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Cannabinoid 1 (CB_1) receptors coupled to cholinergic motorneurones inhibit neurogenic circular muscle contractility in the human colon

[1]Nicholas M. Hinds, [2]Katja Ullrich & *,[2]Scott D. Smid

[1]School of Chemical & Biomedical Sciences, Faculty of Arts Health & Sciences, Central Queensland University, Bruce Highway, Rockhampton, QLD 4702, Australia and [2]Faculty of Health Sciences & Medicine, Bond University, Robina, QLD 4229, Australia

1 The effects of cannabinoid subtype 1 (CB_1) receptor activation were determined on smooth muscle, inhibitory and excitatory motorneuronal function in strips of human colonic longitudinal muscle (LM) and circular muscle (CM) *in vitro*.

2 Electrical field stimulation (EFS; 0.5–20 Hz, 50 V) evoked a relaxation in LM and CM precontracted with a neurokinin-2 (NK-2) selective receptor agonist (β-ala^8-neurokinin A; 10^{-6} M) in the presence of atropine (10^{-6} M); this was unaltered following pretreatment with the CB_1-receptor selective agonist arachidonyl-2-chloroethylamide (ACEA; 10^{-6} M).

3 In the presence of nitric oxide synthase blockade with *N*-nitro-L-arginine (10^{-4} M), EFS evoked a frequency-dependent 'on-contraction' during stimulation and an 'off-contraction' following stimulus cessation. On-contractions were significantly inhibited in CM strips by pretreatment with ACEA (10^{-6} M). These inhibitory effects were reversed in the presence of the CB_1 receptor-selective antagonist *N*-(piperidine-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (10^{-7} M).

4 ACEA did not alter LM or CM contractile responses to acetylcholine or NK-2 receptor-evoked contraction.

5 Immunohistochemical studies revealed a colocalisation of CB_1 receptors to cholinergic neurones in the human colon based on colabelling with choline acetyltransferase, in addition to CB_1 receptor labelling in unidentified structures in the CM.

6 In conclusion, activation of CB_1 receptors coupled to cholinergic motorneurones selectively and reversibly inhibits excitatory nerve transmission in colonic human colonic CM. These results provide evidence of a direct role for cannabinoids in the modulation of motor activity in the human colon by coupling to cholinergic motorneurones.
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Abbreviations: ACEA, arachidonyl-2-chloroethylamide; ACh, acetylcholine; AM251, *N*-(piperidine-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; β-ala^8-NKA, β-ala^8-neurokinin A 4–10; CB_1, cannabinoid receptor subtype 1; CB_1-IR, CB_1 receptor immunoreactivity; CB_2, cannabinoid receptor subtype 2; ChAT, choline acetyltransferase; CM, circular muscle; COX, cyclooxygenase; DMPP, 1,1-dimethyl-4-phenylpiperazinium iodide; EFS, electrical field stimulation; FITC, fluorescein isothiocyanate; NANC, nonadrenergic noncholinergic; NK-2, neurokinin 2; NOS, nitric oxide synthase; LM, longitudinal muscle; L-NNA, N_ω-nitro-L-arginine; RRX, rhodamine red X

Introduction

The discovery of specific membrane-bound cannabinoid (CB) receptors (to which the psychotropic ing redient of marijuana, Δ^9-THC, acts) in the early 1990s led to the rapid characterisation of the effects of CB receptor-selective ligands on gastrointestinal function. The pharmacological effects of all CB ligands are mediated through at least two known G-protein-coupled receptor subtypes, termed CB_1 and CB_2. CB_1 receptors are found predominantly on central and peripheral neurones (including enteric neurones). The functional correlates to CB_1 receptor activation in the gut are a reduction in motility and secretion in a number of species, including humans (Croci *et al*., 1998b; Izzo *et al*., 2001a; Manara *et al*., 2002), which are likely to involve both centrally and peripherally located CB_1 receptors (Hornby & Prouty, 2004). Importantly, indirect evidence suggests that endogenous CBs (endocannabinoids) exert a tonic influence on intestinal motility, as CB_1 receptor antagonists elicit increased isolated intestinal contractility and *in vivo* defaecation when administered alone in animal models, suggesting some constitutive level of activity (Mancinelli *et al*., 2001; Pertwee, 2001; Pinto *et al*., 2002). Anatomical immunohistochemical studies have demonstrated the localisation of

*Author for correspondence at: Discipline of Pharmacology, School of Medical Sciences, Faculty of Health Sciences, The University of Adelaide, Adelaide, SA 5005, Australia;
E-mail: scott.smid@adelaide.edu.au

CB_1 receptor immunoreactivity (CB_1-IR) in the myenteric and submucosal plexuses of the porcine ileum and colon (Kulkarni-Narla & Brown, 2000), murine gastrointestinal tract (Casu *et al*., 2003), in addition to the guinea-pig and rat myenteric plexus (Coutts *et al*., 2002). A recent study characterised the general anatomical locations of the CB_1 receptor in the human colon, but did not specifically investigate discrete neuroanatomical sites of binding, nor neurochemical coding associated with CB_1-IR (Wright *et al*., 2005). Few studies have investigated the effects of CB ligands on human colonic motility. Of those, one study demonstrated a distinct suppression of neurogenic contractility (Manara *et al*., 2002), while another showed that the endocannabinoid anandamide did not stimulate colonic relaxation (Bartho *et al*., 2002).

