

RESEARCH PAPER

Epithelial propionyl- and butyrylcholine as novel regulators of colonic ion transport

Correspondence Martin Diener, Institut für Veterinär-Physiologie und -Biochemie, Universität Gießen. Frankfurter Str. 100, 35392 Gießen, Germany. E-mail: martin.diener@vetmed.uni-giessen.de

Received 24 February 2016; **Revised** 30 June 2016; **Accepted** 2 July 2016

Sarah Moreno¹, Stefanie Gerbig², Sabine Schulz², Bernhard Spengler², Martin Diener¹ and Sandra Bader¹

¹*Institute of Veterinary Physiology and Biochemistry, Justus-Liebig-University Giessen, and* ²*Institute of Inorganic and Analytical Chemistry, Justus-Liebig-University Giessen*

BACKGROUND AND PURPOSE

The colonic surface epithelium produces acetylcholine, released after the binding of propionate to GPCRs for this short-chain fatty acid (SCFA). This epithelial acetylcholine then induces anion secretion via stimulation of acetylcholine receptors. The key enzyme responsible for acetylcholine synthesis, choline acetyltransferase, is known to be unselective as regards the fatty acid used for esterification of choline. As the colonic epithelium is permanently exposed to high concentrations of different SCFAs produced by bacterial fermentation, we investigated whether choline esters other than acetylcholine, propionylcholine and butyrylcholine, are produced by the colonic epithelium, too, and whether these 'atypical' esters are able to stimulate the acetylcholine receptors involved in the regulation of colonic ion transport.

EXPERIMENTAL APPROACH

Desorption electrospray ionization mass spectroscopy (DESI-MS), Ussing chamber and Ca²⁺-imaging experiments were performed on rat distal colon.

KEY RESULTS

DESI-MS analyses revealed the production of acetylcholine, propionylcholine and butyrylcholine in the surface epithelium. Relative expression rates were 2–3% in comparison with acetylcholine. In Ussing chamber experiments, both atypical choline esters caused a concentration-dependent increase in short-circuit current, that is, stimulated anion secretion. Inhibitor experiments in the absence and presence of the submucosal plexus revealed the involvement of neuronal and epithelial acetylcholine receptors. While butyrylcholine obviously stimulated both nicotinic and muscarinic receptors, propionylcholine predominantly acted on muscarinic receptors.

CONCLUSIONS AND IMPLICATIONS

These results suggest a novel pathway for communication between intestinal microbes producing SCFA and the host via modification of epithelial production of choline esters involved in the paracrine regulation of the colonic epithelium.

Abbreviations

DESI-MS, desorption electrospray ionization mass spectrometry; I_{sc} , short-circuit current; SCFA, short-chain fatty acid

Tables of Links

TARGETS	
GPCRs^a	Ligand-gated ion channels^b
ACh receptors (muscarinic)	Nicotinic ACh receptors
M ₁ receptor	
M ₃ receptor	

LIGANDS	
Acetylcholine	Mecamylamine
Atropine	Pirenzepine
Bumetanide	Telenzepine
Darifenacin	Tetrodotoxin
Hexamethonium	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (^aAlexander *et al.*, 2015a,b).

Introduction

In the gastrointestinal tract, acetylcholine is known not only to be synthesized and released by submucosal and myenteric neurons, but also by colonic epithelial cells (Klapproth *et al.*, 1997, Bader *et al.*, 2014), which are part of the so-called non-neuronal cholinergic system. This system consists mainly of cells with barrier and defence functions such as immune cells or epithelial cells (Wessler *et al.*, 2003). Acetylcholine is released from colonic epithelial surface cells after the binding of propionate to the short-chain fatty acid receptor GPR41 (FFA3) and/or GPR43 (FFA2) (Yajima *et al.*, 2011b). Consequently, acetylcholine binds to epithelial acetylcholine receptors and induces anion secretion (Yajima *et al.*, 2011a).

The key enzyme for acetylcholine synthesis, choline acetyltransferase, which is also expressed in rat colonic surface epithelium (Klapproth *et al.*, 1997, Bader *et al.*, 2014), catalyses the synthesis of acetylcholine from the precursor substances choline and acetyl coenzyme A (acetylCoA). This enzyme has a high specificity for choline, whereas its selectivity for different acyl groups is quite poor. In rat brain, it has been shown that choline acetyltransferase exhibits the same affinity for butyrylCoA and propionylCoA as for acetylCoA (Rossier, 1977), leading to the production of propionylcholine and butyrylcholine, respectively, in the presence of the respective precursor substances.

The colonic epithelium, however, is permanently exposed to high concentrations of acetate, propionate and butyrate, which are produced by bacterial fermentation of carbohydrates within the colonic lumen. The total concentration of short-chain fatty acids in the large intestinal lumen is in the range of 100 mM (Bugaut, 1987), and these substrates are rapidly absorbed by the colonic epithelium (Karasov and Douglas, 2013). So it is possible that epithelial choline acetyltransferase might also produce propionyl- and/or butyrylcholine, in addition to acetylcholine. Thus, in the present study, we analysed the presence of these 'atypical' choline esters within the surface epithelium from rat distal colon using desorption electrospray ionization mass spectrometry and investigated, whether these esters (like native acetylcholine) might be able to alter epithelial functions such as Cl⁻ secretion. In addition to muscarinic receptors of the M₁ and the M₃ subtype (Haberberger *et al.*, 2006, Bader and Diener, 2015), the colonic epithelium also expresses different subunits of nicotinic receptors (α_2 , α_4 , α_5 , α_6 , α_7 , α_{10} and β_4);

hence, the sensitivity of the presumed changes in ion transport to muscarinic and nicotinic receptor blockers was also tested.

Methods

Animals

Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015). Female and male Wistar rats were used and weighed 160–250 g. The animals were bred and housed at the Institute of Veterinary Physiology and Biochemistry of the Justus-Liebig-University and had free access to food and water until the time of experiment. Animals were housed in macrolone type IV cages with 3 animals per cage at 22°C, 50% air humidity and a light: dark cycle of 12 h : 12 h. Animals killed by exsanguination after being stunned by a blow to the head. Experiments were approved by the named animal welfare officer of the Justus Liebig University (administrative number 487_M) and performed according to the German and European animal welfare law.

Solutions

If not indicated differently (as e.g. in ion substitution experiments), all Ussing chamber experiments were carried out in a bathing solution containing (in mmol·L⁻¹): 107 NaCl, 4.5 KCl, 25 NaHCO₃, 1.8 Na₂HPO₄, 0.2 NaH₂PO₄, 1.25 CaCl₂, 1 MgSO₄ and 12.2 glucose. The solution was gassed with 5% (v v⁻¹) CO₂ and 95% (v v⁻¹) O₂ at 37°C and had a pH of 7.4 (adjusted by addition of NaHCO₃/HCl). For the Cl⁻-free buffer, NaCl and KCl were equimolarly substituted by Na gluconate (NaGluc) and K gluconate (KGluc), respectively. For Ca²⁺-imaging experiments, a HEPES-buffered Tyrode solution was used containing (in mmol·L⁻¹): 135 NaCl, 5.4 KCl, 1 CaCl₂, 1 MgCl₂, 12.2 glucose and 10 HEPES; buffered at pH 7.4 with NaOH.

Tissue preparation

The rat distal colon, which is a well-established model for studying epithelial anion secretion, was quickly removed and placed in ice-cold Ussing chamber bathing solution. The large intestine was carefully flushed several times before it was mounted on a thin plastic rod. A circular incision was

made near the distal end with a blunt scalpel. The serosa and muscularis propria were stripped off by hand in order to obtain a mucosal–submucosal preparation. This preparation was either directly used for Ussing chamber experiments or for preparation of the mucosa. For the latter, the mucosa–submucosa was opened along the mesenteric border and placed onto a glass plate with the mucosal surface upwards. The proximal end of the tissue was clamped with a clip between a microscope slide and the upper end of the glass plate. The distal end of the colon was fixed with a blunt object slide. With a fresh and sharp glass slide, the mucosa was carefully separated from the submucosal layer in order to obtain a mucosal preparation. Two colonic segments were prepared from each animal; in general, one segment from each location served as control to study the response to a choline ester in the absence of putative inhibitors and the other segment to study the response in the presence of putative inhibitors. Segments were randomly distributed by a technician; blinding did not seem to be appropriate for these *in vitro* experiments.

Ussing chamber experiments

Both the mucosal and mucosal–submucosal preparations were fixed in a modified Ussing chamber, bathed with a volume of 3.5 mL on each side. The tissue was incubated at 37°C and short-circuited by a computer-controlled voltage-clamp device (Ingenieur Büro für Mess- und Datentechnik Mussler, Aachen, Germany), corrected for solution resistance. The exposed surface of the tissue was 1 cm².

