

Neural FFA3 activation inversely regulates anion secretion evoked by nicotinic ACh receptor activation in rat proximal colon

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Key points

- Luminal short-chain fatty acids (SCFAs) influence gut physiological function via SCFA receptors and transporters.
- The contribution of an SCFA receptor, free fatty acid receptor (FFA)3, to the enteric nervous system is unknown.
- FFA3 is expressed in enteric cholinergic neurons.
- Activation of neural FFA3 suppresses Cl⁻ secretion induced by nicotinic ACh receptor activation via a G_{i/o} pathway.
- Neural FFA3 may have an anti-secretory function by modulating cholinergic neural reflexes in the enteric nervous system.

Abstract The proximal colonic mucosa is constantly exposed to high concentrations of microbially-produced short-chain fatty acids (SCFAs). Although luminal SCFAs evoke electrogenic anion secretion and smooth muscle contractility via neural and non-neural cholinergic pathways in the colon, the involvement of the SCFA receptor free fatty acid receptor (FFA)3, one of the free fatty acid receptor family members, has not been clarified. We investigated the contribution of FFA3 to cholinergic-mediated secretory responses in rat proximal colon. FFA3 was immunolocalized to enteroendocrine cells and to the enteric neural plexuses. Most FFA3-immunoreactive nerve fibres and nerve endings were cholinergic, colocalized with protein gene product (PGP)9.5, the vesicular ACh transporter, and the high-affinity choline transporter CHT1. In Ussing chambered mucosa–submucosa preparations (including the submucosal plexus) of rat proximal colon, carbachol (CCh)-induced Cl⁻ secretion was decreased by TTX, hexamethonium, and the serosal FFA3 agonists acetate or propionate, although not by an inactive analogue 3-chloropropionate. Serosal application of a selective FFA3 agonist (*N*-[2-methylphenyl]-[4-furan-3-yl]-2-methyl-5-oxo-1,4,5,6,7,8-hexahydro-quinoline-3-carboxamide; MQC) dose-dependently suppressed the response to CCh but not to forskolin, with an IC₅₀ of 13 μM. Pretreatment with MQC inhibited nicotine-evoked but not bethanechol-evoked secretion. The inhibitory effect of MQC was reversed by pretreatment with pertussis toxin, indicating that FFA3 acts via the G_{i/o} pathway. Luminal propionate induced Cl⁻ secretion via the cholinergic pathway, which was reduced by MQC, as well as by TTX, hexamethonium or removal of the submucosal plexus. These results suggest that the SCFA-FFA3 pathway has a novel anti-secretory function in that it inhibits cholinergic neural reflexes in the enteric nervous system.

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Abbreviations CCh, carbachol; CF3-MQC, *N*-(2-methylphenyl)-4-[5-(2-trifluoromethoxy-phenyl)-furan-2-yl]-2-methyl-5-oxo-1,4,5,6,7,8-hexahydro-quinoline-3-carboxamide; CGRP, calcitonin gene-related peptide; ChAT, choline acetyltransferase; CHT1, high-affinity choline transporter-1; CSMG, celiac superior mesenteric ganglia; DAPI, 4',6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; FK, forskolin; GI, gastrointestinal; GLP, glucagon-like peptide; GPCR, G protein-coupled receptor; G_t , tissue conductance; I_{sc} , short-circuit current; IR, immunoreactivity; FFA3, free fatty acid receptor 3; MQC, *N*-[2-methylphenyl]-[4-furan-3-yl]-2-methyl-5-oxo-1,4,5,6,7,8-hexahydro-quinoline-3-carboxamide; NaAc, sodium acetate; NaP, sodium propionate; NPY, neuropeptide Y; PGP, protein gene product; PTX, pertussis toxin; PYY, peptide YY; SCFA, short-chain fatty acid; VACHT, vesicular ACh transporter; VIP, vasoactive intestinal peptide.

Introduction

Short-chain fatty acids (SCFAs) are 1–6 carbon monocarboxylic acids. Acetic, propionic and butyric acids are gut microbial fermentation products of dietary fibre. Intracellular SCFAs are the major energy source for colonocytes, although luminal SCFAs are also an indicator of luminal microbial fermentative activity. Alteration of gut microbial populations is implicated in the pathogenesis of gastrointestinal (GI) or metabolic disorders, including obesity, inflammatory bowel disease and irritable bowel syndrome (Schwartz *et al.* 2001; Cani & Delzenne, 2011; Soldavini & Kaunitz, 2013). Therefore, clarifying the sensory mechanisms for and physiological responses to SCFAs advances medical knowledge, potentially leading to SCFA-based therapies. The de-orphanization and identification of the transmembrane SCFA receptors, namely free fatty acid receptor (FFA)2 and FFA3, has facilitated the accumulation of data regarding their expression, localization and function. FFA3 is a $G_{i/o}$ -coupled G protein-coupled receptor (GPCR), whereas FFA2 activates the $G_{q/11}$ and $G_{i/o}$ pathways (Brown *et al.* 2003; Le *et al.* 2003). Other FFA family members, FFA1 and FFA4, comprise long-chain fatty acid receptors not activated by SCFAs. FFA2 and FFA3 are implicated in the regulation and function of the gut immune system, in energy metabolism, and in the enteroendocrine system, which is consistent with their broad expression (Nohr *et al.* 2013). In the GI tract, FFA2 is abundantly expressed in neutrophils and adipocytes, whereas FFA3 is detected in enteroendocrine cells and in enteric neurons (Nohr *et al.* 2013). Although gut hormones are synthesized within enteroendocrine cells, which differ between the small and large intestine, FFA3 is consistently co-expressed with glucagon-like peptide (GLP)-1 throughout the intestine (Kaji *et al.* 2012a; Nohr *et al.* 2013). To date, the only reported effect of FFA3 activation in the GI mucosa is an increase in GLP-1 release from L cells in response to perfused SCFAs (Tolhurst *et al.* 2012). This is consistent with the first report of FFA3-deficient mice that have a low plasma concentration

of peptide YY (PYY), which is also produced in L cells (Samuel *et al.* 2008).

Luminally applied SCFAs acutely increase intestinal motility and the transmural potential difference *in vivo* and *in vitro* in rat small and large intestine (Wall *et al.* 1976; Yajima & Sakata, 1986; Richardson *et al.* 1991; Grider & Piland, 2007). From studies of isolated colonic segments, the contractile and secretory effects of SCFAs are primarily mediated by neural and non-neural cholinergic pathways (Yajima, 1985; Yajima *et al.* 2011a). Nevertheless, repeated luminal infusion of SCFAs inhibits colonic motility and fluid secretion via PYY release in rat colon (Squires *et al.* 1992; Cherbut *et al.* 1998). Furthermore, SCFAs reduce electrical stimulation-evoked neurally-mediated contractions of isolated rat colon, which are independent of the presence of mucosa (Dass *et al.* 2007), indicating that SCFAs can inversely regulate enteric neural activity. High concentrations (~100 mM) of mixed SCFAs are generated in the cecal lumen as a result of microbial metabolism (Bergman, 1990). Accordingly, the proximal colon absorbs SCFAs via monocarboxylate transporters (MCTs) as a primary colonocyte energy source and as substrates of hepatocyte metabolism (Iwanaga & Kishimoto, 2015). Thus, SCFA sensing in the proximal colon probably reduces motility and secretion, which facilitates fermentation of the luminal content and the absorption of luminal SCFAs. The mechanisms by which luminal SCFAs evoke gut physiological responses are confounded by the presence not only of membrane receptors, but also epithelial cell SCFA transporters and metabolic enzymes. We have recently reported that the rat proximal duodenum possesses an active SCFA absorption mechanism (Kaji *et al.* 2015b) and that luminal FFA2 and FFA3 agonists activate duodenal bicarbonate secretion via gut hormone- and SCFA transporter-dependent pathways (Akiba *et al.* 2015). Because the absorption-dependent secretory pathway involves activation of capsaicin-sensitive afferent nerves (Akiba *et al.* 2015), we hypothesize that SCFA receptors are functionally expressed in neurons innervating the intestinal mucosa. Despite the ubiquitous expression of FFA3 in autonomic and sensory neurons (Kimura *et al.*

2011; Won *et al.* 2013; Nohr *et al.* 2015), the means by which neuronal FFA3 activation affects GI function have not been clarified.