The capacity for CBs to modulate gastrointestinal motility and secretion has led to their regard as potential therapies for a wide range of gastrointestinal disorders, including diarrhoea, gastroesophageal reflux disease and inflammatory bowel disease (Di Carlo & Izzo, 2003). With this in mind, the aims of the current study were to determine the effects of CB_1 receptor activation on myogenic and neurogenic excitatory and inhibitory motor function in the human colon. This was determined functionally, by measuring the responses of isolated muscle strips of human colonic muscle to various stimuli in the presence of CB_1 receptor agonists. In addition, immunohistochemical localisation of the CB_1 receptor was determined across the mucosal and muscularis layers of the human colon and its localisation compared to a marker of cholinergic neurones.

Methods

Tissue collection and preparation

Experiments were performed on colonic tissue taken from patients referred to the Rockhampton Base and Mater Misericordiae Hospitals, Rockhampton and The John Flynn Hospital, Tugun. All studies were performed in accordance with the guidelines of the Central Queensland University (CQU), Rockhampton Health Service District, Bond University and John Flynn Hospital Human Research Ethics Committees.

Specimens of human colon were prepared following colectomy from a maximum of 20 patients with intestinal carcinoma (mean age 69.3 ± 9.1 years, range 57–83 years). The samples collected were obtained from macroscopically normal regions within the sigmoid colon not involved with malignancy, and were not affected by colitis, fibrosis or inflammation as assessed using independent standard histological techniques. Specimens of full thickness tissue were placed in ice-cold (4°C) carbogenated (95% O_2, 5% CO_2) Kreb's solution of the following composition (mM): NaCl 118, $NaHCO_3$ 25, KCl 4.6, $MgSO_4$ 1.2, NaH_2PO_4 1.3, glucose 11, $CaCl_2$ 2.5. Samples were transferred to the research laboratory within 30 min of resection.

After removal of the mucosa and submucosa, tissue was divided under sharp dissection in the longitudinal and circular orientations to give uniform bands of longitudinal (LM) and circular muscle (CM) of dimensions 3 mm × 15 mm, together with the attached myenteric plexus. Isolated muscle strips were mounted in Kreb's-filled organ baths at 37°C and continuously bubbled with carbogen. One end of the sutured tissue was fastened to a glass tissue support, while the other end was attached to an isometric force transducer (FT03, Grass Instruments, Quincy MA). Platinum wire electrodes were attached to the tissue support and positioned on either side of the tissue to enable electrical field stimulation (EFS). The tissue was placed under an initial tension of 1.0 g and left to equilibrate for 60 min, with replacement of Kreb's solution every 15 min. Once equilibrated, tissue strips were exposed to acetylcholine (ACh; 10^{-4} M) in order to establish responsiveness of the tissue. This concentration of ACh typically evoked stable, submaximal contractions, and preparations which responded poorly to ACh at this stage were discarded from further studies. The remaining tissue was snap frozen and stored at −70°C for further histochemical and immunohistochemical analysis.

Isometric muscle responses were amplified and digitised *via* an analogue/digital interface (Quad Bridge and PowerLab 4/20; AD Instruments, Sydney, Australia) prior to being acquired onto a personal computer. All tension measurements were acquired and managed onto a computer hard drive using Chart™ software (version 4.0.4, AD Instruments).

Excitatory (contractile) motor responses

In order to examine excitatory contractile responses, the nitric oxide synthase (NOS) inhibitor N_ω-nitro-L-arginine (L-NNA; 10^{-4} M) was added as a pretreatment to LM and CM preparations, where a contact time of at least 10 min was allowed before further treatments.

In order to investigate the effects of CB ligands on neurogenic contractility, EFS was delivered to the tissue *via* square wave pulses from an electrical stimulator (SD9, Grass-Telefactor, RI, U.S.A.). Contraction of colonic muscle strips in response to increasing frequencies of electrical stimulation was examined (0.5–20 Hz, 50 V, 1.5 ms pulse width, duration 5 s), with tissue allowed to return to baseline tension for 2–3 min before stimulating at higher frequencies.

In addition, LM and CM responses to increasing concentrations of ACh were also measured to investigate the effects of CB drugs on myogenic responses, with final bath concentrations of 10^{-8}–10^{-4} M achieved using a cumulative protocol. Maximum contraction was recorded and the strips washed out with fresh Kreb's solution, left to return to baseline tension and L-NNA (10^{-4} M) reapplied.

Inhibitory (relaxation) motor responses

To ensure nonadrenergic noncholinergic (NANC) conditions, separate experiments were performed in the presence of bretylium (10^{-6} M) and atropine (10^{-6} M), which was equilibrated with the tissue for 1 h prior to further experimentation. Muscle strips were then exposed to a test concentration of the neurokinin 2 (NK-2) receptor-specific agonist β-ala^8-neurokinin A 4–10 (β-ala^8-NKA) to elicit contraction prior to EFS. A final bath concentration of 10^{-6} M β-ala^8-NKA evoked submaximal contraction in CM and LM strips and was subsequently chosen as the test concentration for precontraction. β-ala^8-NKA produced a stable contraction in isolated tissue preparations within 30 s of administration and this became the minimum contact time for eliciting contraction

prior to EFS in all experiments. LM and CM preparations precontracted with β-ala^8-NKA (10^{-6} M) were followed by sequential EFS (0.5–20 Hz, 50 V, 1.5 ms pulse width, duration 5 s), which evoked muscle relaxation. Muscle tension was allowed to return to a stable plateau of contraction before tissue was stimulated with higher frequencies. The EFS-evoked relaxation was compared with the response to a supramaximal test concentration of isoprenaline (10^{-4} M), which elicits direct myogenic relaxation. This was still possible under NANC conditions, as bretylium blocks sympathetic nerve terminals and not smooth muscle β-adrenergic receptors.