The short-circuit current (I_{sc}) was continuously recorded, and tissue conductance (G_t) was measured every minute by applying a current pulse of $\pm 50 \mu\text{A}\cdot\text{cm}^{-2}$ with a duration of 200 ms. I_{sc} is expressed as $\mu\text{Eq}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$, that is, the flux of a monovalent ion per time and area with $1 \mu\text{Eq}\cdot\text{h}^{-1}\cdot\text{cm}^{-2} = 26.9 \mu\text{A}\cdot\text{cm}^{-2}$. Substances were administered after an equilibration period of about 60 min. The maximal increase in I_{sc} evoked by an agonist is given as difference to the baseline just before administration of the drug. When a drug was administered repeatedly to the same tissue (as indicated in the Results), the compartment to which the substance had been administered was washed three times with 5x the chamber volume, before the drug was administered again.

Imaging experiments at submucosal ganglionic cells

Relative changes in the cytosolic Ca^{2+} concentration of neurons in intact submucosal ganglia were measured using the Ca^{2+} -sensitive fluorescent dye fura-2 as described previously (Bell *et al.*, 2015). The isolated submucosae were transferred to an experimental chamber mounted on an inverted microscope (IX-50; Olympus, Hamburg, Germany) equipped with an epifluorescence set-up and an image analysis system (Till Photonics, Martinsried, Germany). The emission above 420 nm was measured from several regions of interest, each the size of one cell. The cells were excited alternatively at 340 and 380 nm, and the ratio of the emission signal at both excitation wavelengths was calculated.

Data were sampled at 0.2 Hz. The submucosal specimens were loaded for 60 min with $6\cdot 10^{-6} \text{ mol}\cdot\text{L}^{-1}$ fura-2 acetoxymethylester (fura-2 AM; Molecular Probes, Eugene,

USA) in the presence of $0.012 \text{ g}\cdot\text{L}^{-1}$ Pluronic®-F127 (Molecular Probes) in the dark at room temperature. Fura-2 AM which was not taken up by the cells was washed out. All experiments were carried out at room temperature.

The baseline of the fluorescence ratio of fura-2 was measured for several minutes before butyrylcholine or acetylcholine was superfused. The rate of superfusion was $1.5 \text{ mL}\cdot\text{min}^{-1}$. The administration of the choline esters was followed by a washing period and a viability control with KCl (30 mM), which also served to distinguish neurons from glia cells within the submucosal ganglia. A response to a choline ester was accepted by definition when two conditions were fulfilled simultaneously: (i) the amplitude of the change exceeded the 4-fold standard deviation of the scattering in the fura-2 ratio during the control period just prior to addition of the drug. (ii) The amplitude of the change in the fura-2 ratio exceeded an absolute value of 0.1.

Desorption electrospray ionization (DESI)-MS experiments

For DESI-MS measurements, mucosal preparations from rat distal colon were fixed on glass slides (with the mucosal side orientated upwards) using cyanoacrylate adhesive. DESI-MS experiments were carried out with a home-built DESI ion source coupled to an orbital trapping mass spectrometer (Exactive, Thermo Fisher Scientific GmbH, Bremen, Germany). The DESI ion source was operated at 4 kV spray voltage, a nitrogen pressure of $6 \times 10^5 \text{ Pa}$ and a solvent (acetonitrile) flow rate of $5 \mu\text{L}\cdot\text{min}^{-1}$. The sample-to-sprayer distance was about 2 mm. The distance between sprayer and MS inlet was about 3–5 mm and the spray angle was 65° to the sample surface. The source was equipped with a computer-controlled moving stage manufactured by Danaher Precision Systems (Salem, USA). The stage was operated by SERVO DESIGN KIT software V 5.22 (Galil Motion Control, Rocklin, USA), which also triggered the mass spectrometric measurement.

The mass spectrometric analysis was performed in positive ion mode in a mass-to-charge range of 150 to 500. The mass resolution was set to 50 000 at m/z 200, and the mass accuracy obtained was better than $\pm 4 \text{ ppm}$. For spatially resolved analysis of the mucosa preparations, a line-scan was performed over the sample surface from one side to the other side (with a velocity of about $200 \mu\text{m}\cdot\text{s}^{-1}$). Mucosal samples from four independent experiments were analysed in triplicate; for statistical analysis, the mean of each triplicate was considered as one data point. Accurate masses were calculated from the chemical composition for choline ($m [M^+] = 104.10699$), acetylcholine ($m [M^+] = 146.11756$), propionylcholine ($m [M^+] = 160.13321$) and butyrylcholine ($m [M^+] = 174.14886$); spectra were analysed in a mass window of $\pm 5 \text{ ppm}$.

Drugs

Bumetanide and mecamlamine were dissolved in ethanol (final maximal concentration $2.5 \text{ mL}\cdot\text{L}^{-1}$). Darifenacin and J104129 (Tocris, Bristol, UK) were dissolved in DMSO (final maximal DMSO concentration $2.5 \text{ mL}\cdot\text{L}^{-1}$). Tetrodotoxin was dissolved in 20 mM citrate buffer. Atropine, butyrylcholine, hexamethonium, pirenzepine,

propionylcholine and telenezpine were dissolved in distilled water. If not indicated differently, drugs were purchased from Sigma, Taufkirchen, Germany.

Statistics

Results are given as mean \pm SEM of the number (n) of investigated tissues. For all Ussing chamber experiments, a group size of $n = 6$ was designed; in some experimental series, it was elevated up to eight if there was a larger variability. For the comparison of two groups either Student's t -test or Mann–Whitney-U-test was applied. An F -test was used to decide which test method should be used. Both paired and unpaired two-tailed Student's t -tests were applied as appropriate. When means of more than two groups had to be compared, an ANOVA was performed. If there was no significant variance in homogeneity and an F -test indicated that variances between the groups were significantly larger than within the groups, a least-significance difference *post-hoc* test was performed. $P < 0.05$ was considered to be statistically significant. Statistical comparisons were performed using the statistical software WINSTAT 2012.1 (R. Fitch Software, Bad Krozingen, Germany). The concentrations necessary to evoke an increase in 50% of the maximal I_{sc} response (EC_{50}) were calculated by fitting the concentration response data to a Hill equation using GRAPHPAD PRISM 5 (Graphpad Software, La Jolla, USA). The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015).

Results

Atypical choline esters are locally synthesized by colonic epithelial cells

In order to determine whether choline acetyltransferase catalyses the synthesis of propionylcholine and butyrylcholine in colonic epithelial cells, in addition to ACh, DESI-MS experiments were performed on mucosa preparations from rat distal colon. DESI utilizes a spray of electrically charged droplets applied under high pressure (a so called electrospray) to extract, desorb and ionize analytes from the sample (i.e. the colonic epithelium). Afterwards, the desorbed ions were sucked into the mass spectrometer and analysed. A spatially resolved analysis of the sample was achieved by scanning over the sample surface.

High signal intensities were – as expected – found for choline (present in high amounts in biological membranes), within the region of the mucosa fixed to the glass slide (Figure 1, 1st row). Acetylcholine was detected in the same region. In addition, distinct signals for propionylcholine and butyrylcholine were found in the mucosa (Figure 1). Peak intensities for each substance in Figure 1 are shown as relative abundance (in %) in relation to the most intense signal observed for each m/z window. Comparing the three choline esters, mean DESI ion signal intensities across the line scan were propionylcholine $2.6 \pm 0.64\%$ ($n = 4$) and butyrylcholine $2.3 \pm 0.54\%$ ($n = 4$), both normalized to the mean signal intensity of acetylcholine (100%). In other words, the two atypical esters were produced by the colonic surface epithelium, but the concentration of the 'classical' choline ester,

acetylcholine, within the epithelium was about 40 times higher, assuming an equal desorption/ionization yield for the three esters. Because we did not evaluate ionization efficiencies, the relative quantification seen is only a rough estimate.

Concentration-dependent increase in I_{sc} induced by atypical choline esters

Despite their lower local concentration within the epithelium in comparison with acetylcholine, the DESI-MS data clearly indicate that propionylcholine as well as butyrylcholine are produced in colonic epithelium. Therefore, we investigated whether these choline esters might stimulate acetylcholine receptors in the colonic wall. The response to acetylcholine is mediated by epithelial ACh receptors as well as by ACh receptors on secretomotor neurons innervating the epithelium (Diener *et al.*, 1989). Therefore, two preparations from rat distal colon were used, that is, mucosal–submucosal preparations with an intact submucosal plexus and mucosal preparations, where the submucosa has been ablated. All tissues had a positive basal I_{sc} .

Propionylcholine as well as butyrylcholine (both administered to the serosal side) evoked a concentration-dependent increase in I_{sc} in mucosal–submucosal as well as in mucosal preparations from rat distal colon (Figure 2). Because desensitization was observed after administration of these agonists at concentrations above 50 μM , the choline esters were administered only once to an individual tissue, when they were used in concentrations $\geq 100 \mu\text{M}$.

