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Original research article

The endocannabinoid anandamide regulates the peristaltic reflex by reducing neuro-neuronal and neuro-muscular neurotransmission in ascending myenteric reflex pathways in rats

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ABSTRACT

Background: Endocannabinoids (EC) and the cannabinoid-1 (CB_1) receptor are involved in the regulation of motility in the gastrointestinal (GI) tract. However, the underlying physiological mechanisms are not completely resolved. The purpose of this work was to study the physiological influence of the endocannabinoid anandamide, the putative endogenous CB1 active cannabinoid, and of the CB_1 receptor on ascending peristaltic activity and to identify the involved neuro-neuronal, neuro-muscular and electrophysiological mechanisms.

Methods: The effects of anandamide and the CB_1 receptor antagonist SR141716A were investigated on contractions of the circular smooth muscle of rat ileum and in longitudinal rat ileum segments where the ascending myenteric part of the peristaltic reflex was studied in a newly designed organ bath. Additionally intracellular recordings were performed in ileum and colon.

Results: Anandamide significantly reduced cholinergic twitch contractions of ileum smooth muscle whereas SR141716A caused an increase. Anandamide reduced the ascending peristaltic contraction by affecting neuro-neuronal and neuro-muscular neurotransmission. SR141716A showed opposite effects and all anandamide effects were antagonized by SR141716A (1 μM). Anandamide reduced excitatory junction potentials (EJP) and inhibitory junction potentials (IJP), whereas intestinal slow waves were not affected.

Conclusions: CB_1 receptors regulate force and timing of the intestinal peristaltic reflex and these actions involve interneurons and motor-neurons. The endogenous cannabinoid anandamide mediates these effects by activation of CB1 receptors. The endogenous cannabinoid system is permanently active, suggesting the CB_1 receptor being a possible target for the treatment of motility related disorders.
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Introduction

The endocannabinoid system (ECS) is present in the GI tract of numerous species including rodents and humans [20,33]. GI motility is known to be modulated by the ECS [9,37]. Under both, physiological and pathophysiological conditions, there seems to be a permanent activation of the classical cannabinoid receptors [10,20,25]. One receptor, the cannabinoid receptor 1 (CB_1), is located presynaptically and is involved in the control of cholinergic and nitrergic neurotransmission in the GI tract [24,27,34]. The other classical cannabinoid receptor, the cannabinoid receptor 2 (CB_2) unfolds its actions largely in pathophysiological states [12,21,22,42].

The endogenous stimulus for cannabinoid receptors in physiological states in the GI tract is unknown. Presently five different

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endocannabinoid candidates with different affinities to the CB_1 receptor are known [8,11,14]. In the small intestine of rodents high amounts of anandamide (AEA) and 2-acylglycerol (2-AG) were reported, consistent with the notion that endocannabinoids play a major role in the control of GI motility [16]. From these two compounds anandamide is the one with the highest affinity toward the CB_1 receptor [29,31].

There is conflicting data on a constitutively active ECS in the GI tract which regulates GI motility. Dependent on agonists used and on GI regions examined, effects range from no effect to an increase of GI motility *in vitro* [28,43,45] and accordingly an accelerated GI transit [2,3] was reported. If the hypothesis of a constitutively active ECS holds true, GI transit in CB_1 receptor deficient mice should be increased and exactly this was demonstrated recently [4,5,45]. Therefore it seems imperative to characterize the exact effect of the possible endogenous ligands mediating this effect as there is potential for future drugs targeting these mechanisms when changes in motility are wanted [26,32]. Thus the present study focuses on the characterization of the effects of the endogenous cannabinoid anandamide on mechanisms involved in the regulation of intestinal peristalsis, like cholinergic neuro-muscular-interaction, the peristaltic reflex and intracellular electrophysiological neurotransmission patterns *in vitro* and their consequences on intestinal transit *in vivo*. Since the mechanisms underlying the peristaltic reflex like ascending myenteric neurotransmission can be studied best in rats, we used rats in our experiments. A newly designed baffled organ bath was used to characterize possible effects on the peristaltic reflex and to characterize whether neuro-neuronal transmission, neuro-muscular transmission or both are involved.