In the present study, we demonstrate for the first time that FFA3 is expressed in enteric neurons and in mucosal and submucosal varicosities expressing cholinergic markers. We further show that neural FFA3 activation suppresses carbachol (CCh)- or luminal propionate-induced Cl^- secretion via the $\text{G}_{i/o}$ pathway in rat proximal colon.

Methods

Animals

Male Sprague–Dawley rats weighing 200–250 g (Harlan, San Diego, CA, USA) were fed a pellet diet and water *ad libitum*. All studies were performed with approval of the Veterans Affairs Institutional Animal Care and Use Committee. Rats were fasted overnight with free access to water before the experiments. Animals were killed by terminal exsanguination under deep isoflurane anaesthesia, followed by thoracotomy.

Antibody production

Antibody was produced in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Hokkaido University School of Medicine. An antibody against α -calcitonin gene-related peptide (CGRP) was raised in guinea-pigs using CGRP_{1–37} of mature mice and rats (i.e. SCNTATCVTH RLAGLLSRSGGVVKDNFVPTNVGSEAF; 83–119 amino acid residues; GenBank AF330212), which was fused to glutathione S-transferase (GST). Immunization and affinity-purification of antibodies have been reported previously (Fukaya *et al.* 2006). The specificity of the α -CGRP antibody was confirmed by absorption with rat CGRP_{1–37}. An antibody against rat FFA3 (RK1103) was raised in accordance with our previous method for FFA2 antibody production (Akiba *et al.* 2015). Briefly, cDNA fragment encoding C-terminal 28 amino acids of rat FFA3 (296–319 amino acid residues; GenBank NM 001108912) was cloned using a pGEM-T Easy Vector System I kit (Promega, Madison, WI, USA). The cDNA fragment was subcloned into pGEX4T-2 plasmid and *Escherichia coli* BL21 for expression of GST fusion proteins in accordance with the manufacturer's instructions (Pharmacia Biotech AB, Uppsala, Sweden). Fusion proteins, emulsified with Freund's complete or incomplete adjuvant (Difco, Detroit, MI, USA), were injected s.c. into a female New Zealand white rabbit at 2 week intervals. Anti-serum sampled 2 weeks after the sixth injection was affinity-purified using CNBr-activated Sepharose 4B coupled with GST-free polypeptides that were obtained by in-column thrombin

digestion of fusion proteins. The FFA3 antibody RK1103 was characterized by immunostaining of rat FFA2- or rat FFA3-expressing HeLa and HEK298T cells and by western blotting of rat colonic samples as described previously (Akiba *et al.* 2015). The protein samples were extracted from the colonic submucosal layer, which was detached from the mucosa after meticulously removing the muscle layers using fine surgical forceps (Natsume Seisakusho Co., Ltd, Tokyo, Japan) under a stereomicroscope. All procedures were performed in ice-cold Krebs buffer.

Chemicals

N-(2-methylphenyl)-4-(furan-3-yl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydro-quinoline-3-carboxamine (MQC) and *N*-(2-methylphenyl)-4-[5-(2-trifluoromethoxy-phenyl)-furan-2-yl]-2-methyl-5-oxo-1,4,5,6,7,8-hexahydro-quinoline-3-carboxamide (CF3-MQC) were synthesized, purified and verified in Laboratory of Organic Chemistry, School of Pharmaceutical Sciences, University of Shizuoka, Japan, in accordance with the published chemical structures (Ulven, 2012). AR420626 was obtained from Cayman Chemical Company (Ann Arbor, MI, USA). Pertussis toxin (PTX) and rat CGRP_{1–37} was obtained from Tocris Bioscience (Ellisville, MO, USA). TTX was purchased from Calbiochem (EMD Millipore, Billerica, MA, USA). CCh, sodium propionate (NaP), sodium acetate (NaAc), 3-chloropropionic acid, forskolin (FK), capsaicin, hexamethonium, atropine and other chemicals were purchased from Sigma Chemical (St Louis, MO, USA). TTX was dissolved in citrate buffer (pH 4.5). Indomethacin and capsaicin were dissolved in ethanol. MQC, CF3-MQC and FK were dissolved in dimethyl sulfoxide (DMSO). Other chemicals were dissolved in distilled water.

Immunofluorescence staining and real-time PCR

Rats were deeply anaesthetized by isoflurane inhalation and perfused through the left ventricle of the heart with saline and subsequently with Zamboni's fixative containing 2% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.4). The colonic segments were taken and immersed in the same fixative overnight for 4°C. For frozen sections, small pieces of colon were then submerged in 20% sucrose solution overnight at 4°C, embedded in OCT compound and cut to 8 μm -thickness on amino silane-coated glass slides (Matsunami Glass USA Inc., Bellingham, WA, USA). For whole-mount preparations, the fixed colon was separated into mucosa, submucosa and muscle layers with fine forceps under a stereomicroscope.

Sections and whole-mount preparations were pre-treated with 5% normal donkey serum in phosphate-buffered saline containing 0.3% Triton X-100 for 1 h. Pre-blocked tissues were incubated with primary

antibodies anti-FFA3 (RK1103; $1 \mu\text{g ml}^{-1}$), guinea-pig anti- α -CGRP ($1 \mu\text{g ml}^{-1}$), mouse anti-protein gene product (PGP)9.5 (dilution 1:500; ab8189, Abcam, Cambridge, MA, USA), guinea-pig anti-vesicular acetylcholine transporter (VACHT; dilution 1:500; AB1588, EMD Millipore, Temecula, CA, USA), goat anti-CHT1 (high-affinity choline transporter-1; $0.5 \mu\text{g ml}^{-1}$; Frontier Institute Co., Ltd, Sapporo, Japan), goat anti-calbindin ($\text{CB}_{-28 \text{ kDa}}$; $0.25 \mu\text{g ml}^{-1}$; Frontier Institute Co.) or rabbit anti-VIP (CURE Antibody Core, Los Angeles, CA, USA) for 24–72 h at 4°C . For the pre-absorption test, RK1103 was pre-incubated with GST-free antigen peptide ($10 \mu\text{g ml}^{-1}$) overnight at 4°C . After rinsing in phosphate-buffered saline, fluorescence-conjugated antibodies were reacted for 2 h at room temperature. The tissues were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and covered with EverBrite mounting medium (Biotium, Hayward, CA, USA). Immunofluorescence was imaged and captured using a confocal laser microscope (LSM710; Carl Zeiss GmbH, Jena, Germany).