In comparison with native acetylcholine, about 10 times higher concentrations of the atypical esters were needed to evoke a response comparable with acetylcholine. The concentrations needed to evoke an increase of 50% of the maximal I_{sc} response (EC_{50}), was $9.5 \pm 1.2 \mu\text{M}$ for acetylcholine, $31.7 \pm 1.5 \mu\text{M}$ for propionylcholine and $72.0 \pm 1.1 \mu\text{M}$ for butyrylcholine in the mucosal–submucosal preparations. In the mucosal preparations, the EC_{50} was $9.1 \pm 2.9 \mu\text{M}$ for acetylcholine, $208 \pm 1.2 \mu\text{M}$ for propionylcholine and $105 \pm 1.2 \mu\text{M}$ for butyrylcholine. This clearly indicates a lower affinity of the atypical esters for ACh receptors. However, the maximal increase in I_{sc} was in the same range as that evoked by acetylcholine indicating that they act as full agonists. The increase in I_{sc} was concomitant with an increase in tissue conductance (G_t), which did, however, not reach statistical significance (Table 1). Baseline G_t was significantly lower in the mucosal–submucosal preparations compared with the mucosal preparations ($P < 0.05$, Table 1).

Desensitization

Repeated administration of the atypical choline esters in higher concentrations led to a desensitization of the tissues. The strongest desensitization was observed with propionylcholine (500 μM at the serosal side) in the mucosal–submucosal preparation, whereas for example, the desensitization against butyrylcholine (500 μM at the serosal side) in the mucosal preparation was only modest (Figure 3, Table 2) and did not reach statistical significance in the ANOVA.

Due to the lower production rate (Figure 1) and the lower affinity of atypical choline esters to ACh receptors (Figure 2),

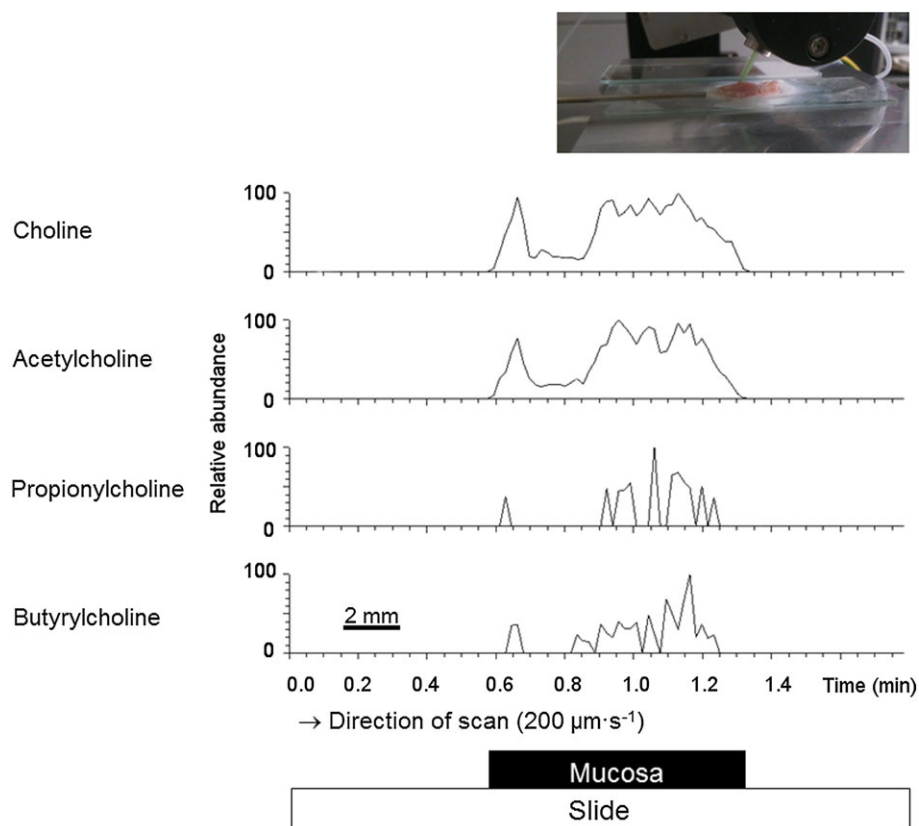


Figure 1

Detection of choline and choline esters in colonic epithelium by DESI-MS. DESI-MS ion signals for choline (1st row, $m [M^+] = 104.10699$), acetylcholine (2nd row, $m [M^+] = 146.11756$), propionylcholine (3rd row, $m [M^+] = 160.13321$) and butyrylcholine (4th row, $m [M^+] = 174.14886$) in mucosal preparations of rat distal colon. A spatially resolved analysis of the sample was achieved by performing a line-scan over the surface of the sample (inset). Relative signal intensities of the analytes are displayed for a mass window of ± 5 ppm, after normalization to the highest signal intensity for each substance, set to 100%. All analytes were detected from the mucosa sample only. Data shown were a selected, representative set out of four independent experiments.

it seems unlikely that they play a physiological role as agonists in regulating epithelial transport. However, they might well act as modulators of cholinergic signalling due to desensitization of the receptors. In order to test this hypothesis, the I_{sc} response evoked by acetylcholine was measured after prior desensitization against an atypical choline ester. For these experiments, propionylcholine was selected because it evoked a stronger desensitization than butyrylcholine (Figure 3). Indeed, after prior desensitization against propionylcholine (administered three times to the serosal side in a concentration of 500 μM with a washing step between each administration), acetylcholine (100 μM at the serosal side) only induced an increase in I_{sc} of $7.64 \pm 1.32 \mu\text{Eq}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$ ($n = 6$) in comparison to $11.51 \pm 0.68 \mu\text{Eq}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$ ($n = 6$) in a control group which had not been pretreated with the atypical choline ester ($P < 0.05$, Figure 4).

Sensitivity of the I_{sc} response evoked by atypical choline esters to tetrodotoxin

In order to test the involvement of secretomotor neurons in the I_{sc} evoked by atypical choline esters, tissues were pretreated with tetrodotoxin (10 μM at the serosal side), that is, a neurotoxin, which suppresses the propagation of action potentials by blocking voltage-dependent neuronal Na^+

channels (Catterall, 1980). In mucosal–submucosal preparations, pre-incubation with tetrodotoxin significantly inhibited the increase in I_{sc} evoked by propionylcholine (500 μM at the serosal side) from $4.86 \pm 0.90 \mu\text{Eq}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$ ($n = 6$) in the absence to $1.86 \pm 0.70 \mu\text{Eq}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$ in the presence of this neurotoxin ($n = 6$, $P < 0.05$ vs. control response $n = 6$, Figure 5, upper row). However, in mucosal preparations, that is, in the absence of the submucosal plexus, tetrodotoxin was ineffective, as the response to propionylcholine (500 μM at the serosal side) amounted to $9.44 \pm 1.29 \mu\text{Eq}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$ in the absence ($n = 6$) and $8.01 \pm 0.70 \mu\text{Eq}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$ ($n = 6$) in the presence of tetrodotoxin (10 μM at the serosal side, difference not significant).

A similar pattern was observed for butyrylcholine. In mucosal–submucosal preparations, a pretreatment with tetrodotoxin (1 μM at the serosal side) significantly inhibited the response in I_{sc} evoked by butyrylcholine (500 μM at the serosal side) from $4.63 \pm 0.58 \mu\text{Eq}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$ under control conditions ($n = 6$) to $0.91 \pm 0.17 \mu\text{Eq}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$ ($P < 0.05$ vs. control response, $n = 6$, Figure 5). In mucosal preparations, tetrodotoxin had no effect on the butyrylcholine-induced increase in I_{sc} , which was $9.94 \pm 3.38 \mu\text{Eq}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$ in its absence ($n = 6$) and $7.74 \pm 1.75 \mu\text{Eq}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$ in the presence of tetrodotoxin ($n = 6$, difference not significant).

