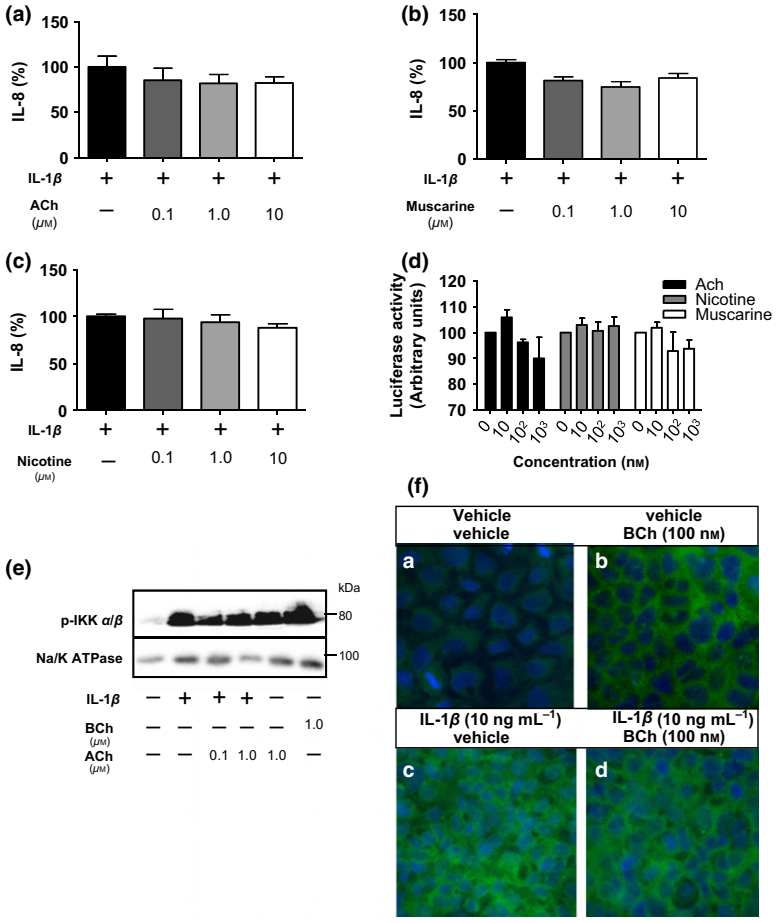

Figure 5 IL-1β-induced activation of canonical NF-kB pathway remains unaffected by AChR agonists. (a, b, c) Chemokine production was measured in supernatants from Caco2 cells after 24 h stimulation with IL-1β (10 ng mL^{-1}), in the presence of ACh (0.1–10 μM) (a), muscarine (0.1–10 μM) (b), or nicotine (0.1–10 μM) (c). (d) Caco2 cells transfected with an NF-kB luciferase reporter were treated with IL-1β (10 ng mL^{-1}) in the presence and absence of cholinergic agonists as indicated. (e) IL-1β (10 ng mL^{-1}) treated Caco2 cells in presence or absence of ACh (0.1–1 μM) or bethanechol (0.1–1 μM) for 24 h, whole cell lysates analysed for pIKKα by Western blotting. (f) Caco2 cells were incubated without (−) or with (+) IL-1β (10 ng mL^{-1}) or bethanechol (0.01–1 μM) for 24 h, followed by p65 protein immunostaining and visualization using confocal microscopy.

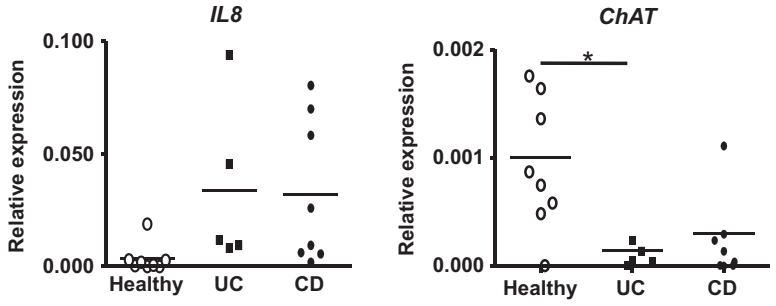

Figure 6 Quantitative reverse transcriptase-PCR (qRT-PCR) analysis of selected genes in intestinal epithelial fraction isolated from patients with inflammatory bowel disease (IBD). mRNA levels for *choline acetyltransferase (ChAT)* and *IL-8*, respectively, in the epithelial fractions isolated from biopsies from inflamed UC, inflamed CD and non-inflamed individuals.

expression of *ChAT* in the epithelial compartment showed significant differences in unaffected, patients with UC and CD, where a marked reduction in *ChAT* mRNA transcripts was observed in both UC and CD inflamed epithelial fractions, compared to unaffected controls. Hence, the metabolism of ACh in diseased epithelia is affected under inflammatory conditions (Figs 6 and 7).