CB effects on motility indices

The effect of CB_1 receptor activation on excitatory and inhibitory myogenic and neurogenic responses was examined in the presence of arachidonyl-2-chloroethylamide (ACEA, 10^{-6} M; CB_1 receptor-selective agonist). ACEA was incubated in the organ bath medium for a period of 10 min prior to the start of the respective protocols. In addition, the effect of ACEA on the neurogenic excitatory response of the CM tissue preparations to EFS was also examined in the presence of *N*-(piperidine-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM251; 10^{-7} M, selective CB_1 receptor antagonist). In these experiments ACEA was re-administered following organ bath washout, allowed to equilibrate for 10 min and then followed by AM251 administration, which was allowed to equilibrate for a further 10 min in the organ bath prior to the stimulus– or concentration–response protocols.

Immunohistochemical studies

Tissue adjacent to that used for functional studies was rinsed in 0.01 M phosphate-buffered saline (PBS, pH 7.2), followed by fixation in ice-cold 2% paraformaldehyde in PBS for 2 h. Fixed tissue was then cryoprotected by dehydrating in graded solutions of sucrose (5, 10 and 20% w/v in PBS) at 4°C for 1 h at each concentration. Tissue was incubated overnight at 4°C in 20% sucrose in PBS, before being embedded in tissue-freezing medium on a cryostat specimen holder and snap frozen in liquid nitrogen. Frozen specimens were stored at −70°C until required and were also processed for routine histological analysis, using a haematoxylin and eosin staining protocol specific for cryogenic sections. For cryogenic sectioning, full-thickness tissue (i.e. including mucosal and submucosal layers) was embedded in TBS tissue-freezing medium (Triangle Biomedical Sciences, Durham, NC, U.S.A.) before tissue sections of 15 μm were cut and slide-mounted (SuperFrost® Plus slides, Menzel-Glaser, Germany). Adjacent sections of colonic tissue were then air-dried for 30 min before being re-hydrated in 0.01 M PBS (pH 7.2) for 15 min. Sections were then incubated at room temperature in 0.4% Triton X-100 and 2% bovine serum albumin in PBS for 30 min to permeabilise the tissue and block nonspecific binding, respectively. After three rinses in PBS, the sections were incubated with 1 : 200, 1 : 600 and 1 : 1000 dilutions of a primary antibody raised in rabbit against the CB_1 receptor (Table 1) or 1 : 10, 1 : 100 and 1 : 1000 dilutions of a goat anti-choline acetyltransferase (ChAT) antibody (Table 1). Both primary antibodies were diluted in 0.4% Triton X-100 and 2% BSA and were incubated at 4°C overnight in a humidified chamber. After rinsing in PBS, tissue sections were incubated with appropriate secondary antibodies, including a donkey anti-rabbit IgG conjugated to Rhodamine Red-X (RRX; Table 1) for CB_1 visualisation and a donkey anti-goat IgG conjugated to fluorescein isothiocyanate (FITC) (Table 1) to visualise ChAT immunoreactivity. Both secondary antibodies were diluted 1 : 50 in PBS and incubated in a humidified chamber at room temperature for 1 h in the dark. Following three rinses in PBS for 15 min, coverslips were mounted with Vectashield mounting medium (Vector Laboratories, CA, U.S.A.).

Control experiments were conducted on adjacent full-thickness tissue sections and included negative control sections in which the primary antibodies were omitted from the staining protocol (replaced by 0.01 M PBS alone) and isotype controls in which rabbit IgG (Sigma-Aldrich) diluted 1 : 200 or goat IgG (Sigma-Aldrich) diluted 1 : 10 were used in place of the primary antibodies, in order to examine nonspecific binding of the IgG molecules. Stained tissue sections were observed using an epifluorescence microscope (Olympus BX 41, Japan) fitted with filter cubes specific for selective observation of rhodamine red X (RRX) and FITC. Images were obtained using the fitted Olympus digital camera (DP70) and processed by DP manager software (version 1,2,1,107, Olympus Optical, Japan).

For double-labelling studies, tissue sections were prepared in the same manner as for single-labelling experiments, except that the primary antibodies were incubated together overnight at 4°C at a 1 : 200 dilution in PBS for the rabbit anti-CB_1 IgG

Table 1 Description of antibodies used for immunohistochemical studies

Antibodies	*Host species*	*Dilution*	*Source*
Primary			
CB_1	Rabbit	1 : 200 1 : 600 1 : 1000	Santa Cruz Biotechnology Inc., CA, U.S.A.
ChAT	Goat	1 : 10 1 : 100 1 : 1000	Santa Cruz Biotechnology Inc., CA, U.S.A.
Secondary			
Rabbit IgG (RRX)	Donkey	1 : 50	Jackson Immunoresearch, PA, U.S.A.
Goat IgG (FITC)	Donkey	1 : 50	Jackson Immunoresearch, PA, U.S.A.

CB_1, cannabinoid receptor 1; ChAT, choline acetyltransferase; RRX, rhodamine red X; FITC, fluorescein isothiocyanate.

and 1 : 100 dilution for the goat anti-ChAT IgG. After rinsing in PBS, sections were simultaneously incubated with the donkey anti-rabbit RRX IgG (1 : 50) and donkey anti-goat FITC IgG (1 : 50) secondary antibodies in the dark for 1 h at room temperature. Images consisting of CB_1 and ChAT immunoreactivity were overlaid using DP manager software to examine colocalisation patterns.