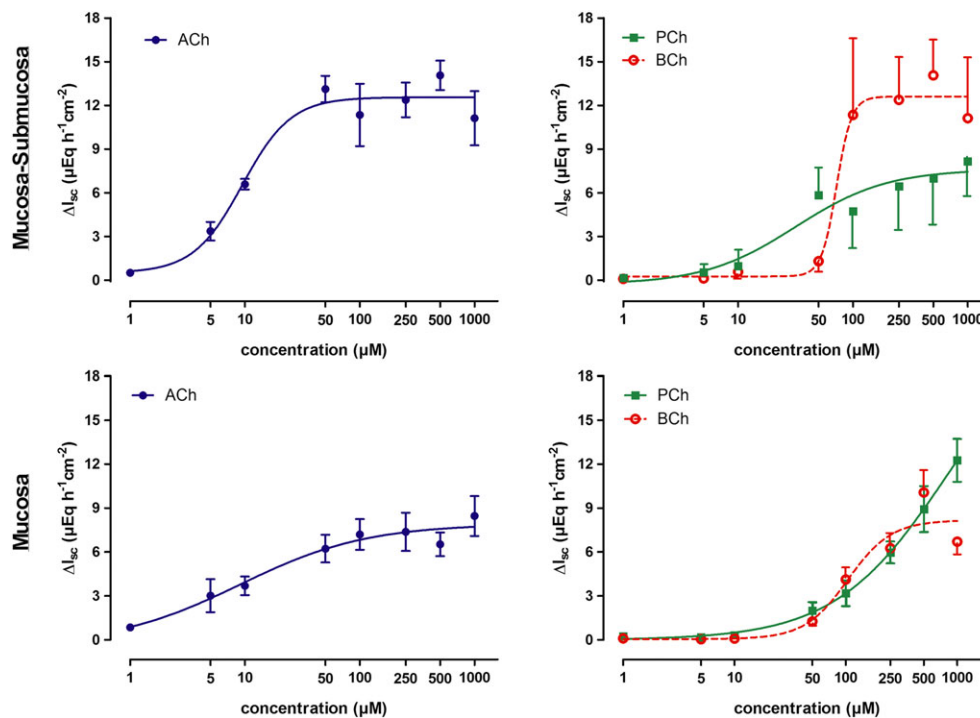


Figure 2

Concentration-dependent increase in I_{sc} across mucosal–submucosal preparations (1st row) and mucosal preparations (2nd row) from rat distal colon induced by serosal administration of acetylcholine, propionylcholine or butyrylcholine. Because of the desensitization of the I_{sc} response at higher concentrations, all three choline esters were only administered once to an individual tissue when used in concentrations $\geq 100 \mu\text{M}$, whereas for lower concentrations, they could be administered repeatedly to the same tissue with a washing step between the individual administrations (Methods). The curves show fits to a Hill equation. Hill coefficients were in the range of 0.8–2.2 with the exception of the response to butyrylcholine in the mucosal–submucosal preparation, where the Hill coefficient was 6.6 (for EC_{50} values, see text). Values are given as increase in I_{sc} (ΔI_{sc}) above baseline just prior administration of the respective agonist concentration and are means (symbols) \pm SEM (vertical lines). $n = 5$ –8. ACh, acetylcholine; BCh, butyrylcholine; PCh, propionylcholine.

Table 1

Tissue conductance (G_t) under baseline conditions and maximal value observed in the presence of propionylcholine ($500 \mu\text{M}$ at the serosal side) or butyrylcholine ($500 \mu\text{M}$ at the serosal side) in mucosal–submucosal (left) or mucosal preparations (right) of rat distal colon

	G_t ($\text{mS}\cdot\text{cm}^{-2}$)		n
	Baseline	Ester	
Propionylcholine			
Mucosa–submucosa	9.3 ± 0.76	11.0 ± 1.0	8
Mucosa	13.7 ± 1.6^a	16.6 ± 2.0	6
Butyrylcholine			
Mucosa–submucosa	11.8 ± 1.1	12.9 ± 1.2	6
Mucosa	16.4 ± 1.4^a	17.8 ± 1.3	6

Values are means \pm SEM.

^a $P < 0.05$ versus baseline G_t in mucosal–submucosal preparations.

Submucosal neurons are excited by butyrylcholine

In order to prove the stimulatory action of atypical choline esters on submucosal neurons suggested by Ussing

chamber experiments with tetrodotoxin (Figure 5), submucosal ganglionic cells were loaded with the Ca^{2+} -sensitive dye, fura-2, and changes in the cytosolic Ca^{2+} concentration were measured. For these experiments butyrylcholine was selected as agonist as its responses were more sensitive to the effects of TTX in the Ussing chamber experiments (Figure 5). When tested on 42 neurons (finally verified as neurons by the fura-2 ratio increase evoked by 30 mM KCl depolarization), 26 neurons (i.e. 60%) responded to butyrylcholine (1 mM) with a significant increase in the fura-2 ratio signal. This increase amounted to 0.74 ± 0.09 ($n = 26$). Acetylcholine (10 mM), subsequently administered after washout of butyrylcholine, evoked an increase in the fura-2 ratio signal of 0.58 ± 0.07 ($n = 24$, Figure 6).

Sensitivity of the I_{sc} response evoked by atypical choline esters to acetylcholine receptor antagonists

Both nicotinic (Bader and Diener, 2015) and muscarinic (Lindqvist *et al.*, 1998, Haberberger *et al.*, 2006, Bader and Diener, 2015) acetylcholine receptors are expressed in the colonic epithelium. Therefore, the sensitivity of the I_{sc} induced by both atypical choline esters against muscarinic and

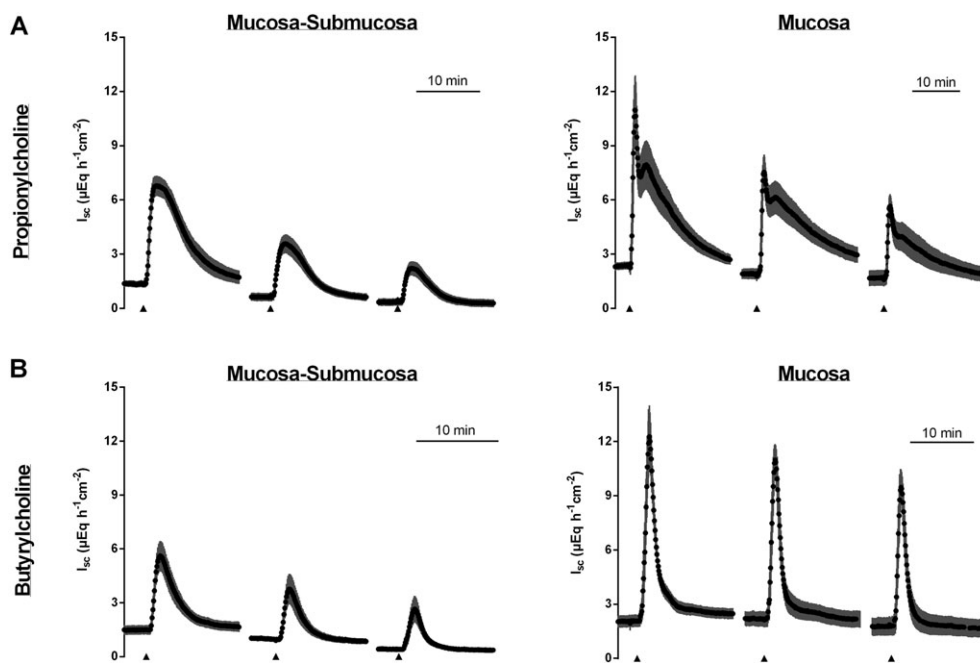


Figure 3

Propionylcholine (500 μM at the serosal side, upper row) or butyrylcholine (500 μM at the serosal side, lower row) were repeatedly administered to mucosal–submucosal (left column) or mucosal (right column) preparations of rat distal colon. The serosal compartment was washed three times with five times the chamber volume before the next administration of the respective choline ester. Values are given as means (black symbols) ± SEM (grey area), *n* = 6 for propionylcholine and *n* = 8 for butyrylcholine. Line interruptions are caused by omission of time intervals in order to synchronize the tracings of individual records to the administration of the respective agonists.

Table 2

Effect of butyrylcholine (500 μM at the serosal side) or propionylcholine (500 μM at the serosal side) on *I*_{sc} across mucosal-submucosal or mucosal preparations of rat distal colon after repeated application to the tissue

	Butyrylcholine			
	Mucosa–submucosa		Mucosa	
	ΔI_{sc} ($\mu\text{Eq}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$)	<i>n</i>	ΔI_{sc} ($\mu\text{Eq}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$)	<i>n</i>
1st administration	4.34 ± 0.77 ^a	8	11.62 ± 1.49 ^a	8
2nd administration	2.94 ± 0.82 ^a	8	10.28 ± 1.03 ^a	8
3rd administration	2.49 ± 0.82 ^a	8	8.85 ± 0.83 ^a	8
	Propionylcholine			
	Mucosa–submucosa		Mucosa	
	ΔI_{sc} ($\mu\text{Eq}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$)	<i>n</i>	ΔI_{sc} ($\mu\text{Eq}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$)	<i>n</i>
1st administration	5.65 ± 0.65 ^a	7	9.69 ± 1.60 ^a	6
2nd administration	3.02 ± 0.55 ^b	7	5.87 ± 0.90 ^b	6
3rd administration	2.03 ± 0.40 ^b	7	4.17 ± 0.44 ^b	6

Butyrylcholine and propionylcholine, respectively, was administered three times to the same tissue, after each administration the tissue was washed three times. Values are means ± SEM and are given as difference to the baseline just before administration of the respective agonist (ΔI_{sc}). Statistical homogeneous groups are indicated by the same letter a, b; ANOVA followed by least-significance difference post hoc test.

nicotinic receptor blockers was tested in mucosal–submucosal and mucosal preparations, that is, preparations with and without an intact submucosal plexus.