Methods

Animals were obtained from Charles River, housed at stable temperatures (22 °C) and a constant photoperiod (12:12-h – light:dark) in plastic cages with free access to tap water and laboratory chow. Male Wistar rats (150 g each) were anesthetized using CO_2 followed by an intraperitoneal sodium pentobarbital injection (100 mg/kg). The ileum was then removed and kept in oxygenated Krebs–Ringer bicarbonate buffer (KRS: NaCl 115.5 mM, $MgSO_4$ 1.16 mM, NaH_2PO_4 1.16 mM, glucose 11.1 mM, $NaHCO_3$ 21.9 mM, $CaCl_2$ 2.5 mM, KCl 4.16 mM). Experiments lasted less than 2.5 h and each muscle strip was used for one experiment only. For PCR experiments we additionally used male C57BL/6 mice (20 g each) which were euthanized by cervical dislocation.

Animal use was approved by the Government of Bavaria and all experiments were performed in agreement with institutional animal care guidelines.

RNA isolation and RT-PCR

Total RNA was extracted from liquid nitrogen frozen mouse and rat brain, longitudinal muscle-myenteric plexus layer, circular muscle layer and mucosa of the ileum. Tissues were homogenized and RNA was isolated as previously described [36], followed by DNase treatment for 15 min at room temperature (1 U DNase I/μg RNA, Invitrogen, Groningen, The Netherlands. Total RNA was reverse transcribed in complementary DNA (cDNA) essentially as described previously [36]. To determine mRNA expression of the CB_1 receptor, we performed PCR using specific CB_1 primers (Table 1). As negative controls, we used isolated RNA amplified without reverse transcriptase or random hexamer primers. Amplification products were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. Bands were excised from the gel, purified by a Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into TOPO PCRII vector (Invitrogen). Nucleotide sequences were deduced by cycle sequencing of the isolated plasmids (Qiagen, QIAprep spin miniprep kit) with T7 sequencing primer (GATC, Konstanz, Germany). Sequences were then analyzed using the BLASTn homology search.

Table 1
Oligonucleotides of sense- (S) and antisense-strand (AS) primers used for RT-PCR.

Name	Sequence 5′ 3′
mCB_1 (S)	GCTCACAGCCACGCAGTTC
mCB_1 (AS)	AACAGTGCTCTTGATGCAGCTTT
rCB_1 (S)	CCTGGGCTGGAACTGCAA
rCB_1 (AS)	CCGAAGACGTCATACACCATGA

Isolated smooth muscle strips

1 cm segments of full-thickness ileum strips were prepared. One end was attached to an electrode holder, while the other end of the strip was slipped through two circular platinum electrodes, which were 0.8 cm apart. The electrode holders were then placed in an organ bath containing 4 mL oxygenated KRS and kept at 37 °C. The free end of the segments was connected to an isometric force transducer (Swegma force-displacement transducer SG4-500; Swegma, Stockholm, Sweden). One gram tension was applied and the preparation was equilibrated for 30 min. Changes in tension were amplified using Hellige couplers and Hellige amplifiers (Hellige, Wien, Austria) and recorded on a personal computer.

First SR141716A, anandamide or both were added to the organ bath and effects on basal tonus and spontaneous activity were recorded. In the next set of experiments effects on stimulated smooth muscle was evaluated. Strips were stimulated with carbachol (CCH 1 μM). SR141716A, anandamide or both were added when the contraction plateau was stable, which was 1 min after CCH addition. Changes in tension were recorded.

In a second series of experiments electrical field stimulation (EFS: 10 s stimulation duration, 40 V, 5 Hz) was applied every 2 min using a Grass S11 stimulator (Grass, Quincy, MA, USA). EFS evoked stable contractions mediated *via* atropine (1 μM) sensitive cholinergic mechanisms which were abolished in presence of tetrodotoxin (100 μM). To test the effect of SR141716A and anandamide, 10 consecutive stimuli were applied and then the drugs were added in increasing concentrations and EFS responses were continuously evoked. A possible effect of vehicles was tested in control experiments.