Real-time RT-PCR was performed as described previously (Akiba *et al.* 2015) with primers for rat FFA3 (sense: 5'-CTACAACGTGTCCCATGTCG-3' and anti-sense: 5'-TTCTGCTCCTTCAGCTCCAT-3') giving rise to a 219 bp product and for β -actin. The FFA3 expression level was presented as the fold induction per 10^3 copies of β -actin by the ΔCt method.

Short-circuit current measurements in Ussing chambered preparations

Mucosa–submucosa preparations were fashioned from the proximal colon as described previously (Kaji *et al.* 2012b). Submucosal layers were further removed using fine surgical forceps for some of the experiments. Two preparations were prepared from each segment by dividing each longitudinally, and these were then mounted between two hemichambers with an aperture = 0.3 cm^2 (Physiologic Instruments, San Diego, CA, USA). Measurements of short-circuit current (I_{sc}) and tissue conductance (G_{T}) were conducted as described previously (Kaji *et al.* 2015a). Positive values for I_{sc} indicate a negative electrical charge flux from serosal \rightarrow luminal bath as a result of anion secretion or cation absorption. Indomethacin ($10 \mu\text{M}$) was added to the luminal and serosal baths to eliminate the effects of endogenous PG production. The tissues were stabilized for ~ 50 min before the effects of CCh and other drugs were investigated.

Statistical analysis

Values are expressed as the mean \pm SEM. The number of animals in each experimental group was ≥ 5 . Statistical analysis was performed using Prism, version 6 (GraphPad

Software Inc., La Jolla, CA, USA) using Student's *t* test or one-way ANOVA followed by Fischer's least significant difference test depending on the number of experimental groups. $P < 0.05$ was considered statistically significant.

Results

Antibody specificity

Celiac superior mesenteric ganglia (CSMG) neurons, used as an FFA3-positive control (Won *et al.* 2013), were immunoreactive for FFA3 antibody RK1103 (Fig. 1A–C), which was abolished by preincubation of antibody with antigen peptide (Fig. 1D). FFA3-immunoreactivity (IR) was absent in the liver (Fig. 1E), which is consistent with low mRNA expression in the liver (Fig. 1I). In HeLa-rFFA3 cells, abundant FFA3-IR was detected in the plasma membrane, whereas mock-transfected or HeLa-rFFA2 cells were faintly stained (Fig. 1F–H), indicating specificity of the antibody and lack of cross-reaction with FFA2. Western blot was performed with proteins extracted from rat colonic submucosa, which was obtained by meticulous removal of the mucosa and the muscle layers using fine forceps. FFA3 antibody RK1103 detected a unique band with apparent size of ~ 38 kDa, close to the predicted molecular mass of FFA3 (36 kDa), which was absorbed by antigen peptide (Fig. 1J).

The specificity of the anti- α -CGRP antibody was tested with adjacent sections of dorsal root ganglia. α -CGRP antibody labelled the cell bodies of small-sized ganglion cells (Fig. 1K), whereas such immunohistochemical labelling disappeared after preincubation of the α -CGRP antibody with rat CGRP_{1–37} peptide (Fig. 1L).

Localization of FFA3 in rat colon

FFA3-IR was detected in the myenteric and submucosal plexuses, as well as intramuscular and mucosal nerve-like fibres, in frozen sections (Fig. 2A). FFA3-IR was present in fibres surrounding crypts and the fibres were double-stained with PGP9.5 to confirm their identity as nerve fibres (Fig. 2B–C). All of the FFA3-IR in proximal colon was abolished by pre-absorption with antigen peptide (Fig. 2D). We further separately stained whole-mount preparations of mucosa, submucosa or circular muscle-stripped longitudinal muscle layers, which includes mucosal, submucosal (Meissner's) or myenteric (Auerbach's) plexus, respectively (Fig. 2E–G). FFA3-IR was detected in the mucosal and submucosal plexuses, located in the nerve fibres and endings rather than in the neuronal cell bodies. In the myenteric plexus, a subset of myenteric neurons expressed cytoplasmic FFA3.

Because choline acetyltransferase (ChAT) has two variants (Chiocchetti *et al.* 2003), we used VACHT and

CHT1 as cholinergic markers, as previously reported in the rat enteric nervous system (Harrington *et al.* 2007). Most of the FFA3-IR nerves colocalized with VAcHT and CHT1 in the mucosal and submucosal plexuses, indicating that FFA3 was expressed in cholinergic nerves (Fig. 3A–F). CHT1 did not colocalize with vasoactive intestinal peptide (VIP) in the mucosal plexus (Fig. 3G), although both nerves were closely approximated, suggesting that FFA3-IR cholinergic nerves and VIP-ergic nerves are distinct populations. We detected α -CGRP-IR, a marker of afferent neurons, in varicosities and nerve fibres but not in submucosal neuronal cell bodies in the proximal colon.

α -CGRP-IR did not colocalize with FFA3-IR (Fig. 3H). Calbindin, which labels neuropeptide Y(NPY)/VIP-ergic secretomotor neurons in the submucosal plexus of rat small intestine (Buchan, 1991), did not colocalize with FFA3-IR in the submucosal ganglia (Fig. 3I).

Effect of FFA3 agonist and CCh on I_{sc} in rat proximal colon

We studied the contribution of neural FFA3 activation with respect to regulation of ion transport in Ussing chambered mucosal–submucosal preparations of rat