In mucosal–submucosal preparations, blockade of nicotinic acetylcholine receptors with hexamethonium (0.1 mM at the serosal side) or mecamylamine (10 μM at the serosal

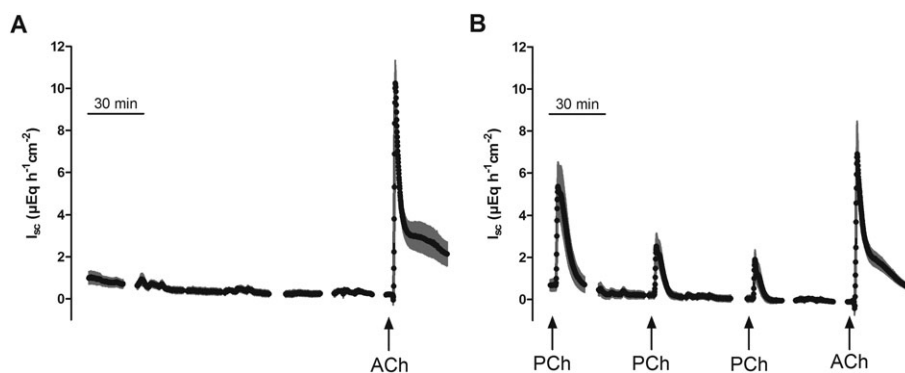


Figure 4

Repeated administration of propionylcholine (PCh; 500 μM at the serosal side; B) leads to desensitization against the subsequent administration of acetylcholine (ACh; 100 μM at the serosal side) in comparison with an untreated control group (A). Values are means (symbols) \pm SEM (lines), $n = 6$. For statistics, see text.

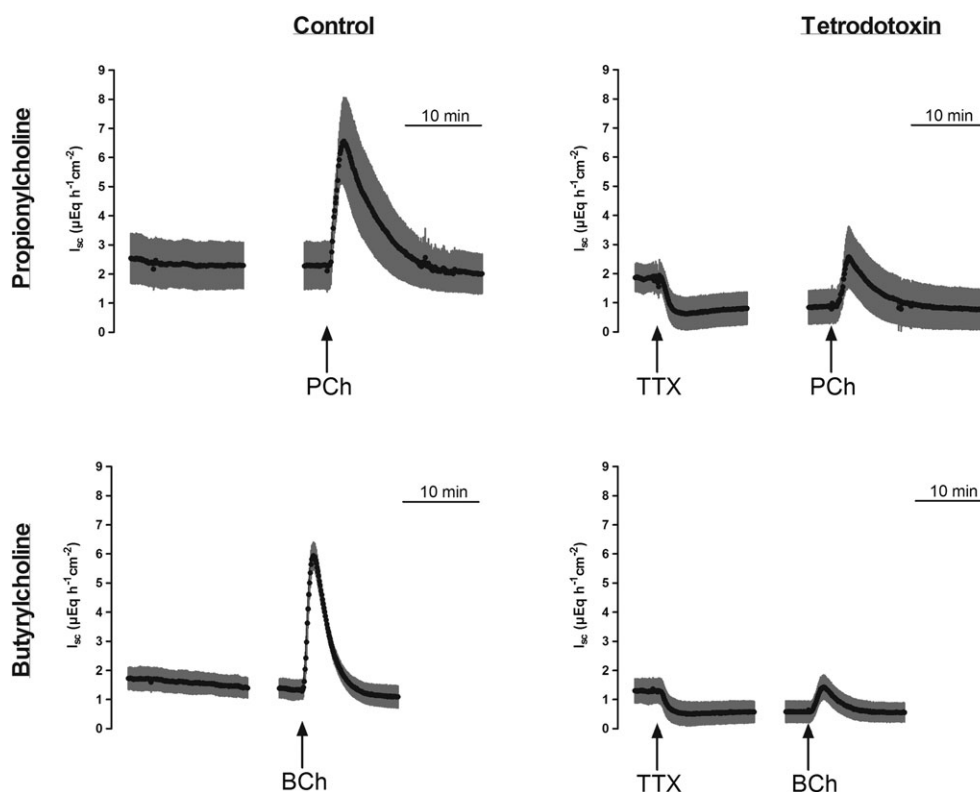


Figure 5

Effect of propionylcholine (500 μM on the serosal side; upper row) or butyrylcholine (500 μM on the serosal side; lower row) in the absence (left column) or presence (right column) of tetrodotoxin (1 μM at the serosal side). Values are given as means (black symbols) \pm SEM (grey area), $n = 6$. Line interruptions are caused by omission of time intervals in order to synchronize the tracings of individual records to the administration of the respective agonist. BCh, butyrylcholine; PCh, propionylcholine; TTX, tetrodotoxin.

side) did not affect the increase in I_{sc} induced by propionylcholine (Table 3). In mucosa preparations, however, the secretory response to this agonist tended to be reduced by about one third by hexamethonium and inhibition reached statistical significance in the case of

mecamylamine, suggesting that propionylcholine stimulates in part epithelial (but not neuronal) nicotinic receptors.

Unselective blockade of muscarinic acetylcholine receptors with atropine (1 μM at the serosal side) significantly reduced the propionylcholine response in both preparations.

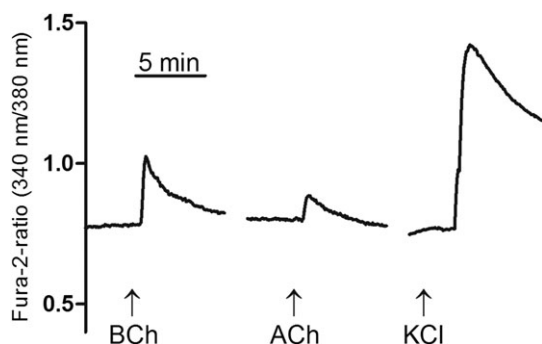


Figure 6

Increase in the cytosolic Ca^{2+} concentration of submucosal neurons measured by the change in the fura-2 ratio signal evoked by butyrylcholine (1 mM) and acetylcholine (1 mM). At the end of experiment, neurons were depolarized by KCl (30 mM) in order to evoke a Ca^{2+} influx via voltage-dependent Ca^{2+} channels. Typical tracing from 26 (out of 42 tested) cells; for statistics, see text. BCh, butyrylcholine.

Inhibition was mimicked by selective M_1 receptor blockers (for references to the inhibitors, e.g. Hammer and Giachetti, 1980, Galvan *et al.*, 1989) such as telenzepine (0.1 μ M at the serosal side) or pirenzepine (1 μ M at the serosal side) and M_3 receptor blockers (for references to the inhibitors, e.g. Mitsuya *et al.*, 1999, Zinner, 2007) such as darifenacine (1 μ M at the serosal side) or J104129 (5 μ M at the serosal side). With the exception of the effect of telenzepine in

mucosal–submucosal preparations, inhibition of the propionylcholine-induced increase in I_{sc} was statistically significant in all cases ($P < 0.05$, Table 3).

A different pattern of inhibition was observed when using butyrylcholine (500 μ M) as agonist. In this case, both nicotinic receptor blockers inhibited a significant part of the epithelial response (measured in mucosa preparations) and – at least in the case of mecamlamine – also strongly inhibited the effect of this agonist in mucosal–submucosal preparations, where a major part of the secretory response is mediated by the excitation of secretomotor submucosal neurons (Figures 5 and 6). Also, muscarinic receptor blockade with atropine, pirenzepine or the two M_3 receptor blockers, darifenacine and J104129, inhibited significantly the increase in I_{sc} evoked by butyrylcholine, whereas telenzepine was ineffective at the concentration used (Table 4). In both preparations, the increase in I_{sc} evoked by propionyl- or butyrylcholine was nearly abolished, when both nicotinic and muscarinic receptors were blocked by a combination of hexamethonium and atropine (Tables 3 and 4), suggesting that their effect is solely mediated by ACh receptors.

Taken together, these inhibitor experiments suggest a stronger effect of butyrylcholine on nicotinic receptors in comparison with propionylcholine. Furthermore, close inspection of the data presented in Tables 3 and 4 reveal an over-additive inhibitory action of atropine and hexamethonium, as both drugs inhibit, for example, the response to butyrylcholine by more than two thirds in mucosal preparations (Discussion).