Discussion

Epithelial permeability plays an important role in onset, severity and relapse of IBD. Cytokine-induced defective intestinal TJ permeability leads to an increased paracellular permeability of luminal antigens and opportunistic pathogens, thereby promoting inflammation. In the context of intestinal inflammation, we have shown the

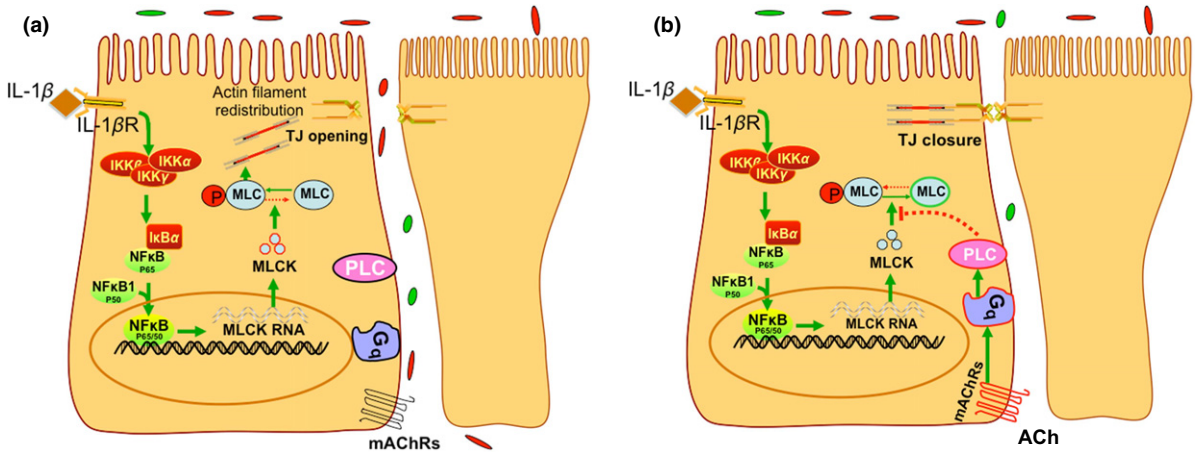

Figure 7 Schematic representation of pathways underlying cholinergic receptor activation and IL-1β-induced epithelial permeability. (a) IL-1R stimulation leads to activation of canonical NF-kB pathway, leading to transcription of MLCK and a subsequent phosphorylation of myosin light chain, resulting in contraction of acto-myosin filaments, TJ reorganization and occludin internalization. (b) Simultaneous mAChR stimulation activates phospholipase C (PLC), thereby blocking phosphorylation of myosin light chain (MLC) and facilitating TJ protein relocation to the plasma membrane, resulting in TJ closure.

restriction of inflammatory responses by vagal efferent activity, referred to as cholinergic anti-inflammatory pathway (The *et al.* 2007, Matteoli *et al.* 2014). While anti-inflammatory effects of cholinergic receptor stimulation on intestinal macrophage activation has been well established, the role of vagal-derived ACh remains questionable due to lack of vagal innervation of the mucosal compartment (Cailotto *et al.* 2014). Hence, cholinergic signalling in the mucosa likely involves enteric or non-neuronal cells rather than vagal terminals. Irrespectively, cholinergic receptor activation has demonstrated to dampen inflammatory activation on peritoneal macrophages and dendritic cells, likely via reducing NF-kB activity and stimulation of STAT3 pathways (Van Der Zanden *et al.* 2009).

Previous studies have shown that *in vitro* IL-1β reduces Caco2 TER and markedly increases the paracellular permeability (Al-Sadi & Ma 2007, Al-Sadi *et al.* 2010, 2013). Given the functional receptor AChR expression pattern in the intestinal epithelium (Hirota & McKay 2006a, Hirota & Mckay 2006b, Cameron & Perdue 2007), it is plausible to envisage a protective role of ACh in modulating proinflammatory cytokine-induced epithelial barrier function. In this study, we aimed to investigate the changes in paracellular permeability induced by cholinergic receptor activation in Caco2 cells, in the presence of pro-inflammatory cytokine IL-1β. Our *in vitro* data demonstrate that cholinergic receptor activation on epithelial cells can attenuate IL-1β-induced paracellular permeability. We found these effects to be mediated via muscarinic receptors on the epithelium, as the protective effects were abolished upon pre-incubation with specific muscarinic receptor antagonist, atropine (50 μM). First, the protective effects were elicited by muscarinic receptor agonist bethanechol and attenuated by atropine. Second nicotinic agonists were without effect. Both muscarinic and nicotinic receptors are expressed on intestinal epithelia, as demonstrated *in vivo* and *in vitro* cell lines [reviewed in (Hirota & McKay 2006a, Hirota & Mckay 2006b)]. Third, muscarinic receptors are involved in colitis pathogenesis (Hirota & McKay 2006a, Hirota & Mckay 2006b), where the role of nicotinic receptors in mediating colitis disease course (Ghia *et al.* 2008) likely involves central activation of muscarinic receptors (Martelli *et al.* 2014), or post-ganglionic enteric neurones (Dhawan *et al.* 2012) rather than nicotinic receptors on epithelia.