Drugs

β-ala^8-NKA was obtained from Auspep (Melbourne, Australia), while ACEA and AM251 were obtained from Tocris Cookson Ltd (Ellisville, MO, U.S.A.). ACh chloride, atropine sulphate, bretylium tosylate, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), isoprenaline hydrochloride, L-NNA and capsaicin were obtained from Sigma-Aldrich (Sydney, Australia). All drugs were dissolved in saline (0.9% w/v), except for ACEA, AM251 and capsaicin, which were dissolved in ethanol. Further drug dilutions were made in deionised water prior to being used. Incubation of tissue with the equivalent organ bath concentration of solvent alone showed no effect on functional indices in colonic tissue.

Data analysis

Increases in tension were measured as either a change in absolute contraction (g) or as a percentage of the maximal contraction elicited by a supramaximal concentration of ACh (10^{-4} M). Relaxation responses were measured as a decrease in tension from the maximal tension achieved by precontraction by β-ala^8-NKA (10^{-6} M) and expressed as a percentage of the maximal relaxation elicited by a supramaximal concentration of isoprenaline (10^{-4} M).

All data were analysed using the graphical and statistical analysis program Prism 2.0b (GraphPad, San Diego, CA, U.S.A.). Statistical analysis was performed using a two-way analysis of variance (ANOVA) followed by Bonferonni post-tests for EFS stimulus–response and NANC relaxation data. EC_{50} (agonist concentration eliciting 50% maximum response) and E_{MAX} (agonist concentration eliciting maximum response) values were generated *via* sigmoidal nonlinear regression of ACh and β-ala^8-NKA concentration–response data and used in paired, two-tailed *t*-tests to enable comparison between control and ACEA treatment groups. The significance was set at a *P*-value <0.05 for all analyses.

Results

Functional organ bath responses

EFS (0.5–20 Hz) of human colon circular (CM) and longitudinal muscle (LM) preparations in the presence of L-NNA evoked regular, frequency-dependent 'on-contractions' during the stimulus and often larger, frequency-independent 'off-contractions' once EFS had ceased (representative response of CM shown in Figure 1a). Contractions evoked by EFS were similar in profile between longitudinal and CM preparations, with the exception of larger amplitudes of response generally in LM preparations compared to CM (Figure 2). Off-contractions were not always evident, but were more often observed in LM preparations.

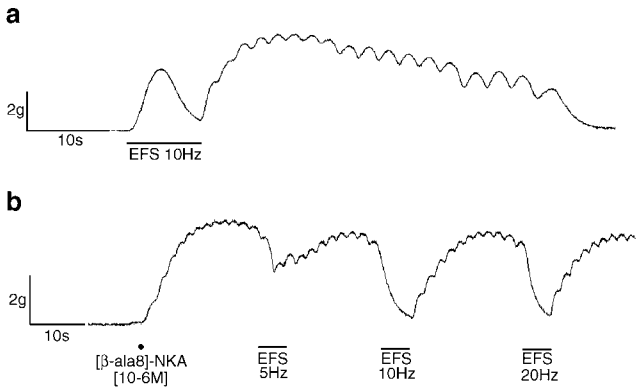

Figure 1 Single representative tracings of isolated muscle strip responses to EFS. (a) CM strip biphasic contraction in response to EFS (10 Hz) in the presence of L-NNA (10^{-4} M), demonstrating 'on-contraction' during stimulus (bar) and off-contraction following stimulus cessation. LM responses were similar in their response profile to CM, but generally larger in amplitude. (b) Longitudinal smooth muscle relaxation in response to EFS (5, 10 and 20 Hz), following precontraction with β-ala^8-NKA (10^{-6} M) under NANC conditions. CM relaxation responses were similar in response profile to LM (L-NNA, N_ω-nitro-L-arginine; NANC, nonadrenergic–non-cholinergic).

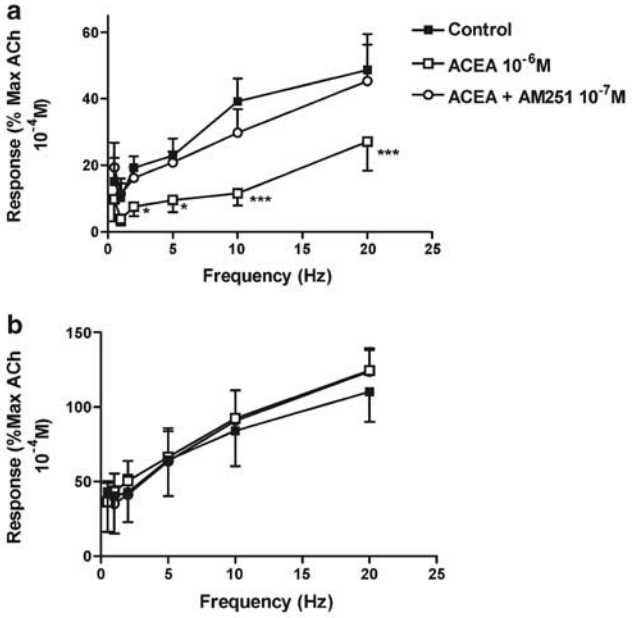

Figure 2 Effects of the CB_1 receptor agonist ACEA (10^{-6} M) on frequency–response curves in response to EFS-evoked 'on-contractions' in human colonic (a) CM and (b) LM. ACEA significantly inhibited neurogenic contractions over the range 2–20 Hz in CM, but not LM. The effects of ACEA in CM were reversed by the CB_1 receptor-selective antagonist AM251 (10^{-7} M) (CB_1, cannabinoid receptor 1, ACEA, arachidonyl-2-chloroethylamide, AM251, *N*-(piperidine-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1 *H*-pyrazole-3-carboxamide; $*P<0.05$, $***P<0.001$). $N=6$ CM; $n=5$ LM.