Table 3

Effects of several acetylcholine receptor antagonists on I_{sc} induced by propionylcholine across mucosal–submucosal as well as mucosal preparations of rat distal colon

Blocker	PCh (– blocker)	PCh (+ blocker)	n	PCh (– blocker)	PCh (+ blocker)	n
	ΔI_{sc} (μ Eq·h ⁻¹ ·cm ⁻²)					
	Mucosa–submucosa			Mucosa		
ACh receptors						
Hexamethonium and atropine	4.43 ± 0.66	0.64 ± 0.22 ^a	8/7	10.30 ± 1.11	0.15 ± 0.02 ^a	6/7
Nicotinic acetylcholine receptors						
Hexamethonium	2.61 ± 0.83	5.72 ± 1.19	6	9.49 ± 1.75	6.34 ± 1.38	6
Mecamylamine	5.02 ± 0.45	5.39 ± 1.16	6	9.63 ± 1.27	5.22 ± 0.78 ^a	6
Muscarinic acetylcholine receptors						
Atropine	9.97 ± 1.88	3.10 ± 0.66 ^a	6	10.82 ± 1.37	1.60 ± 0.44 ^a	6/7
M_1 acetylcholine receptors						
Telenzepine	4.55 ± 1.01	2.78 ± 0.35	6	12.16 ± 1.62	3.79 ± 0.87 ^a	6
Pirenzepine	4.28 ± 0.90	1.69 ± 0.21 ^a	7	9.68 ± 1.45	4.31 ± 0.52 ^a	6/7
M_3 acetylcholine receptors						
Darifenacine	6.84 ± 1.68	1.41 ± 0.41 ^a	6	11.21 ± 1.46	2.45 ± 0.44 ^a	6
J104129	4.30 ± 0.49	0.39 ± 0.10 ^a	6	8.85 ± 1.82	0.47 ± 0.18 ^a	6

The response to propionylcholine (500 μ M at the serosal side) was either tested under control conditions, that is, in the absence of any inhibitor (– blocker), or in the presence of different inhibitors of acetylcholine receptors (+ blocker). Inhibitors used were as follows: hexamethonium (100 μ M), mecamlamine (10 μ M), atropine (1 μ M), telenzepine (0.1 μ M), pirenzepine (1 μ M), darifenacine (10 μ M) and J104129 (5 μ M). All inhibitors were administered to the serosal side. Values are given as increase in I_{sc} (ΔI_{sc}) above baseline just before administration of propionylcholine and are means ± SEM.

^a $P < 0.05$ versus response evoked by propionylcholine in the respective control series. PCh, propionylcholine.

Table 4

Effects of acetylcholine receptor antagonists on I_{sc} induced by butyrylcholine across mucosal–submucosal or mucosal preparations of rat distal colon

Blocker	BCh (– blocker)	BCh (+ blocker)	n	BCh (+ blocker)	BCh (– blocker)	n
	ΔI_{sc} ($\mu\text{Eq}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$)			Mucosa		
	Mucosa–submucosa					
ACh receptors						
Hexamethonium and atropine	5.11 ± 1.10	1.17 ± 0.48 ^a	6	7.50 ± 1.22	0.26 ± 0.05	6
Nicotinic acetylcholine receptors						
Hexamethonium	3.67 ± 0.58	2.39 ± 0.71	8	5.10 ± 1.13	1.51 ± 0.19 ^a	5/6
Mecamylamine	5.18 ± 0.81	0.53 ± 0.13 ^a	6	6.93 ± 1.65	1.30 ± 0.24 ^a	5/6
Muscarinic acetylcholine receptors						
Atropine	8.63 ± 2.30	2.96 ± 0.74 ^a	8/7	10.24 ± 1.87	3.44 ± 0.37 ^a	6
M ₁ acetylcholine receptors						
Telenzepine	3.99 ± 0.71	4.00 ± 1.22	7	8.59 ± 1.67	6.61 ± 1.13	6/7
Pirenzepine	5.92 ± 0.80	3.17 ± 0.55 ^a	7	9.74 ± 1.08	6.21 ± 0.90 ^a	6
M ₃ acetylcholine receptors						
Darifenacine	5.22 ± 0.46	2.76 ± 0.75 ^a	7	9.50 ± 1.59	3.07 ± 0.53 ^a	6
J104129	3.28 ± 0.83	0.43 ± 0.16 ^a	7	6.43 ± 1.51	0.40 ± 0.06 ^a	6/7

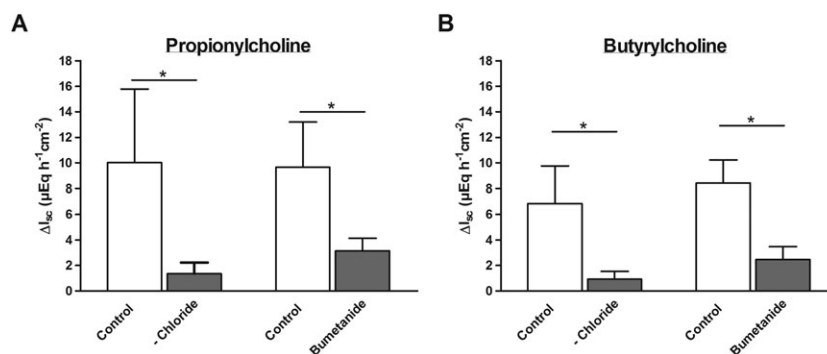
The response to butyrylcholine (500 μM at the serosal side) was either tested under control conditions, that is, in the absence of any inhibitor (– blocker), or in the presence of different inhibitors of acetylcholine receptors (+ blocker). Inhibitors used were: hexamethonium (100 μM), mecamylamine (10 μM), atropine (1 μM), telenzepine (0.1 μM), pirenzepine (1 μM), darifenacine (1 μM) and J104129 (5 μM). All inhibitors were administered to the serosal side. Values are given as increase in I_{sc} (ΔI_{sc}) above baseline just before administration of butyrylcholine and are means ± SEM.

^a $P < 0.05$ versus response evoked by butyrylcholine in the respective control series. BCh, butyrylcholine.

Ionic nature of the I_{sc} response to propionylcholine and butyrylcholine

In order to characterize the ionic nature of the increase in I_{sc} evoked by the two atypical choline esters, experiments were performed in colonic mucosal preparations. To find out if the I_{sc} induced by propionylcholine is due to Cl^- secretion, anion substitution experiments were performed. When

administered under control conditions, that is, with Cl^- ions present on both sides of the epithelium, propionylcholine (500 μM at the serosal side) evoked an increase in I_{sc} of $10.0 \pm 2.35 \mu\text{Eq}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$ ($n = 6$, Figure 7A). However, after substitution of Cl^- on both sides of the tissue with the impermeable anion gluconate, the increase in I_{sc} was significantly reduced and only amounted to $1.35 \pm 0.36 \mu\text{Eq}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$

**Figure 7**

Chloride-dependence of the I_{sc} response evoked by propionylcholine (500 μM at the serosal side, A) or butyrylcholine (500 μM at the serosal side, B) across colonic mucosal preparations. For each experimental series the response to propionylcholine or butyrylcholine was tested after chloride substitution by gluconate (on the mucosal and serosal side; -Chloride) or in the presence of bumetanide (100 μM at the serosal side), respectively, and compared with the response under control conditions (open bars). Values are given as increase in I_{sc} (ΔI_{sc}) above baseline just before administration of the respective choline ester and are means ± SEM; $n = 6$ for all series except the control response to the bumetanide series for propionylcholine ($n = 5$) or the series with butyrylcholine in the presence of bumetanide ($n = 7$). * $P < 0.05$ versus response evoked by propionylcholine or butyrylcholine in the respective control series.

($P < 0.05$ vs. control response in the presence of Cl^- , $n = 6$, Figure 7A). The same was observed when the tissue was pre-incubated with bumetanide (100 μM at the serosal side), an inhibitor of the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ -cotransporter (Kaplan *et al.*, 1996), which is responsible for the basolateral uptake of Cl^- during Cl^- secretion (Barrett and Keely, 2000). The blockade of this transporter resulted in a significant inhibition of the propionylcholine-induced increase in I_{sc} from $9.65 \pm 1.59 \mu\text{Eq}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$ ($n = 5$) to $3.14 \pm 0.40 \mu\text{Eq}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$ ($n = 6$, $P < 0.05$, Figure 7A). As a consequence, these series of experiments indicate that the I_{sc} evoked by propionylcholine is due to Cl^- secretion. Similarly, also the increase in I_{sc} evoked by butyrylcholine (500 μM at the serosal side) was significantly reduced by both manoeuvres (Figure 7B) indicating that both agonists – like native acetylcholine – evoke the secretion of Cl^- across the colonic epithelium.