Consistent with our present findings, previous studies have also suggested that IL-1β-induced increase in Caco2 permeability is mediated by a reduced occludin and ZO-1 protein expression and a reduction in claudin-3 expression (Al-Sadi *et al.* 2010, Ivanov *et al.* 2010, Keita & Söderholm 2010). We observed that ACh did not significantly affect ZO-1 or claudin-3 expression but markedly antagonized IL-1β-induced degradation of occludin protein expression. The observed changes in transepithelial permeability were not a consequence of cell proliferation or wound healing response of epithelium to pro-inflammatory cytokines, as no changes in cell proliferation or wound healing responses upon pre-incubation with cholinergic agonists were observed, suggesting that cholinergic agonists exclusively modulate paracellular permeability by stimulating occludin expression in enterocytes. Previously IL-1β has been shown to activate canonical (classical) and non-canonical (alternative) NF-κB pathways in enterocytes, ultimately leading to the transcription of

NF-κB-dependent genes, such as *CXCL8* and *MLCK*. The canonical branch of NF-κB pathway is mediated by MEKK-1 pathway, phosphorylation of serine residues in IKK-α and IKK-β resulting in activation and nuclear translocation of NF-κB p65/p50 dimer (Chen 2002). On the other hand, the non-canonical pathway requires NIK activation and p52 nuclear translocation. Studies indicate a strong association between p65 translocation, MLCK transcription and a subsequent TJ opening indicates a major role of canonical pathway in barrier function. Our present data show that neither ACh nor bethanechol affected IL-1β-induced phosphorylation of IKK-α/β. Furthermore, IL-1β induced p65 nuclear translocation, NF-κB luciferase activity and *CXCL8* expression remained unaffected upon pre-incubation with cholinergic agonists. Collectively these data suggest, unlike in macrophages (Wang *et al.* 2002), AChRs do not interfere with activation of the canonical NF-κB pathway by IL-1β in enterocytes. Thus, our data suggest a protective mechanism of muscarinic receptor activation in cytokine induced barrier dysfunction, as summarized in Fig 7.

MLCK has been shown to be an essential protein in inducing the opening of intestinal epithelial TJ barrier via phosphorylation of MLC leading to the contraction of acto-myosin filaments (Cunningham & Turner 2012). Two isoforms of MLCK are expressed by the intestinal epithelium, MLCK1 and MLCK2. MLCK1 is expressed along villus epithelium and a specific deletion thereof has been reported to increase permeability, while MLCK2 is expressed throughout the crypt–villus axis, and its role in barrier function is yet unknown(Graham *et al.* 2011). In intestinal resections and biopsies, ileal epithelium MLCK and p-MLC expression was reportedly high in patients with inactive Crohn's disease, and even higher in those with active disease, further substantiating a pivotal clinical role of MLCK in IBD (Chen *et al.* 2014). Thus, it is interesting to speculate that therapeutic targets aimed at blocking transcription of MLCK or phosphorylation of MLC may be of value in IBD. To this end, we show that mAChR activation does not influence IL-1β-induced MLCK transcription or protein expression, either in acute or chronic phase. Interestingly, we observed an inhibition of IL-1β-induced phosphorylation of MLC, upon pre-incubation with mAChR agonists. Hence, a plausible mechanism of TJ protection by ACh may be that cholinergic signalling pathways interfere with enzymatic activity of MLCK, whether this is due to post-translational modification or through alteration of the MLCK catalytic domains (Cunningham & Turner 2012), or rather an interaction with phosphatase activity, needs further investigation.