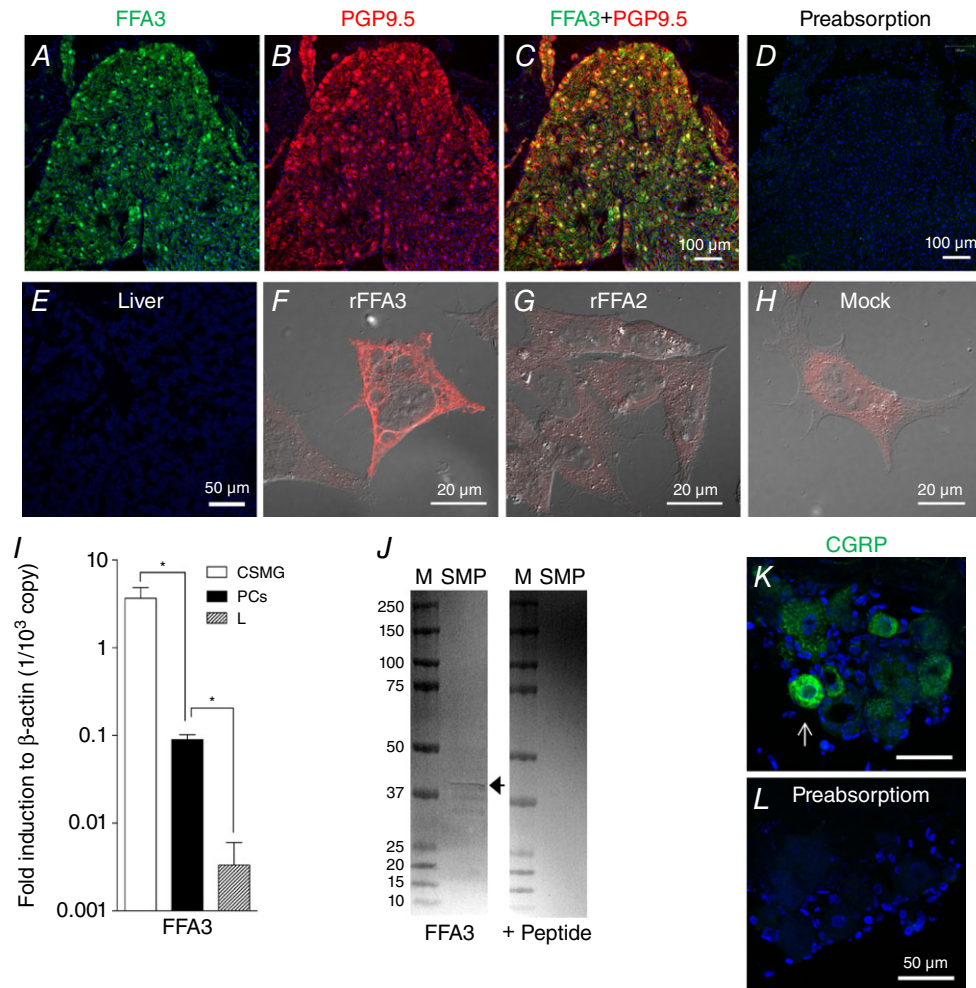


Figure 1. Antibody characterization

A and **B**, frozen sections of CSMG were double-stained with antibodies to FFA3 (**A**) (green) and PGP9.5 (**B**) (red). **C**, merged image showing FFA3 and PGP9.5 colocalization. **D**, preabsorption of FFA3 antibody in the section of CSMG. **E**, liver section as a negative control had no FFA3-IR. Nuclei were counterstained with DAPI (blue). **F–H**, FFA3-IR (red) in rat FFA3- (**F**), rat FFA2- (**G**) or mock- (**H**) transfected HeLa cells. After a 48 h culture, the transfected cells were fixed by 4% paraformaldehyde for 15 min and reacted with FFA3 antibody. **I**, FFA3 mRNA expression assessed by real-time RT-PCR in the CSMG, in the muscle layer of proximal colon (PCs) and in the liver (L). * $P < 0.05$. **J**, western blot using FFA3 antibody ($1 \mu\text{g ml}^{-1}$) detected a ~ 38 kDa band (arrow) in the extracted protein from rat colonic submucosa (SM) and absorbed by antigen peptide ($10 \mu\text{g ml}^{-1}$). **M**, standard proteins with molecular size (kDa) on the left. **K** and **L**, immunohistochemistry with α -CGRP antibody in serial sections of rat dorsal root ganglia. α -CGRP-IR (arrow, **K**) was absorbed by antigen peptide ($5 \mu\text{g ml}^{-1}$, **L**).

proximal colon. The serosal application of CCh ($10 \mu\text{M}$) in the absence of pretreatment rapidly increased I_{sc} , accompanied by G_{t} increase, peaking at ~ 2 min, followed by a gradual return to basal levels (Fig. 4A). The I_{sc} increase evoked by CCh in rat proximal colon exclusively reflects Cl^- secretion (Kaji *et al.* 2015a). The selective FFA3 agonist MQC itself, when applied into the luminal or serosal bath after the stabilization period, had no effect on basal I_{sc} , whereas pretreatment with MQC decreased the peak value of CCh-induced ΔI_{sc} but did not change

the time course of the response (Fig. 4A). CCh-induced ΔI_{sc} was suppressed by serosal MQC in a dose-dependent manner (Fig. 4B) with a 65% maximal inhibition and a calculated $\text{IC}_{50} = 13 \mu\text{M}$ by fitting to a sigmoid curve by the least squares method, which is comparable to the previously reported affinity of MQC as measured in a cell expression system ($6 \mu\text{M}$) (Hudson *et al.* 2014). These results suggest that activation of FFA3 expressed on the submucosal plexus inhibits cholinergic-mediated anion secretion.

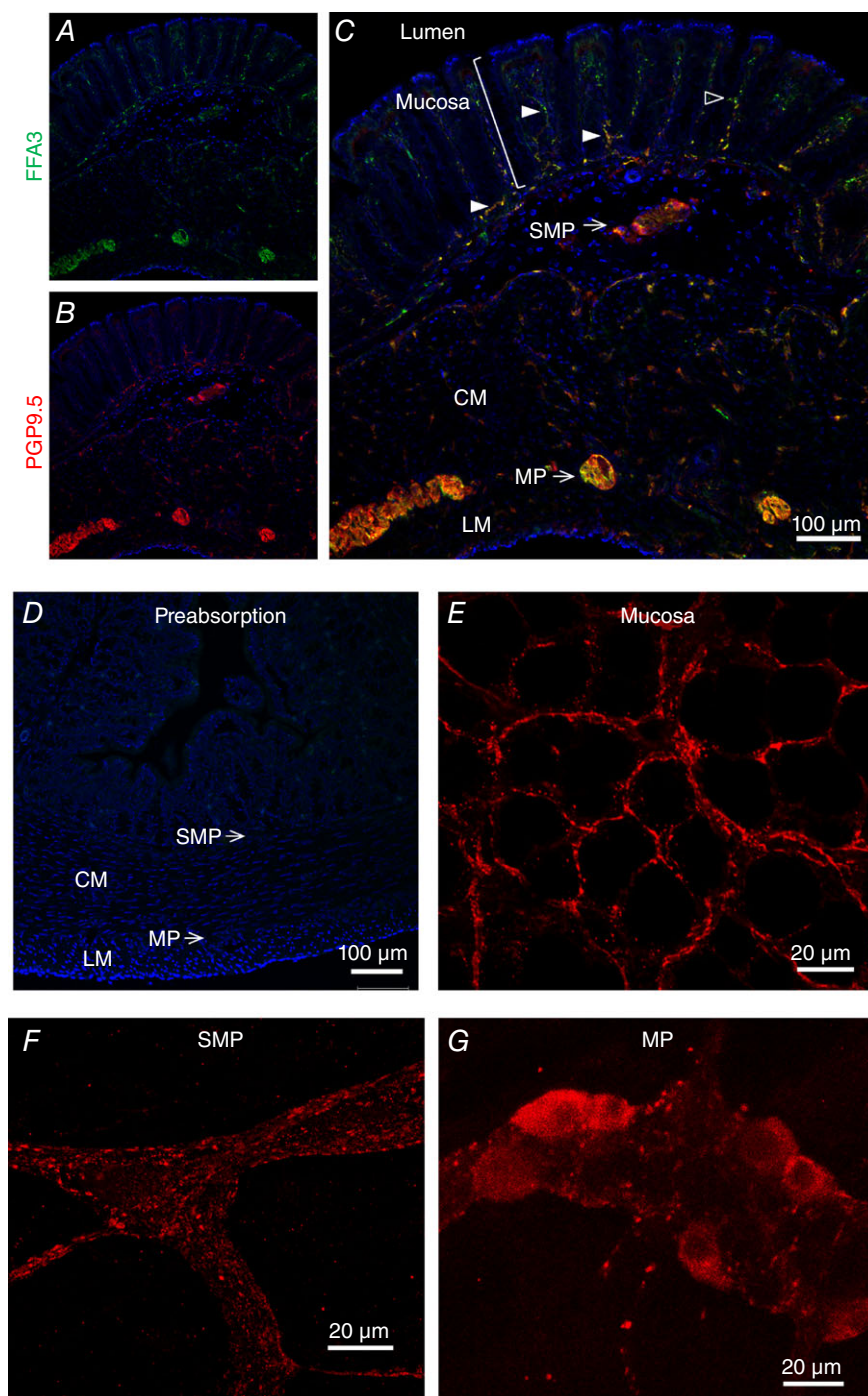


Figure 2. Localization of FFA3-IR in rat proximal colon

A–C, frozen sections were double-stained with FFA3 (A) and with PGP9.5 (B). The merged image (C) showed that FFA3 colocalized with PGP9.5 in subepithelial nerve fibres (solid arrowheads), in the submucosal plexus (SMP), in intramuscular nerves in circular (CM) and longitudinal (LM) muscle, and in myenteric plexus (MP). Scattered FFA3-positive epithelial cells had morphology resembling enteroendocrine cells (open arrowhead). D, preabsorption abolished FFA3-IR. Nuclei were counterstained with DAPI (blue). E–G, whole mounts of mucosa (E), submucosa (F) and myenteric plexus (G) of proximal colon were stained with FFA3 antibody (red).