Discussion

Acetylcholine is not only a neurotransmitter, but is also released as a paracrine mediator from the so-called non-neuronal cholinergic system. This system includes mainly barrier-forming epithelial cells, for example, in skin, airways or gastrointestinal tract and cells with a defence function such as macrophages or granulocytes, which express and release acetylcholine (Wessler *et al.*, 2003). Both the classical acetylcholine synthesizing enzyme, choline acetyltransferase, as well as the mitochondrial enzyme carnitine acetyltransferase have been suggested to be involved in the synthesis of non-neuronal acetylcholine in urothelium (Lips *et al.*, 2007). In the rat colon, experiments with inhibitors of both enzymes indicate that only choline acetyltransferase, expressed in the surface epithelium of the large intestine of different species (Klapproth *et al.*, 1997), but not the carnitine acetyltransferase (Bader *et al.*, 2014), is responsible for the epithelial production of this signalling molecule.

Choline acetyltransferase, however, is known to be quite unselective concerning the fatty acid residue used for esterification of choline and does not distinguish between acetylCoA, propionylCoA or butyrylCoA as a substrate (Rossier, 1977). Furthermore, colonic bacteria permanently produce high amounts of short-chain fatty acids like acetate, propionate and butyrate during the fermentation of carbohydrates such as cellulose which cannot be digested by mammalian enzymes during their passage through the small intestine (Bugaut, 1987). As short-chain fatty acids are rapidly absorbed via non-ionic diffusion or via anion transporters (e.g. Hadjiagapiou *et al.*, 2000), it seemed of interest in the present study to investigate whether propionylcholine or butyrylcholine are produced within the colonic epithelium. Indeed, scanning the mucosa with DESI-MS clearly revealed – besides acetylcholine – the presence of propionyl- and butyrylcholine in the analytes released from the mucosa (Figure 1). It seems reasonable to assume that the electrospray applied under high pressure and high voltage destroys the apical membranes so that the intracellular content of the esters is measured. This is further supported by the observation that all three esters were also detected, when a DESI-MS analysis was performed in mucosal homogenates (data not shown).

Despite their relatively low physiological expression (about 2–3% in comparison with native acetylcholine), the ability of these atypical esters to induce changes in ion transport across the colonic epithelium was tested in two preparations from rat distal colon, mucosal–submucosal and mucosal preparations. The functional difference between both is determined by the presence or absence of the submucosal plexus, which contains the main secretomotor neurons innervating the colonic epithelium (Andres *et al.*, 1985). In both preparations, the two atypical esters evoked an increase in I_{sc} (Figure 2). Anion substitution experiments and sensitivity to bumetanide (Figure 7) indicate that the increase in I_{sc} evoked by atypical choline esters was due to chloride secretion. In the presence of the submucosal plexus, the effect of both esters was significantly reduced, when the tissue was pretreated with tetrodotoxin (Figure 5), suggesting a stimulation of submucosal secretomotor neurons, which was confirmed by Ca^{2+} -imaging experiments at submucosal ganglia (Figure 6). Nevertheless, in general, the maximal increase in I_{sc} evoked by these esters tended to be smaller in mucosal–submucosal preparations in comparison with mucosal preparations with the ablated submucosa (c.f. the control responses in Tables 2–4). This might suggest that a part of these esters might be degraded, for example, by cellular metabolism during diffusion across the submucosa, preventing these esters from reaching their epithelial action sites.

Due to the lower production rate (Figure 1) and the lower affinity of the atypical esters for ACh receptors (Figure 2), it seems unlikely that they play a physiological role as agonists in regulating epithelial transport, although the concentration–response experiments show that they act as full agonists at their receptors (Figure 2). However, they might well function as modulators of cholinergic signalling due to desensitization of the receptors against the dominant signalling molecule, acetylcholine, released from enteric neurons or from colonic epithelium. This hypothesis is indicated by the fact that prior desensitization against propionylcholine significantly inhibited the action of a subsequent administration of acetylcholine (Figure 4). This is in analogy to the effect of choline, which is a low affinity agonist at ACh receptors eliciting biological responses due to binding at the receptors only at very high, unphysiological concentrations. Nevertheless, choline is thought to modulate cholinergic signalling in the brain via desensitization of $\alpha 7$ nicotinic receptors by choline (Alkondon *et al.*, 1997); similarly in the rat colon, where it desensitizes muscarinic receptors (Bader and Diener, 2015). So despite their low production rate (Figure 1), these esters might well modulate cholinergic signalling under physiological conditions. However, it must be noted that for the experiment depicted in Figure 4, a quite high concentration of propionylcholine (500 μM at the serosal side) was selected in order to produce a maximal desensitization, that is, the molar ratio of propionylcholine to acetylcholine in this experiment was far from that observed in physiological conditions, where the local production of acetylcholine is much larger than that of propionylcholine. Nevertheless, under *in vivo* conditions, a continuous release of choline esters is expected as the epithelium is in permanent contact with the colonic content, in which short-chain fatty acids represent the predominant

anions (Bugaut, 1987). It remains to be investigated whether manoeuvres modifying the pattern of short-chain fatty acid production within the colonic lumen (e.g. dietary modulation of fibre content or treatment with antibiotics) might change the molar ratios of choline ester production within the epithelium and the functional response evoked by stimulation of their release after stimulation of the epithelial receptors for short-chain fatty acids (Yajima *et al.*, 2011b).

Both esters have a distinct profile at ACh receptors. Muscarinic receptors are found in colonic epithelial cells. However, they also express different subunits (i.e. $\alpha 2$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\alpha 7$, $\alpha 10$ and $\beta 4$) of nicotinic receptors (Bader and Diener, 2015) previously thought to be only characteristic of excitable cells. The butyrylcholine-induced increase in I_{sc} was strongly inhibited by mecamylamine, a nonselective blocker of nicotinic receptors (Table 4). This inhibition was particularly marked in the presence of the submucosa. In the mucosal-submucosal preparation, mecamylamine reduced the I_{sc} response to this ester by about 90% suggesting that butyrylcholine is able to stimulate nicotinic receptors on enteric neurons as well as epithelial nicotinic receptors. In contrast, in the case of propionylcholine, mecamylamine was ineffective in mucosal-submucosal preparations and reduced the response to this ester by only about 45% in the mucosal preparations, suggesting that this ester might only stimulate nicotinic receptors expressed by epithelial cells. Experiments with the nonselective muscarinic receptor blocker atropine supported by data obtained with darifenacin and J104129, clearly indicate that a part of the action of both atypical esters is mediated by muscarinic receptors (Tables 3 and 4), especially those of the M_3 subtype, which is the predominant muscarinic receptor involved in cholinergic induction of epithelial anion secretion (Haberberger *et al.*, 2006). Surprisingly, in some experimental series, the 'selective' subtype blockers exerted a stronger inhibitory action than the nonselective muscarinic blocker atropine (e.g. the inhibitory effect of J104129, Tables 3 and 4). This might either indicate an additional effect of J104129 on other muscarinic receptors or might be caused by an insufficient concentration of atropine.

Thus, in principle, like acetylcholine both atypical choline esters are able to act on both types of ACh receptors albeit with differences in their relative efficacy. Interestingly, butyrylcholine is considered in most studies as a nicotinic receptor agonist (e.g. Andreev *et al.*, 1984), whereas actions on muscarinic receptors – apart from a study in the first half of the last century (Hunt and Renshaw, 1934) – seem to have been neglected.

Close inspection of the data presented in Tables 3 and 4 reveal that the effect of muscarinic and nicotinic receptor blockade seems to be over additive. For example, blockade of nicotinic receptors by mecamylamine reduces the I_{sc} response evoked by propionylcholine by 45%, and blockade of muscarinic receptors with atropine inhibits about 85% of the current increase. This observation might indicate some kind of potentiation between muscarinic and nicotinic signalling at the level of the epithelium, which is equipped with both types of ACh receptors, leading to a synergistic stimulation of anion secretion, when both receptor types are stimulated simultaneously.

We know from studies with carbachol that the main anion channel mediating Cl^- secretion induced by this ACh receptor agonist is the CFTR. It is not activated itself by the increase in the cytosolic Ca^{2+} concentration, but instead the driving force for Cl^- movement is increased by hyperpolarization of the membrane via activation of Ca^{2+} -dependent K^+ channels (Strabel and Diener, 1995). This is supported by the transient activation of Ca^{2+} -dependent apical Cl^- channels (Hennig *et al.*, 2008). Although it was behind the scope of the present study to confirm this for propionyl- and butyrylcholine, the clear mediation of the response to both esters by ACh receptors (Tables 3 and 4) gives reason to assume that the same channels underlie the increase in I_{sc} evoked by these esters.