The cannabinoid receptor 1 (CB_1) agonist ACEA (10^{-6} M) inhibited the electrically evoked 'on-contractions' to a significant extent in CM, but not LM preparations (Figure 2). ACEA pretreatment did not have any effect on baseline

tension. The inhibition of the electrically evoked on-contractions by ACEA in CM was antagonised by a concentration of the CB_1 receptor antagonist AM251 (10^{-7} M) shown previously to reverse the effects of CB_1 receptor agonism (Sterin-Borda *et al*., 2005). Reversal of the inhibitory effect of ACEA was observed for the 'on-contractions' in the presence of AM251 (Figure 2a).

ACh (10^{-8}–10^{-4} M) caused both CM and LM strips to contract in a concentration-dependent manner. Neither EC_{50} nor E_{MAX} values were significantly different between control and ACEA treatment groups for both CM and LM strips (Table 2).

EFS (0.5–20 Hz) was applied to muscle strips following precontraction with β-ala^8-NKA (10^{-6} M). EFS elicited frequency-dependent muscle relaxation in both CM and LM strips (representative response of LM shown in Figure 1b; group data of LM and CM responses graphed in Figure 3). In both CM and LM strips maximal relaxation was achieved between 10 and 20 Hz. While there was evidence of a modest potentiation of EFS-evoked relaxations in CM preparations in the presence of ACEA (Figure 3a), this was not statistically significant.

Isoprenaline was employed as an agonist eliciting direct myogenic relaxation. This was still possible under NANC conditions, as bretylium blocks sympathetic nerve terminals and not smooth muscle β-adrenergic receptors. Isoprenaline (10^{-4} M) produced maximal relaxation in both CM and LM preparations. The extent of relaxation produced by isoprenaline did not change significantly between control and ACEA-treated tissue preparations (data not shown).

Histochemical and immunohistochemical analysis

Haematoxylin and eosin staining of full-thickness specimens of human colonic tissue was undertaken adjacent to the regions used for functional and immunohistochemical studies. Histological assessment revealed an intact epithelium containing many goblet cells within uniform crypts of Lieberkühn, which extended to the muscularis mucosae. Between the crypts, the lamina propria contained neutrophils and other immune cells associated with the normal immunological surveillance of the tissue (not shown). The muscularis externa consisted of a typical arrangement of outer longitudinal and inner circular smooth muscle fibres, with interspersed myenteric plexus. There was no evidence of neoplasia, fibrosis, overt inflammation or other discernible structural abnormality in any of the tissue samples analysed.

CB_1 receptor immunoreactive nerve fibres were observed in all major ganglionated nerve plexuses in the human colon, but were predominantly distributed within myenteric ganglia (Figure 4a), whilst a distinct structure in the CM also labelled (Figure 5b). Neurones expressing CB_1-IR were also identified in the submucosal plexus, mucosa and in serosal tissue surrounding the LM layer (not shown). Specific staining was absent in control tissue sections which had the primary rabbit anti-CB_1 antibody omitted (not shown); however, some nonspecific staining existed in these tissue sections. Similar findings were obtained in tissue treated with rabbit IgG in place of the rabbit anti-CB_1 receptor antibody. Nerve fibres throughout the tissue preparation appeared to express a background level of fluorescence; however, these fibres could be readily distinguished from the higher intensity of the CB_1 receptor immunoreactive neurones.

The distribution of ChAT showed a broad distribution in all layers of the human colon, including the myenteric and submucosal plexuses (not shown), as well as the longitudinal and CM layers. Intense ChAT immunoreactivity was identified extending from the myenteric plexus between the septa of the CM and as varicose nerve fibres extending throughout the muscle tissue (Figure 4b). Immunoreactivity for ChAT was

Table 2 Effect of ACEA (10^{-6} M) on acetylcholine-mediated contraction ACh (10^{-8}–10^{-4} M) in circular and longitudinal muscle strips of human colon

	Control	*ACEA*	P-*value (NS)*
Circular muscle			
Log EC_{50}	-5.86 ± 0.13	-5.46 ± 0.18	0.08
E_{MAX}	2.53 ± 0.46	3.61 ± 0.71	0.87
Longitudinal muscle			
Log EC_{50}	-6.02 ± 0.32	-5.83 ± 0.35	0.30
E_{MAX}	5.30 ± 0.64	5.56 ± 0.77	0.76

Mean $E_{MAX}\pm$s.e.m. (g) and mean log $EC_{50}\pm$s.e.m.; NS = not statistically significant, $P>0.05$.