Effect of TTX and ACh receptor antagonists on CCh-induced I_{sc} increase

Pretreatment with serosal TTX, hexamethonium, MQC (100 μM) or another FFA3 agonist AR420626 (10 μM) decreased CCh-induced ΔI_{sc} by 45%, 61%, 59% or 61% respectively (Fig. 4C). The non-selective muscarinic receptor antagonist atropine abolished CCh-induced ΔI_{sc} , indicating that cholinergic-induced Cl^- secretion is mediated by muscarinic receptors, whereas the neural reflex via nicotinic ACh receptors mediates $\sim 60\%$ of the response. A combination of TTX and MQC had no additional effect on CCh-induced Cl^- secretion, suggesting that FFA3 activation suppresses the response to CCh via a TTX-sensitive neural pathway. Pretreatment with MQC suppressed nicotine-induced secretion by 55% but had no effect on bethanechol-induced secretion (Fig. 4D). Because bethanechol is a selective muscarinic agonist, these results suggest that the TTX-inhibitable portion of CCh-evoked secretion is mediated by nicotinic

receptors expressed on cholinergic nerves, which are inhibited by pre-activation of FFA3.

We confirmed the involvement of the neural pathway in MQC-induced inhibition using mucosa-only preparations, in which submucosal neurons have been excluded. In Ussing chambered mucosal preparations, CCh-induced ΔI_{sc} was unaltered by TTX or MQC treatment (Fig. 4E), further suggesting that TTX-sensitive submucosal neurons are involved in the inhibitory effect of MQC on CCh-induced secretion.

Effect of PTX on FFA3-mediated cholinergic inhibition

We next studied the inhibitory mechanisms of FFA3 paying particular attention to the $G_{i/o}$ pathway using PTX in mucosa-submucosa preparations. Serosal application of a submaximal concentration of MQC (30 μM) decreased CCh-evoked ΔI_{sc} by 42%. Pretreatment with serosal PTX (500 ng ml^{-1}) for 45 min before MQC application restored the response to CCh in the presence of MQC (Fig. 4F),

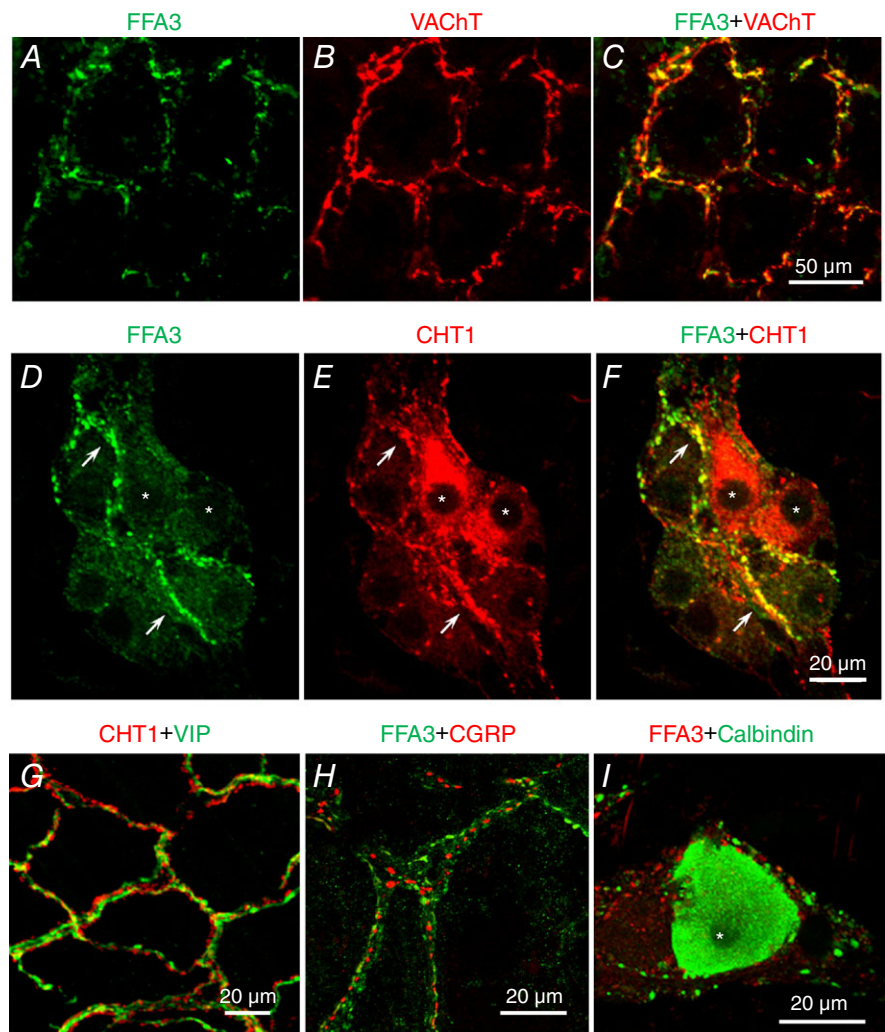


Figure 3. Localization of FFA3-IR in cholinergic nerves in whole mounts of mucosa and in submucosal plexus
 A–C, FFA3 (A) and VAcHT (B) in mucosal plexus. Both FFA3- and VAcHT-IR nerves (merged image in C) surrounded the crypts. D–F, FFA3 (D) and CHT1 (E) expression in the submucosal plexus. Most FFA3-IR nerve fibres and endings expressed CHT1 (arrows in F), whereas FFA3-IR was faint in CHT1-IR neuron somata (asterisks). G, CHT1 (red) and VIP (green) expressed in mucosal plexus. VIP-IR and CHT1-IR were detected in individual nerve fibres. H and I, FFA3 and α -CGRP (H) or calbindin (I) in submucosal plexus. Calbindin-IR neuron (asterisk) had no FFA3-IR.