Investigation of the myenteric part of the peristaltic reflex in vitro

Ascending myenteric reflex pathways were studied in ileum segments using an established model [38,44]. Segments of terminal ileum (10 cm long) were dissected and the mesenteric fat was removed. Segments were flushed but not filled with KRS, placed in horizontal orientation in an organ bath filled with KRS and bubbled with carbogen (95% O_2 – 5% CO_2) at 37 °C. The ends of the segments were tied over a polyvinyl tubing (diameter 1.8 mm) without stretching of the segments. Two platinum electrodes, which were 1 cm apart, were placed on either side 1 cm proximal to the anal end of the ileum. The specialty of this organ bath was that it can be divided by a baffle into an anal stimulation chamber and an oral recording chamber with the recording sites in chamber 2. Fig. 1 gives a schematic drawing of this newly designed organ bath. The first recording site was 4 cm orally of the stimulation site; the second recording site was 7 cm orally of the stimulation

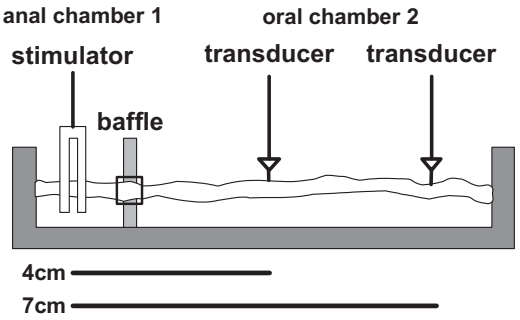

Fig. 1. Schematic drawing: segments of isolated rat ileum were studied in a chambered organ bath. The reflex was initiated by electrical stimulation in the anally located chamber 1. Force and timing of the ascending reflex pathways of the myenteric part of the peristaltic reflex of rat ileum were recorded at two sites in the oral chamber 2. Chambers were separated by a baffle only allowing the ileum to pass. This baffle separates stimulation and recording sites.

site. The denseness of the baffle was tested by application of methylene blue at the end of each experiment. Field stimulation impulses to evoke neural responses were applied using a Grass S9 stimulator (Grass, Quincy, MA, USA). The ileum segments were fixed in horizontal orientation in the organ bath and the muscular contractions following the electrical neuronal stimulations were recorded using isometric force transducers attached to the ileum wall with a thin thread (Swegma force-displacement transducer SG4-500; Swegma, Stockholm, Sweden). The impulse signals were recorded simultaneously with the contraction curves using Hellige amplifiers (Hellige, Wien, Austria) and a Beckman A/C coupler (Beckmann, Berlin, Germany). After an equilibration of 30 min the segments were stimulated every 2 min for 10 s with 40 V, 5 Hz and 2 ms pulse duration. This periodic stimulation was maintained throughout the experiments. When a stable response was established (identical contractions to at least three consecutive stimuli) the experiments were started. The first 3 contractions served as an internal control and changes to control were expressed in %. Drugs were added in chamber 1 in 15 min intervals in a cumulative increasing manner. Solvents had no effect on basal tonus or electrically induced contractions in respective control experiments.

Tissue preparation for electrophysiological experiments

Ileum and colon were removed through an abdominal midline incision and placed in oxygenated KRS. The ileum and colon were opened along the mesenteric border, washed of remaining fecal material and pinned out in a Sylgard-lined (Dow Corning Corp., Midland, MI, USA) dissecting dish. Mucosa and submucosa were removed, resulting in sheets of muscle layers with attached myenteric plexus. The recording chamber was constantly perfused (5 ml/min^{-1}; Kwik Pump, World Precision Instruments) with oxygenated KRS. Tissues were then equilibrated for 90 min.

Intracellular recording

Intracellular recordings of smooth muscle cells of the circular muscle layer of ileum and proximal colon were performed as detailed recently [30,39]. Throughout all experiments nifedipine (1 μM) was present. Successful impalement of circular muscle cells with the glass microelectrode was indicated by a sharp negative deflection in membrane potential followed by spontaneously occurring inhibitory junction potentials. Neurons were then stimulated with single pulses (15 V; 0.3 ms duration) with platinum electrodes arranged perpendicularly to the circular muscle layer (Grass S11 stimulator and Grass SIU59 stimulus isolation unit; Grass Instruments, Quincy, MA, USA). EJP and IJP were elicited in rat colon by field stimulation of intrinsic neurons as detailed earlier [35,41,44]. Briefly electrical stimulation of the neurons resulted in changes in smooth muscle cell membrane potentials. To record excitatory junction potentials (EJP), the stimulation was conducted 5 mm anally of the recording electrode, to record the intestinal slow wave activity and the colonic inhibitory junction potential (IJP) the stimulation electrodes were placed perpendicular to the circular muscle and the stimulation electrodes were 1 cm apart. Evoked electrical events were amplified (DUO 733 microelectrode amplifier; World Precision Instruments, Sarasota, FL, USA) and digitalized with an analog-to-digital converter (SCB 68 interface; National Instruments, Austin, TX, USA). Permanent recordings of membrane potentials were made using the LABVIEW 5.0 program (National Instruments, USA). Drugs were added to the perfusate in increasing concentrations.