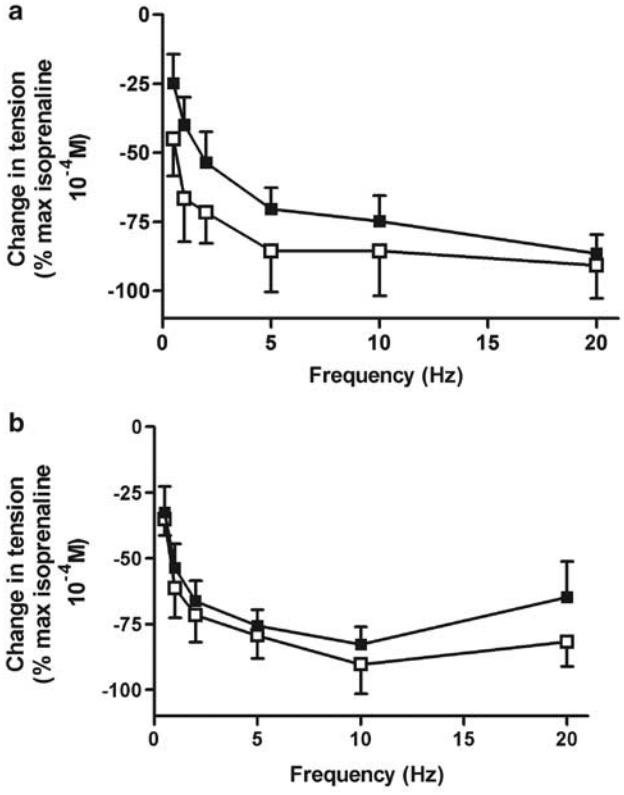

Figure 3 Effect of the CB_1 receptor agonist ACEA on frequency–response curves in response to relaxation elicited by EFS in human colonic (a) CM and (b) longitudinal smooth muscle strips precontracted with β-ala^8-NKA (10^{-6} M). ACEA did not alter the relaxation response to EFS in either CM or LM preparations (CB_1, cannabinoid receptor 1; ACEA, arachidonyl-2-chloroethylamide). $N=6$ CM; $n=8$ LM.

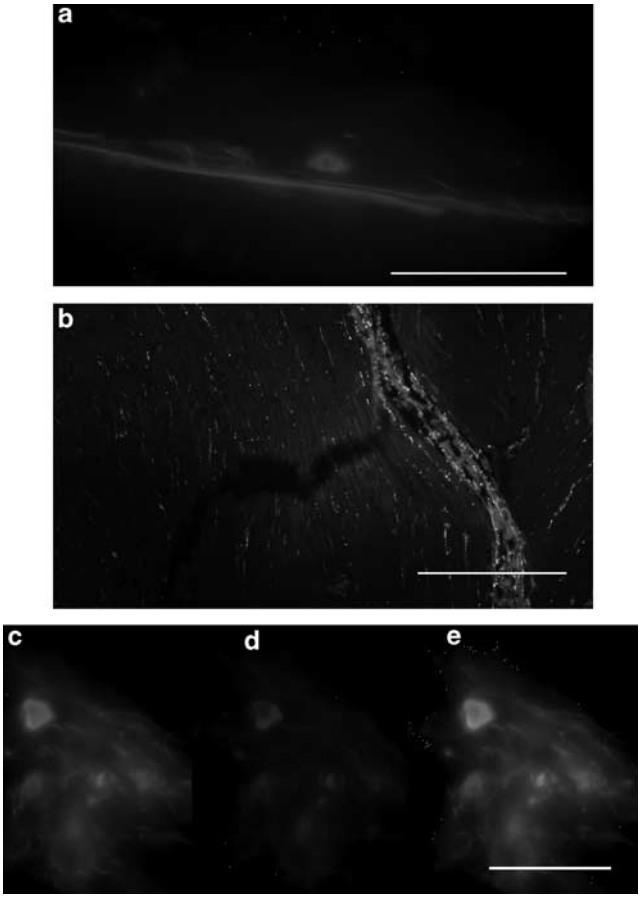

Figure 4 Immunohistochemical images of CB_1 and ChAT immunoreactivity in the human colon. (a) CB_1 immunoreactivity in the colonic myenteric plexus; (b) ChAT-positive nerve fibres extending throughout the CM layer. Coimmunoreactivity of ChAT (c) and the CB_1 receptor (d) in colonic myenteric neurones is demonstrated in (e) (scale: (a) 100 μm, (b) 250 μm, (c) 50 μm).

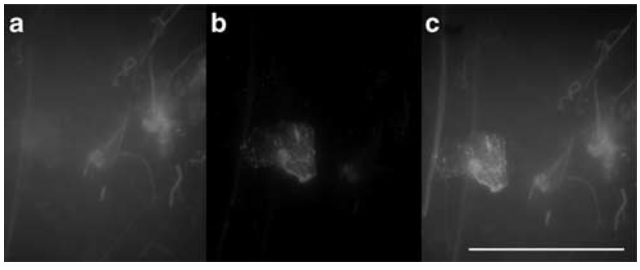

Figure 5 Unidentified structure in human colonic CM, demonstrating a web-like, mesh appearance and strong, punctate immunolabelling for the CB_1 receptor (b), but not ChAT (a). CB_1 immunoreactivity did not colocalise with ChAT (c) (scale = 250 μm) (CB_1, cannabinoid receptor 1; ChAT, choline acetyltransferase).

absent in tissue sections in which the primary antibody was omitted (not shown). A low level of nonspecific staining persisted in the negative controls as well as tissue sections incubated with goat IgG (1 : 10) in place of the primary antibody (not shown). The background fluorescence of nerve fibres was easily distinguished from the higher-intensity fluorescence of ChAT immunoreactivity.

In double-labelling experiments combining immunolabelling to both the CB_1 receptor and ChAT, many myenteric neurones that were immunoreactive for CB_1 receptor were also immunoreactive towards ChAT (Figure 4e). Nerve fibres expressing CB_1-R/ChAT immunoreactivities were also observed in the CM layer and the submucosa (not shown). A distinct but unidentified structure in the muscle layer showed CB_1-IR without being colocalised with ChAT (Figure 5). As was found in the single-labelling experiments, no specific staining was observed in the omission and IgG control sections (not shown).