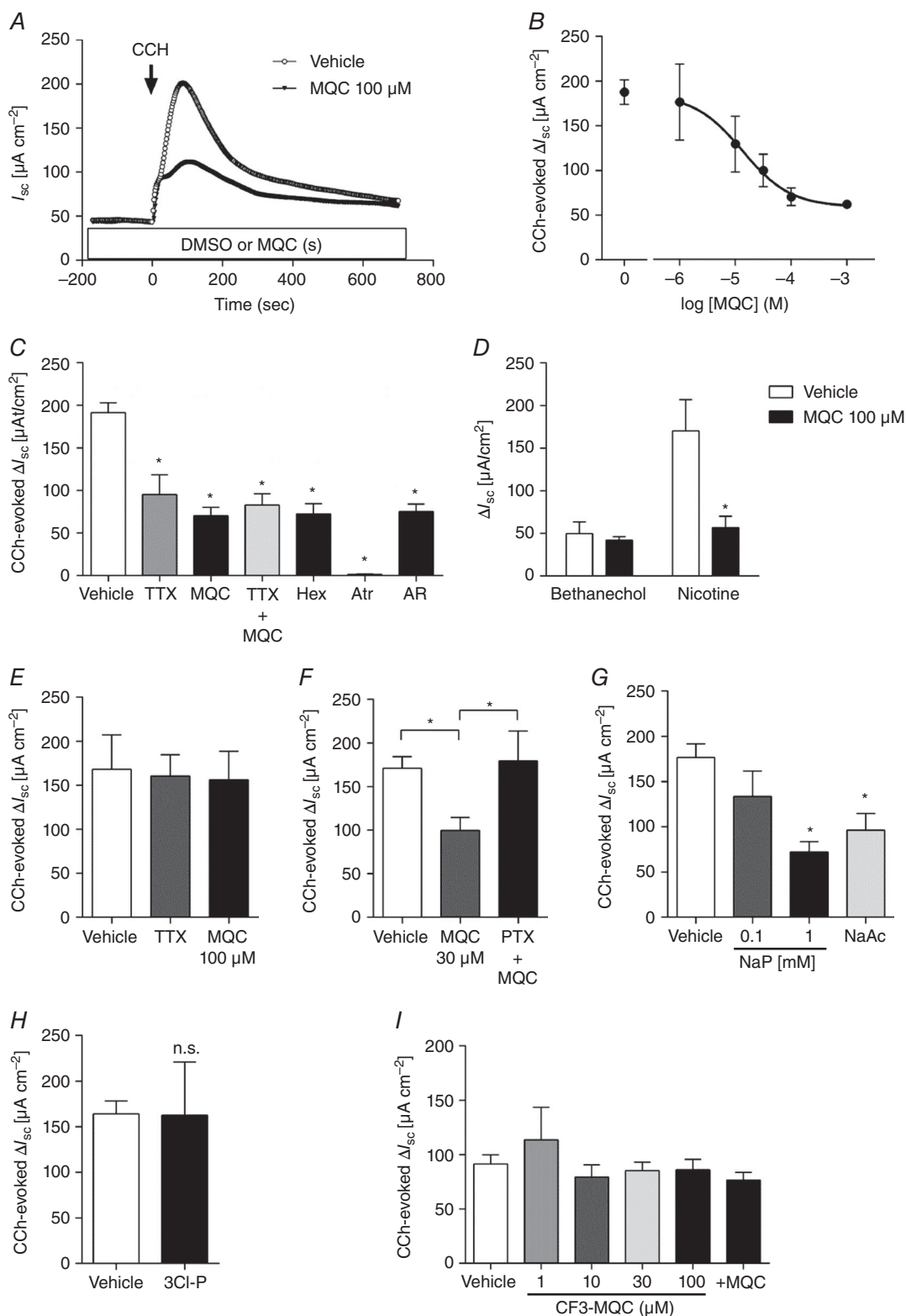


Figure 4. Effect of FFA3 ligands on CCh-induced I_{sc} increases in Ussing-chambered rat proximal colon
 Preparations were incubated with inhibitors during the stabilization period, whereas MQC or AR420626 was added 15 min before CCh application. All chemicals were added into the serosal bath. *A*, representative I_{sc} traces in mucosa–submucosa preparations in response to CCh ($10 \mu\text{M}$) in the presence or absence of MQC. *B*, concentration-dependent inhibition of MQC on CCh-evoked ΔI_{sc} in mucosa–submucosa preparations. A single

indicating that the anti-secretory effect of FFA3 activation is mediated by the $G_{i/o}$ pathway.

Effect of serosal SCFAs on CCh-induced I_{sc} increase

We further examined whether serosal SCFAs mimic the inhibitory effect of MQC on CCh-induced I_{sc} increase. The sodium salts of the endogenous FFA3 ligands propionate (NaP) and acetate (NaAc), pH adjusted to 7.4 with 1 N HCl, were serosally applied in mucosa–submucosa preparations. Pretreatment with 1 mM NaP or NaAc suppressed CCh-induced Cl^- secretion as did MQC (Fig. 4G). Conversely, 3-chloropropionate, an inactive propionate derivative (Yajima *et al.* 2011a), did not affect CCh-induced ΔI_{sc} (Fig. 4H).

Effect of FFA3 antagonists on CCh-induced I_{sc} increase

To confirm the specificity of FFA3 agonist MQC, we tested the synthetic FFA3 antagonist CF3-MQC (Ulven, 2012) in mucosa–submucosa preparations. Serosal application of CF3-MQC (1–100 μM) did not affect basal I_{sc} or the response to CCh (Fig. 4I). Pretreatment with CF3-MQC (100 μM) prevented the inhibitory effect of serosal MQC (30 μM) on CCh-induced Cl^- secretion (Fig. 4I, +MQC), suggesting that MQC acts as an FFA3 agonist.

Effect of MQC on luminal NaP-induced I_{sc} increase

Because luminal NaP evokes epithelial ACh release followed by colonic Cl^- secretion (Yajima *et al.* 2011a), we examined the effect of MQC on NaP-induced secretion in rat proximal colon. In mucosa–submucosa preparations, luminal NaP increased I_{sc} , accompanied by increased G_t , peaking at ~ 1.5 min, and subsequently returning to basal levels (Fig. 5A). NaP-induced ΔI_{sc} was reduced 65% by pre-treatment with TTX or with hexamethonium, and by 97% with atropine (Fig. 5B). Removal of the submucosa mimicked the inhibitory effect of TTX and hexamethonium (Fig. 5B), suggesting that the TTX-sensitive neural pathway is reliant on nicotinic receptors expressed in the submucosal plexus and that TTX-sensitive and -insensitive cholinergic pathways mediate NaP-induced Cl^- secretion. Serosally or mucosally applied MQC (1 mM) decreased the response

to NaP (Fig. 5C and D), suggesting that luminal NaP stimulates the mucosal plexus, which is inhibited by pre-activation of FFA3.

Effect of MQC on FK-induced I_{sc} increase

To examine whether neural FFA3 activation suppresses other secretory responses in mucosa–submucosa preparations, we used FK, a potent secretagogue that activates the cAMP pathway. Serosal application of FK (5 μM) consistently increased I_{sc} in the presence or absence of MQC (30 μM –1 mM), indicating that FFA3 activation is not involved in cAMP-mediated secretory responses and that MQC does not directly damage the mucosa (Fig. 5E).

Discussion

In this study, we have demonstrated that the SCFA receptor FFA3 was present in the mucosal, submucosal and myenteric plexuses of rat proximal colon. Most of the FFA3-positive nerves coexpressed the cholinergic neuronal markers, VAcHT and CHT1, but did not colocalize with VIP, calbindin or α -CGRP that innervated the colonic wall. In Ussing chambered mucosal and mucosa–submucosa preparations, synthetic and natural FFA3 agonists inversely regulated nicotinic ACh receptor-mediated Cl^- secretion via TTX- and PTX-sensitive pathways. Our findings suggest that SCFAs, via neural FFA3 activation, have a novel anti-secretory function.