Drugs

Anandamide (Tocris, Ellisville, USA) and SR141716A (donation from Sanofi-Aventis, Montpellier, France) were solved in one drop of Tween 80 and 1 ml DMSO (5%) and then further diluted in saline up to final concentrations. Charcoal, gum Arabic, evans blue, atropine, carbachol, nifedipine, hexamethonium and tetrodotoxin (TTX) were obtained from Sigma–Aldrich, Taufkirchen, Germany.

Statistics

Results are expressed as mean (±SEM) and were compared using ANOVA followed by Dunnet's *post hoc* testing (SigmaStat, Jandel Scientific, San Rafael, CA, USA). A $p < 0.05$ was considered as statistically significant.

Results

Detection of CB_1 mRNA by RT-PCR

Presence of mRNA for CB_1 in the longitudinal muscle layer with attached myenteric plexus (LM/MP), the circular muscle layer and the mucosa of mouse and rat ileum was determined by RT-PCR with specific primers. RNA was isolated from the tissues, reverse transcribed into cDNA and PCR amplification was performed. Single bands at the expected size were obtained for murine (450 bp) and rat CB_1 (351 bp) cannabinoid receptors in the circular muscle layer and the longitudinal muscle/neuron layer of the ileum, whereas no amplification products was obtained in the mucosa (see Fig. 2). Sequencing of the cloned RT-PCR products confirmed expression in both species.

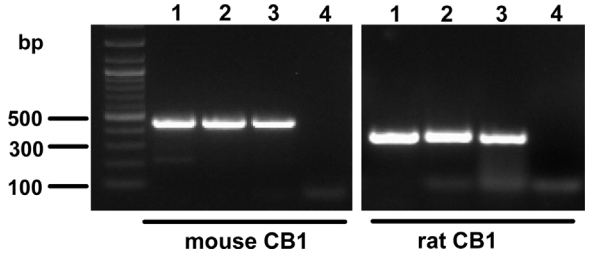

Fig. 2. RT-PCR for CB1 cannabinoid receptors using total RNA isolated from mouse and rat brain (lane 1, positive control), the longitudinal muscle-myenteric plexus layer (LM/MP, lane 2), the circular muscle layer (lane 3) and the mucosa (lane 4) of the ileum. Single bands with the expected size of approximately 450 bp and 350 confirmed the expression of CB1 in the LM/MP and the circular muscle layer, whereas the mucosa (lane 4) showed no specific PCR product.

Influence of SR141716A and anandamide on ileal smooth muscle preparations

Neither SR141716A (1 μM) nor anandamide (1 μM) had an effect on basal tonus or spontaneous activity of rat ileal smooth muscle (data not shown; $n = 6$). Neither SR141716A (1 μM) nor anandamide (1 μM) had a significant effect on CCH (1 μM) stimulated smooth muscle (CCH + SR141716A: $-3.1 \pm 4.6\%$; CCH + anandamide: $2.1 \pm 2.2\%$; $n = 6$).

Electrical field stimulation caused cholinergic mediated twitch contraction which were stable for 3 h and were abolished in presence of the muscarinic receptor antagonist atropine (1 μM) or the neuronal blocker TTX (1 μM). Anandamide (0.1 nM–1 μM) reduced those twitch contractions in a concentration dependent manner, with a maximal inhibition of $-46.8 \pm 5.7\%$ ($n = 6$; $p < 0.05$ *vs.* control) (Fig. 3). SR141716A (0.1 nM–1 μM) did not change the contraction amplitude at the lower concentrations but caused an increase of contraction amplitude at the highest concentration used. Continuous SR141716A (1 μM) presence prevented the anandamide effect on the twitch contractions (maximal anandamide inhibition in presence of SR141716A: $-18.4 \pm 4.2\%$; $n = 6$; $p < 0.05$ *vs.* anandamide alone) (Fig. 3).

Influence of atropine and hexamethonium on the ascending peristaltic reflex responses

Within 15 min after initiating the rhythmic electrical stimulation the intestinal segments responded with stable ascending contractions which could be characterized by contraction amplitude and area under the contraction curve. Additionally latency, defined as the interval between onset of electrical stimulation and occurrence of the contractile response, was recorded. The recording sites were 4 and 7 cm orally from the stimulation site. The latency was 5.33 ± 0.58 s for the 4 cm recording site and 9.8 ± 0.78 for the 7 cm recording site.