Discussion

In the present study, we have sought to determine the effects of the CB_1 receptor agonist, ACEA, on enteric neuronal and colonic longitudinal and CM function in humans. In addition, immunohistochemical studies examined the distribution of the CB_1 receptor in the human colon in relation to a marker of cholinergic neurons, ChAT.

Properties of neurogenic contractility in isolated human colonic muscle strips

EFS of human colonic muscle strips in the presence of an NOS inhibitor evoked two distinct types of contractions, an 'on-contraction' during the period of electrical stimulation and an 'off-contraction' initiated after the stimulus had ceased. This pattern of neurogenic contractility in response to EFS has been previously described in isolated human colonic tissue (Mitolo-Chieppa *et al*., 1998; 2001; Tomita *et al*., 1998; McKirdy *et al*., 2004), where the off-contraction has been likened to the equivalent of the oral ascending contractile component of peristalsis in the isolated tissue preparation (Mitolo-Chieppa *et al*., 2001). The experimental conditions partly determine the extent of on-contraction, as EFS would normally concomitantly activate both excitatory cholinergic and inhibitory nitrergic transmission *in vitro*, producing a degree of physiological antagonism of both types of contraction (McKirdy *et al*., 2004). Evidence suggests that both types of contractions are neurogenic in origin, as both are abolished by tetrodotoxin (Tomita *et al*., 1998).

Effects of CB_1 receptor agonists on excitatory motor responses

The CB_1 receptor agonist ACEA inhibited the on-contractions of human colonic circular, but not longitudinal, muscle preparations. The relative lack of CB_1 immunolabelling in the LM layer, combined with a lack of functional effect of ACEA on longitudinal neurogenic contractility, suggests that CB_1 receptors modulate motility primarily through an action within the CM layer of the human colon. The innervation and regulation of LM is more variable and less well understood than that of CM, but studies do suggest a high proportion of cholinergic motorneurones in LM layers (Bornstein *et al*., 2004). The findings of the present study suggest that functional CB_1 receptors are localised primarily to circularly projecting cholinergic motorneurones in the human colon, which is at variance with previous studies (Manara *et al*., 2002). The reasons for such differences are not clear, but functionally may

relate to differences in experimental conditions, including the use of different CB_1 receptor agonists and other pharmacological pretreatments, especially with the use of indomethacin in other studies. Indomethacin has been shown to directly inhibit fatty acid amide hydrolase, the enzyme responsible for the degradation of endogenous CBs such as anandamide (Fowler *et al*., 2003). As such endocannabinoids are produced and exert functional inhibition of motility during EFS in isolated gastrointestinal preparations (Izzo *et al*., 1998), there may be a potentiation of CB effects from EFS in the presence of such a pretreatment. Indomethacin has also been demonstrated to potentiate neurogenic excitatory LM contractions in human colon through both pre-and postjunctional sites *via* cyclooxygenase (COX) inhibition (Fornai *et al*., 2005). This may have also contributed to the facilitation of, or otherwise revealed an effect of, CBs in longitudinal preparations 'sensitised' to contraction. Indeed, the differential expression of COX isoforms between circular and LM layers (Fornai *et al*., 2005) infers a potentially complex role for prostanoids in the control of gastrointestinal motility and the use of indomethacin needs to be considered judiciously in this setting.

The inhibitory effect of ACEA on EFS-evoked contractions was reversed when ACEA was incubated in the presence of the CB_1 receptor-selective antagonist AM251. This finding suggests that the inhibitory action of ACEA was being achieved through selective activation of CB_1 receptors and is in keeping with previous studies which have demonstrated a reversal of CB agonist-evoked inhibition of neurogenic cholinergic contractility following pretreament with a CB_1-receptor antagonist (Coutts & Pertwee, 1997; Croci *et al*., 1998b; Izzo *et al*., 1998; Manara *et al*., 2002).

ACEA inhibited neither the maximal contraction of ACh nor the NK-2 receptor-selective agonist, β-ala^8-NKA. Similarly, the potency of ACh in evoking 50% of the maximal contraction was unaffected by ACEA in either LM or CM. As both agents evoke contraction primarily by activating receptors directly on the smooth muscle (Croci *et al*., 1998a, b), the results indicate that the inhibitory action of ACEA on cholinergic transmission is achieved primarily by acting at prejunctional or presynaptic CB_1 receptors. These findings are consistent with previous studies which have described the prejunctional locus of the inhibitory effect of CBs on neurogenic ACh release from a variety of visceral preparations (Coutts & Pertwee, 1997; 1998; Croci *et al*., 1998b; Izzo *et al*., 1998; Spicuzza *et al*., 2000). In addition, our immunohistochemical studies support a neuronal site of location of the CB_1 receptor.

Effects of CB_1 receptor agonists on inhibitory (relaxation) motor responses

Following precontraction and under NANC conditions, EFS caused frequency-dependent relaxation of both circular and LM preparations. Previous studies have demonstrated that the EFS-evoked NANC relaxation is mediated primarily by nitric oxide (Tomita *et al*., 1998; Zyromski *et al*., 2001) with possible corelease of ATP, vasoactive intestinal peptide and pituitary adenylate cyclase-activating peptide (Keef *et al*., 1993; Bornstein *et al*., 2004). Evidence of a small but nonsignificant enhancement of EFS-evoked relaxation in the presence of ACEA may be a permissive effect due to inhibition of a residual or atropine-resistant component of stimulated release of ACh, a neurokinin or serotonin. Alternatively, CB_1 receptor activation may facilitate inhibitory motor pathways in the colon, leading to a more pronounced relaxation response. This has been demonstrated previously using methanandamide in the isolated guinea-pig ileum (Heinemann *et al*., 1999). A direct myogenic facilitation of relaxation cannot be excluded, but is unlikely, as ACEA did not evoke direct relaxation of human colonic tissue and isoprenaline-evoked relaxation was unaffected by ACEA pre-treatment (data not shown).