Classically, cholinergic neurones have been believed to be a major source of ACh in the intestine. The ENS is a prominent neuronal source of ACh, with approximately 26% of enteric neurones in the human intestine being ChAT positive (Neunlist *et al.* 2003). Interestingly, analysis of changes in subpopulations showed a significantly smaller proportion of ChAT positive neurones in inflamed (18.8%) and non-inflamed (15.8%) areas of UC (Neunlist *et al.* 2003). However, an interaction between the cholinergic ENS fibres and epithelium in humans is unclear. While ENS-derived ACh effects on transcellular barrier function of enterocytes has been earlier shown by the group of Perdue, lack of ENS fibre-enterocyte synapses has been reported by others (Porter *et al.* 1996, Cameron & Perdue 2007). It is well understood that close apposition is crucial for a cholinergic synapse, owing to an abundance of acetylcholinesterases and rapid hydrolysis of ACh upon release. However, evidences suggest that an abundance of non-neuronal sources (e.g. lymphocytes) of ACh may exist in the gut (Wessler & Kirkpatrick 2008), suggesting greater bioavailability of ACh than previously appreciated. In fact, investigations have reported that through release of ACh colonic epithelial cells can modulate cell proliferation through autocrine signalling, and that ChAT overexpression in epithelial cells was associated with hyperproliferation and tumour growth (Klapproth *et al.* 1997, Cheng *et al.* 2008, Pettersson *et al.* 2009). We therefore analysed epithelial and subepithelial fractions isolated from inflamed patients with IBD, which revealed a significant downregulation of choline acetyltransferase (ChAT) expression, compared to non-inflamed controls. Studies (Collins *et al.* 1992) have documented more than 90% reduction in ACh release from myenteric plexus preparations, upon pre-incubation of tissues with IL-1β for 1 h. Whether IL-1β has the same effect on non-neuronal cells, that is epithelial cells, needs further investigation. This suggests that effects of IL-1β on the epithelium are not restricted to TJ proteins but also extend to suppression of homeostatic mechanisms, such as cholinergic signalling. Although we cannot conclusively prove that these patients had impaired barrier function, in the light of our *in vitro* data we speculate that chronic inflammatory processes significantly impact ACh synthesis and metabolism, thereby impeding the bioavailability of ACh to the epithelium. However, we have to be very critical and cautious of findings from a small clinical study, not only do these patients have varied clinical history, differences might be attributed to multiple factors such as diverse drug regimen, diets and intestinal flora, all of which are known mediators of cholinergic signalling.

In conclusion, we define a novel aspect of cholinergic signalling and inflammation in the context of barrier function in IBD. Our data indicate that ACh via activation of mAChRs antagonize IL-1β-induced phosphorylation of MLC and the subsequent degradation of occludin. However, at this point, it is uncertain which muscarinic receptor mediates barrier function. Additionally, we propose that low expression of ChAT in the epithelial fraction is correlated with inflammation in patients with UC and CD, suggesting low bioavailability of ACh underlying impaired barrier function in colitis. While the source of ACh to the intestinal epithelium remains unclear, in the light of present evidences, use of cholinomimetic therapeutics to target barrier epithelial permeability deserves merit. An attractive option to achieve clinical intervention of this cholinergic protective activity in disease setting is the use of nutritional activation of the cholinergic anti-inflammatory pathway (Lubbers *et al.* 2010, de Haan *et al.* 2013). When dietary lipids, proteins and peptides enter the gastrointestinal tract, especially the duodenum, neuroendocrine hormones such as cholecystokinin (CCK) are released. CCK is essentially involved in activating the anti-inflammatory cholinergic pathway. CCK is released upon formation of chylomicrons and binds to CCK receptors located on vagal afferents, after which the autonomic nervous system is activated resulting in peripheral tissue release of ACh. Another example of therapeutic use of cholinergic protective potential is the clinical intervention in autonomic activity using implantable devices that stimulate vagal nerve activity (Bonaz 2007, Clarençon *et al.* 2014). Although the working mechanism of such vagal nerve stimulation therapies is to be established, these trials are intriguingly exploring novel avenues of therapy of IBD. Our study thus adds to an additional mechanism of autonomic nervous regulation of IBD pathology.

Physiological relevance

This paper describes the physiological relevance of non-neuronal acetylcholine synthesis, and cholinergic receptors, in epithelial cells of mouse and human origin. We describe the potential of cholinergic signalling to dampen cytokine-induced barrier dysfunction in epithelia, a system that may contribute to the immune homeostasis in the healthy intestine, but also to pathogenesis of inflammatory bowel diseases once it fails.

This paper appears as contribution to the 2nd International meeting on Nerve Driven Immunity: Neurotransmitters and Neuropeptides In The Immune System and In Neuroimmune Dialogues, August 20–21, 2014. Nobel Forum, Karolinska Institute, Stockholm.

Conflict of interest

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