Taken together, the current experiments suggest the possibility of a new form of communication between the intestinal microbiome and the colonic epithelium via the delivery of different short-chain fatty acids for the synthesis of choline esters by the epithelial choline acetyltransferase. Depending on the relative concentrations of acetate, propionate or butyrate within the colonic lumen and the resulting pattern of choline esters synthesized by the epithelium, the sensitivity of ACh receptors might be altered by production of lower-affinity choline esters, such as, propionyl- or butyrylcholine, which reduce the sensitivity of ACh receptors to the high affinity agonist, acetylcholine, due to desensitization. Modulation of colonic fermentation with pre- or probiotics is often used therapeutically to treat colonic disorders, thus this pathway might well have clinical relevance for humans or implications for animal experiments with regard to a sufficient fibre content of the diet. In another part of the gastrointestinal tract, the forestomach of ruminants, it is well known that the composition of the food strongly affects the luminal production pattern of short-chain fatty acids (Church, 1988). Therefore, we are currently investigating whether different feeding conditions, which should modify colonic production of short-chain fatty acids due to a different content of plant fibres, has an impact on non-neuronal cholinergic signalling in the colon of rodents.

Acknowledgements

The diligent technical contributions of Mrs. B. Brück, E. Haas, B. Schmidt and A. Stockinger are gratefully acknowledged. Supported by the LOEWE research focus "Non-neuronal cholinergic systems" and a Just'us grant (to S.B.) of the University Giessen.

Author contributions

S.M., S.G. and S.B. designed the study, performed experiments and interpreted data and drafted the manuscript. S.S., B.S. and M.D. designed the study, interpreted data and drafted the manuscript. All authors approved the version to be submitted.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This [Declaration](#) acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

References

- Alexander SPH, Davenport AP, Kelly E, Marrion N, Peters JA, Benson HE *et al.* (2015a). The Concise Guide to PHARMACOLOGY 2015/16: G protein-coupled receptors. *Br J Pharmacol* 172: 5744–5869.
- Alexander SPH, Peters JA, Kelly E, Marrion N, Benson HE, Faccenda E *et al.* (2015b). The Concise Guide to PHARMACOLOGY 2015/16: Ligand-gated ion channels. *Br J Pharmacol* 172: 5870–5903.
- Alkondon M, Pereira EF, Cortes WS, Maelicke A, Albuquerque EX (1997). Choline is a selective agonist of $\alpha 7$ nicotinic acetylcholine receptors in the rat brain neurons. *Eur J Neurosci* 9: 2734–2742.
- Andreev AA, Veprintsev BN, Vulfius CA (1984). Two-component desensitization of nicotinic receptors induced by acetylcholine agonists in *Lymnaea stagnalis* neurones. *J Physiol* 353: 375–391.
- Andres H, Bock R, Bridges RJ, Rummel W, Schreiner J (1985). Submucosal plexus and electrolyte transport across rat colonic mucosa. *J Physiol* 364: 301–312.
- Bader S, Diener M (2015). Novel aspects of cholinergic regulation of colonic ion transport. *Pharmacol Res Perspect* 3: e00139.
- Bader S, Klein J, Diener M (2014). Choline acetyltransferase and organic cation transporters are responsible for synthesis and propionate-induced release of acetylcholine in colon epithelium. *Eur J Pharmacol* 733: 23–33.
- Barrett KE, Keely SJ (2000). Chloride secretion by the intestinal epithelium: molecular basis and regulatory aspects. *Annu Rev Physiol* 62: 535–572.
- Bell A, Althaus M, Diener M (2015). Communication between mast cells and rat submucosal neurons. *Pflügers Arch Eur J Physiol* 467: 1809–1823.
- Bugaut M (1987). Occurrence, absorption and metabolism of short chain fatty acids in the digestive tract of mammals. *Comp Biochem Physiol* 86B: 439–472.
- Catterall WA (1980). Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. *Annu Rev Pharmacol Toxicol* 20: 15–43.
- Church DC (1988). The ruminant animal. In: *Digestive physiology and nutrition*, 2nd edn. Prentice Hall: London.
- Curtis MJ, Bond RA, Spina D, Ahluwalia A, Alexander SP, Giembycz MA *et al.* (2015). Experimental design and analysis and their reporting: new guidance for publication in BJP. *Br J Pharmacol* 172: 3461–3471.
- Diener M, Knobloch SF, Bridges RJ, Keilmann T, Rummel W (1989). Cholinergic-mediated secretion in the rat colon: neuronal and epithelial muscarinic responses. *Eur J Pharmacol* 168: 219–229.
- Galvan M, Boer R, Schudt C (1989). Interaction of telenzepine with muscarinic receptors in mammalian sympathetic ganglia. *Eur J Pharmacol* 167: 1–10.
- Haberberger R, Schultheiss G, Diener M (2006). Epithelial muscarinic M1 receptors contribute to carbachol-induced ion secretion in mouse colon. *Eur J Pharmacol* 530: 229–233.
- Hadjigapiou C, Schmidt L, Dudeja PK, Layden TJ, Ramaswamy K (2000). Mechanism(s) of butyrate transport in Caco-2 cells: role of monocarboxylate transporter 1. *Am J Physiol Gastrointest Liver Physiol* 279: G775–G780.
- Hammer R, Giachetti A (1980). Muscarinic receptor subtypes M1 and M2. Biochemical and functional classification. *Life Sci* 31: 2991–2998.
- Hennig B, Schultheiss G, Kunzelmann K, Diener M (2008). Ca^{2+} -induced Cl^{-} efflux at rat distal colonic epithelium. *J Membrane Biol* 221: 61–72.
- Hunt R, Renshaw RR (1934). Further studies of the methyl cholines and analogous compounds. *J Pharmacol Exp Ther* 51: 237–262.
- Kaplan MR, Mount DB, Delpire E (1996). Molecular mechanisms of NaCl cotransport. *Annu Rev Physiol* 58: 649–668.
- Karasov WH, Douglas AE (2013). Comparative digestive physiology. *Comprehen Physiol* 3: 741–783.
- Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). NC3Rs Reporting Guidelines Working Group. *Br J Pharmacol* 160: 1577–1579.
- Klapproth H, Reinheimer T, Metzgen J, Münch M, Bittinger F, Kirkpatrick CJ *et al.* (1997). Non-neuronal acetylcholine, a signalling molecule synthesized by surface cells of rat and man. *Naunyn Schmiedebergs Arch Pharmacol* 355: 515–523.
- Lindqvist SM, Sharp P, Johnson IT, Satoh Y, Williams MR (1998). Acetylcholine-induced calcium signaling along the rat colonic crypt axis. *Gastroenterology* 115: 1131–1143.
- Lips KS, Wunsch J, Zarghooni S, Bschleipfer T, Schukowski K, Weidner W *et al.* (2007). Acetylcholine and molecular components of its synthesis and release machinery in the urothelium. *Eur Urol* 51: 1042–1053.
- McGrath JC, Lilley E (2015). Implementing guidelines on reporting research using animals (ARRIVE etc.): new requirements for publication in BJP. *Brit J Pharmacol* 172: 3189–3193.
- Mitsuya M, Mase T, Tsuchiya Y, Kawakami K, Hattori H, Kobayashi K *et al.* (1999). J-104129, a novel muscarinic M3 receptor antagonist with high selectivity for M3 over M2 receptors. *Bioorg Med Chem* 7: 2555–2567.
- Rossier J (1977). Acetyl-coenzyme A and coenzyme A analogues. Their effects in rat brain choline acetyltransferase. *Biochem J* 165: 321–326.
- Southan C, Sharman JL, Benson HE, Faccenda E, Pawson AJ, Alexander SP *et al.* (2016). The IUPHAR/BPS Guide to PHARMACOLOGY in 2016: towards curated quantitative interactions between 1300 protein targets and 6000 ligands. *Nucl Acids Res* 44: D1054–D1068.
- Strabel D, Diener M (1995). Evidence against direct activation of chloride secretion by carbachol in the rat distal colon. *Eur J Pharmacol* 274: 181–191.
- Wessler I, Kilbinger H, Bittinger F, Unger R, Kirkpatrick CJ (2003). The non-neuronal cholinergic system in humans: expression, function and pathophysiology. *Life Sci* 72: 2055–2061.

Yajima T, Inoue R, Matsumoto M, Yajima M (2011a). Non-neuronal release of ACh plays a key role in secretory response to luminal propionate in rat colon. *J Physiol* 589: 953–962.

Yajima T, Inoue R, Yajima M, Tsuruta T, Karaki S, Hira T *et al.* (2011b). The G-protein on cholesterol-rich membrane microdomains

mediates mucosal sensing of short- chain fatty acid and secretory response in rat colon. *Acta Physiol* 203: 381–389.

Zinner N (2007). Darifenacin: a muscarinic M3-selective receptor antagonist for the treatment of overactive bladder. *Exp Opin Pharmacother* 8: 511–523.