Immunohistochemical localisation of the CB_1 receptor and colocalisation with ChAT

CB_1-IR was distributed in nerve cell bodies and nerve fibres in select regions of the myenteric plexus, submucosa and in a number of distinct structures in the muscle layers. These findings are consistent with the reported distribution of CB_1-IR in the porcine (Kulkarni-Narla & Brown, 2000), mouse (Pinto *et al*., 2002; Casu *et al*., 2003), rat and guinea-pig colon (Coutts *et al*., 2002). These data are also supported by the recent immunohistochemical localisation of CB_1-IR in human colon (Wright *et al*., 2005), although the weak signal localised to circular and LM in that study was not demonstrated in the present study and it should be noted that many functional studies do not support a role for CB_1 receptors acting postjunctionally on smooth muscle.

The distribution of CB_1-IR was limited compared to the broad distribution of the cholinergic marker, ChAT. Immunoreactivity towards ChAT was detected broadly in the human colon, particularly in the myenteric plexus and submucosa. ChAT-positive nerve fibres occurred sporadically throughout the entire thickness of the submucosa, although these fibres were not arranged in any distinct nerve plexus. ChAT immunoreactive nerve fibres were also identified in the myenteric plexus and these fibres frequently extended into the circular and LM layers. ChAT-positive nerve fibres also coursed in thick concentric bands between the septa of the CM, with varicosities identified in both muscle layers but particularly in the CM. This pattern of distribution is similar to previous studies using human colon tissue (Porter *et al*., 1997; 2002; Schneider *et al*., 2001). Cells in the mucosa that demonstrated intense immunoreactivity towards ChAT are most likely to represent enteroendocrine cells (Porter *et al*., 1996).

Double-labelling studies examining the colocalisation of the CB_1 receptor and ChAT revealed that CB_1-IR was highly colocalised with immunoreactivity for ChAT in the myenteric plexus, submucosa and nerve fibres, extending predominantly into the circular and LM layers. Distinct neural populations that were immunoreactive for either ChAT or the CB_1 receptor alone were also identified. It is likely that, in these neural populations, the CB_1 receptor and ChAT were colocalised with other neurotransmitters such as NOS, VIP or substance P (Kulkarni-Narla *et al*., 1999; Kulkarni-Narla & Brown, 2000), reflecting the plurichemical nature of neurotransmission in the human colon (Porter *et al*., 1997).

CB_1-IR was occasionally found in distinct, yet unidentified, structures in the CM layer. The fine mesh-like webbing was interspersed with densely labelled puncta in close proximity to a tract of dense labelling that did not colocalise with ChAT. It would be provocative to suggest that the morphology may be consistent with a sensory structure such as an intramuscular

array, one of two types of terminal specialisations of extrinsic primary afferent neurones present in the gastrointestinal tract (Phillips & Powley, 2000). However, the structures identified in the present study are not dissimilar to such sensory terminals identified in the rat colon (Wang & Powley, 2000). Intrinsic primary afferent neurons also cannot be excluded, as nerves with Dogiel Type II morphology have been shown to project to CM in the human colon (Wattchow *et al*., 1997). Future studies utilising anterograde labelling of extrinsic nerves together with immunohistochemistry may discern the nature and chemical coding of these structures.

The capacity for CB_1 receptor activation to reduce neurogenic contractility in the human colon provides support for the development of CBs as therapeutic agents in hypermotility disorders. However, such therapies may also be of value in the treatment of a wide spectrum of GI disorders such as irritable bowel syndrome (IBS), diarrhoea, diverticulosis and gastroesophageal reflux disease (Holzer, 2001; Hunt & Tougas, 2002; Di Carlo & Izzo, 2003). The localisation of the CB_1 receptor in mucosal and submucosal neurones, as also described in the human colon in a recent study (Wright *et al*., 2005), may suggest a role in the modulation of mucosal secretory function. The CB_1 receptor agonist WIN 55,212-2 has been demonstrated to reduce electrically evoked secretory responses in the rat and guinea-pig ileum by acting directly at CB_1 receptors on enteric nerves (Tyler *et al*., 2000; MacNaughton *et al*., 2004).

CBs exhibit antiemetic, orexigenic and analgesic effects in addition to purportedly suppressing the development of colorectal malignancy (Ligresti *et al*., 2003). CB_1 receptor upregulation in inflammatory conditions (Izzo *et al*., 2001a; Siegling *et al*., 2001) and the recent finding that CBs ameliorate the development of colonic inflammation (Massa *et al*., 2004) implicate CBs as modulators of the neuroimmune axis in the gastrointestinal tract (Gongora *et al*., 2004; Kraft *et al*., 2004). The recent finding that a CB_2 receptor agonist was capable of reducing the enhanced gastrointestinal transit following an inflammatory stimulus (Mathison *et al*., 2004) suggests that further studies examining the role of both CB_1 and CB_2 receptors in human gastrointestinal disease are necessary in order to reveal the true therapeutic value of CBs.

In conclusion, this study has demonstrated that CB_1 receptor activation inhibits the neurogenic contraction to EFS in the human colon; this effect is attributed to inhibition of cholinergic motorneuronal ACh release. This finding is supported by immunohistochemical studies revealing a high level of colocalisation between the CB_1 receptor and ChAT in enteric neurones of the human colon.

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