Mucosal and submucosal cholinergic-mediated pathways are important for regulating colonic ion secretion (Diener *et al.* 1989). In colonocytes, ACh and VIP are Ca^{2+} - and cAMP-dependent secretagogues, respectively, synergistically potentiating epithelial ion secretion (Neunlist *et al.* 1998). Consistent with the lack of colocalization with ChAT and VIP in mice intestine (Foong *et al.* 2014), our whole-mount immunohistochemistry demonstrated that CHT1 colocalized with FFA3 and not with VIP in the branching nerves surrounding crypts in rat proximal colon, suggesting that FFA3-positive cholinergic nerves are separate from the VIP-ergic nerves. On the other hand, in rat small intestine, ChAT labels the majority of submucosal neurons, of which half are immunoreactive for NPY,

concentration of MQC was applied to each preparation. C, CCh-evoked ΔI_{sc} in the presence or absence of MQC (100 μM), AR420626 (10 μM), TTX (1 μM), hexamethonium (Hex: 10 μM) or atropine (Atr: 10 μM) in mucosa–submucosa preparations. * $P < 0.05$ vs. vehicle. D, effect of bethanechol (100 μM) or nicotine (100 μM) on I_{sc} in the presence or absence of MQC (100 μM) in mucosa–submucosa preparations. * $P < 0.05$ vs. vehicle. E, effect of MQC and TTX (1 μM) on CCh-evoked ΔI_{sc} in mucosal preparations. F, effect of MQC in the presence or absence of PTX (500 ng ml $^{-1}$) in mucosa–submucosa preparations. * $P < 0.05$. G and H, effect of NaP, NaAc (1 mM) or 3-chloropropionate (3Cl-P, 1 mM) on CCh-evoked ΔI_{sc} in mucosa–submucosa preparations. * $P < 0.05$ vs. vehicle. n.s., not significant. SCFA solutions were adjusted to pH 7.4. I, effect of CF3-MQC with or without MQC on CCh-evoked ΔI_{sc} in mucosa–submucosa preparations. A single concentration of CF3-MQC was applied to each preparation. MQC (30 μM) was added 10 min after the application of CF3-MQC (100 μM).

VIP and calbindin (Mann *et al.* 1999). In this study, CHT1 detected only 20% (21 plexuses from two rats) of PGP9.5-IR submucosal neurons in the proximal colon, suggesting that neurotransmitter distribution differs among animal species and intestinal regions. From double-staining studies of FFA3 with calbindin, FFA3-IR was present only in nerve fibres and scarcely

detected in the neuronal somata. Although CGRP-ergic secretomotor neurons are identified in rat small intestine and distal colon (Buchan, 1991; Mitsui, 2010), we failed to detect α -CGRP in the submucosal neurons. Rat myenteric and submucosal neurons express β -CGRP, which is a gene product almost homologous to, but distinct from, α -CGRP, rather than α -CGRP (Sternini &

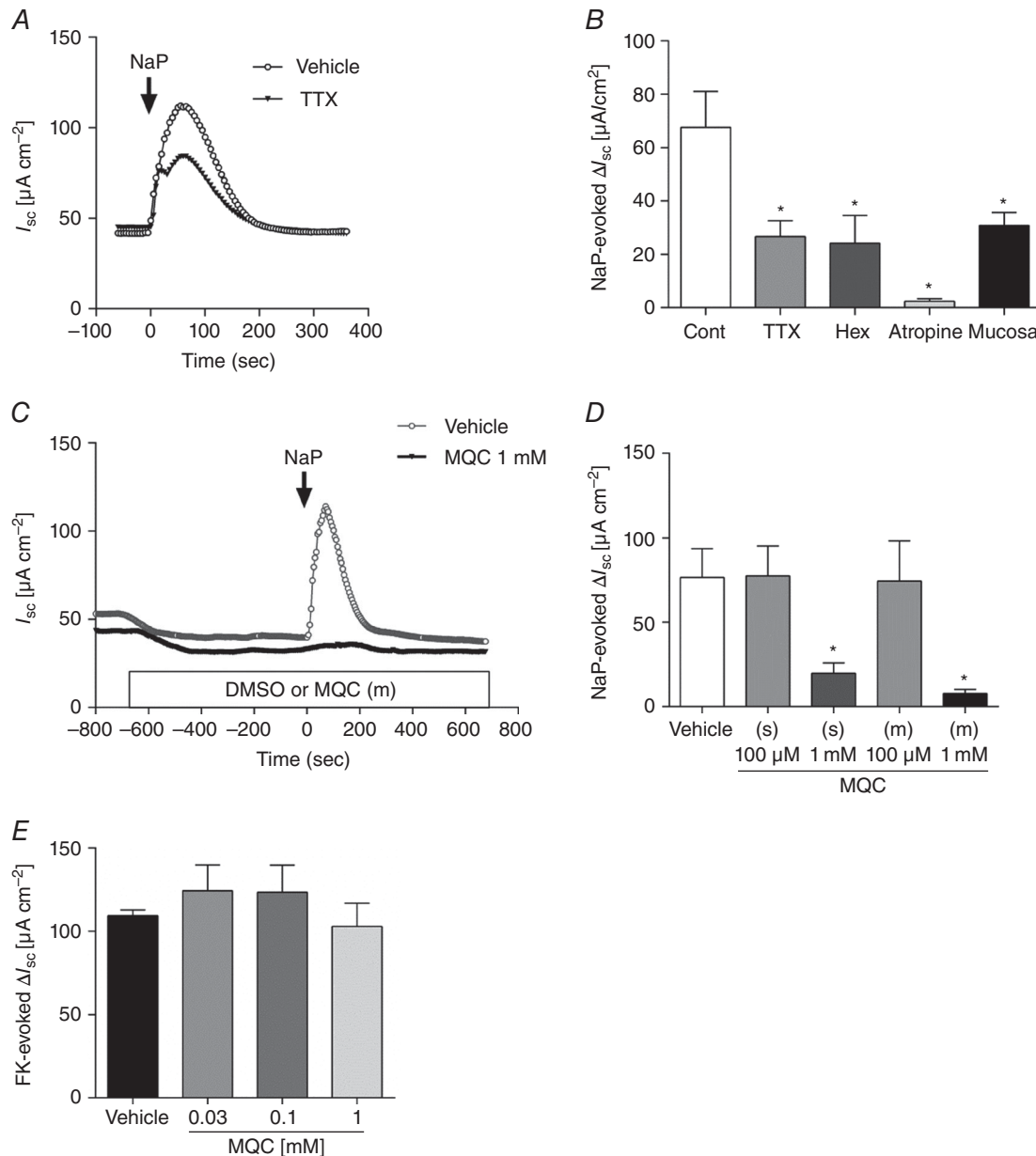


Figure 5. Effect of MQC on NaP- or FK-evoked ΔI_{sc}

A, representative I_{sc} traces in response to luminal NaP (5 mM) in the presence (solid triangles) or absence (open circles) of TTX (1 μM). B, luminal NaP-evoked ΔI_{sc} in the presence or absence of TTX (1 μM), hexamethonium (100 μM) or atropine (10 μM), or in the submucosa-stripped mucosal preparations (Mucosa). C, representative I_{sc} traces in mucosa–submucosa preparations. NaP (5 mM) was added into the luminal bath following luminal DMSO (grey) or MQC (1 mM, black). MQC decreased basal I_{sc} , as did DMSO (vehicle) and inhibited the response to NaP. D, NaP-evoked ΔI_{sc} in the presence or absence of MQC in the serosal (s) or luminal (m) bath 10 min before the application of NaP (5 mM). * $P < 0.05$ vs. vehicle. E, FK (5 μM) was added into the serosal bath and ΔI_{sc} was measured in the presence or absence of individual concentrations of serosal MQC.

Anderson, 1992). Our antibody directed against α -CGRP might not recognize β -CGRP under the experimental conditions used in the present study. By contrast, α -CGRP expression is greater than β -CGRP expression in the dorsal root and nodose ganglia (Sternini & Anderson, 1992). Accordingly, our data suggest that α -CGRP-IR nerves represent extrinsic afferent neurons and do not express FFA3, at least in rat proximal colon. FFA3-positive cholinergic nerve fibres and endings probably originated from myenteric neurons and/or extrinsic neurons. Cholinergic vagal nerve endings are present in rat proximal colonic ganglia (Berthoud *et al.* 1991) and ACh production is present in rat nodose and dorsal root ganglia (Palouzier *et al.* 1987; Bellier & Kimura, 2007), which include FFA3-positive afferent neurons (Nohr *et al.* 2015). Enteric neural circuits, including internal primary afferent neurons and/or interneurons that secondarily excite secretomotor neurons, are proposed to be present in guinea-pig ileum and in rat distal colon (Christofi *et al.* 2004; Reed & Vanner, 2007). Therefore, further investigations are required to identify the origin of the FFA3-IR nerves, as well as their contribution to the neural circuit, which innervate and terminate in mucosal and submucosal plexuses in rat proximal colon.

Our Ussing chamber study demonstrated that the selective FFA3 agonists MQC or AR420626 reduced cholinergic-mediated secretion via the submucosal plexus. The anti-secretory effect of MQC was concentration-dependent and PTX-sensitive, consistent with $G_{i/o}$ -coupled FFA3 activation. TTX reduced CCh-evoked responses by half, as did hexamethonium, AR420626, MQC or a combination of TTX and MQC, suggesting that FFA3 activation suppresses a nicotinic receptor-mediated response. Because atropine abolished CCh-induced secretion, muscarinic receptors were the end target of cholinergic-mediated secretion via direct and indirect pathways. Pre-treatment with MQC consistently inhibited nicotine-induced secretion but did not affect bethanechol-induced secretion in mucosa-submucosa preparations. Furthermore, neither MQC, nor TTX altered CCh-evoked secretion in the mucosal preparation, where submucosal neurons were eliminated, suggesting that MQC has no direct interaction with muscarinic receptors expressed on epithelial cells. Because ligands activate nicotinic receptors at the site of neurotransmitter release via a TTX-sensitive mechanism (Schneider *et al.* 2000), cholinergic neuronal axons expressing FFA3, which emanate from the myenteric neurons located in the submucosal plexus, are possibly activated by CCh and nicotine, and release ACh. In addition to the synthetic agonists, serosal SCFAs suppressed CCh-evoked Cl^- secretion. The physiological concentration of SCFAs in the subepithelial and submucosal space is unknown; however, it is probably in the micromolar to millimolar range as a result of

the absorption of luminal SCFAs via enterocyte MCTs followed by transcellular transport into the submucosal space. Therefore, cholinergic-mediated Cl^- secretion may be physiologically controlled by luminal SCFAs via FFA3 activation. By contrast, 3-chloropropionate did not alter basal I_{sc} and CCh-evoked I_{sc} increase. This analogue inhibits NaP-induced ACh production and subsequent anion secretion in rat distal colon (Yajima *et al.* 2011a) and also inhibits lactate transport in rabbit renal brush border membrane (Nord *et al.* 1983). These observations suggest that 3-chloropropionate is probably competitive with SCFAs for MCT, but is not itself an FFA3 ligand, and also that the serosal SCFA-induced anti-cholinergic effect is not a result of MCT inhibition.

Although luminal NaP stimulates epithelially-derived ACh release (Yajima *et al.* 2011a), FFA3-selective agonists in the concentrations that we tested did not directly evoke Cl^- secretion even with luminal application. Luminal NaP-induced secretion was inhibited by TTX, hexamethonium or removal of the submucosa, and abolished by atropine in rat proximal colon, which is consistent with a study of secretion in rat distal colon (Yajima, 1988). Similar to the effect of CCh, luminal NaP stimulates epithelial muscarinic receptors and a TTX-sensitive pathway, which is suppressed by FFA3 activation. Because higher concentrations of MQC were needed to reduce the response to luminal NaP than to serosal CCh, luminal NaP probably releases ACh, which diffuses to the lamina propria where it stimulates mucosal cholinergic axons, followed by neuronal nicotinic receptor activation and activation of the submucosal neural reflex, resulting in the release of additional ACh, which in turn stimulates Cl^- secretion. Our results suggest that activation of mucosal cholinergic nerves by luminal NaP is suppressed by neuronal FFA3 activation. $G_{q/11}$ -coupled GPCRs expressed on the apical membrane of colonocytes are probably SCFA sensors that stimulate epithelial ACh release (Yajima *et al.* 2011b). Therefore, $G_{i/o}$ -coupled FFA3 is probably not involved in the stimulatory effects of luminal SCFAs.

Luminal SCFAs stimulate anion secretion via the cholinergic pathway, whereas basolateral SCFAs suppress cholinergic-mediated secretion via FFA3 activation. This apparently contradictory response to SCFAs might represent a negative feedback system that homeostatically regulates colonic anion secretion during SCFA absorption. From a pharmacological standpoint, SCFAs may have a novel anti-cholinergic function via FFA3 activation, serving to curtail vagal hyperactivity. Activation of FFA3 suppresses peripheral synaptic transmission by pre-synaptic Ca^{2+} influx inhibition in rat prevertebral ganglia (Won *et al.* 2013). Furthermore, FFA3 activation inhibits glucose-dependent insulin secretion via $G_{i/o}$ *in vivo* and *in vitro* in mice pancreas (Priyadarshini & Layden, 2015; Tang *et al.* 2015). FFA3 activation, which modulates

transmitter release via inhibition of Ca^{2+} influx into neurons and endocrine cells, probably exerts its anti-cholinergic action by presynaptic ACh release.

We also detected FFA3-IR in intramuscular nerves and in a subpopulation of myenteric neurons. A lack of FFA3 accelerates the intestinal transit rate and decreases the absorption rate of luminal SCFAs (Samuel *et al.* 2008). In the GI tract, neural FFA3 may be involved in regulation of the rate of nutrient absorption via the slowing of intestinal transit and inhibition of secretion during the digestive phase following a meal. The high concentrations of plasma acetate (~ 1 mM) present after alcohol consumption (Korri *et al.* 1985) or of the ketone β -hydroxybutyrate present during starvation or diabetic ketoacidosis (6–10 mM) serve as endogenous FFA3 agonists (Won *et al.* 2013), suggesting that the enteric cholinergic reflex may be disrupted under such conditions. Because ACh availability is increased under stress conditions (Kita *et al.* 1986) and cholinergic signalling mediates stress-induced increases in intestinal ion transport and permeability in rats (Saunders *et al.* 1997), stress-induced diarrhoea or diarrhoea-predominant irritable bowel syndrome could be a therapeutic target for the FFA3 agonists.

In conclusion, neural FFA3 activation counters cholinergic secretion in the mucosal and submucosal plexuses of rat proximal colon. FFA3, which senses luminal bacteria-derived SCFA probably 'fine-tunes' the activity of the enteric nervous system. We propose that FFA3 is a key modifier of the cholinergic reflex that helps maintain physiological levels of secretion and motility.

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Additional information

Competing interests

The authors declare that they have no competing interests.

Author contributions

IK, YA and JDK were responsible for the study concept and design, and for the original draft. IK, KK, MW and TI were responsible for antibody production. AK and KI were responsible for chemical design and synthesis. IK, YA and SK were responsible for collection, assembly and analysis of data. IK, YA, MW, TI, AK and JDK were responsible for data interpretation. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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