When atropine was added, contractions were reduced at both recording sites (atropine 1 μM: 4 cm: $-72.4 \pm 6.8\%$*; 7 cm: $-65.1 \pm 7.6\%$*; $n = 6$; *$p < 0.05$). Latency between initiation of stimulation and onset of contraction was unchanged (atropine 1 μM: 4 cm: $+9.4 \pm 4.1\%$; 7 cm: $+4.4 \pm 4.1\%$; $n = 6$). When hexamethonium was added contractions were significantly reduced (hexamethonium 10 μM: 4 cm: $-61.3 \pm 6.5\%$*; 7 cm: $-75.5 \pm 6.3\%$*; $n = 6$; *$p < 0.05$). Latency between initiation of stimulation and onset of contraction was prolonged (hexamethonium 10 μM: 4 cm: $+19.4 \pm 4.9\%$*; 7 cm: $+24.1 \pm 5.6\%$*; $n = 6$; *$p < 0.05$).

Influence of SR141716A and anandamide on the ascending peristaltic reflex responses

When SR141716A (1 nM–1 μM) was added into the chamber the area under the curve was significantly increased in a concentration dependent manner ($n = 6$). For the maximal concentration of SR141716A used (1 μM) the increase was $+14.1 \pm 2.4\%$* at the 4 cm distance and $+22.2 \pm 3.5\%$* at the 7 cm distance ($n = 6$; *$p < 0.05$) (Fig. 4). Additionally latency was reduced by SR141716A (1 nM–1 μM) (SR141716A (1 μM): 4 cm: $-21.4 \pm 4.8\%$*; 7 cm: $-13.1 \pm 1.3\%$*; $n = 6$; *$p < 0.05$) (Fig. 4).

When anandamide (1 nM–10 μM) was added into the chamber the area under the curve was significantly reduced in a concentration dependent manner ($n = 6$). For the maximal concentration of anandamide used (10 μM) the reduction was $-48.4 \pm 5.1\%$* at the 4 cm distance and $-49.4 \pm 5.7\%$* at the 7 cm distance ($n = 6$; *$p < 0.05$) (Fig. 5a and b). Additionally latency was increased by anandamide (1 nM–10 μM) (anandamide (10 μM): 4 cm: $+28.3 \pm 5.6$*; 7 cm: $+29.8 \pm 8.8\%\%$*; $n = 6$; *$p < 0.05$) (Fig. 5a and b).

In presence of SR1741716A (1 μM) the effects of anandamide were significantly reduced. The remaining effects of anandamide reached statistical significance only for changes in AUC at the highest concentration used (anandamide 10 μM: 4 cm: $-22.8 \pm 8.9\%$*; 7 cm: $-25.4 \pm 6.3\%$*; $n = 6$; *$p < 0.05$) (Fig. 5c).

Electrophysiology rat ileum and colon

Small intestinal slow waves are not changed by anandamide or SR141716A

The small intestinal slow wave activity in the ileum was not altered by anandamide or SR141716A (membrane potential:

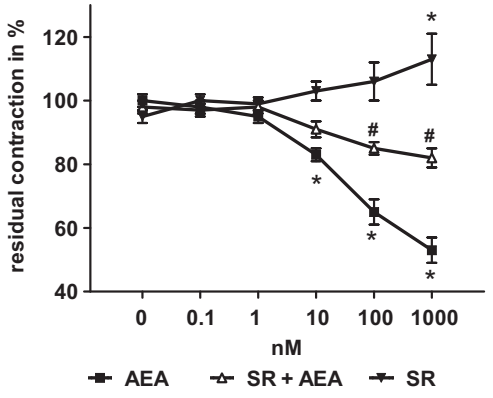

Fig. 3. The endogenous cannabinoid anandamide (0.1–1000 nM) reduced electrically induced twitch contractions (40 V; 5 Hz; 1 ms pulse duration; 10 s stimulation duration) in ileum smooth muscle preparations compared to pre-drug application controls. The CB1 receptor antagonist SR141716A (0.1–1000 nM) increased the contraction amplitude at the highest concentration applied whereas SR141716A (1 μM) presence prevented the anandamide effect ($n = 6$–10; *$p < 0.05$ *vs.* control; #$p < 0.05$ anandamide *vs.* SR141716A + anandamide).

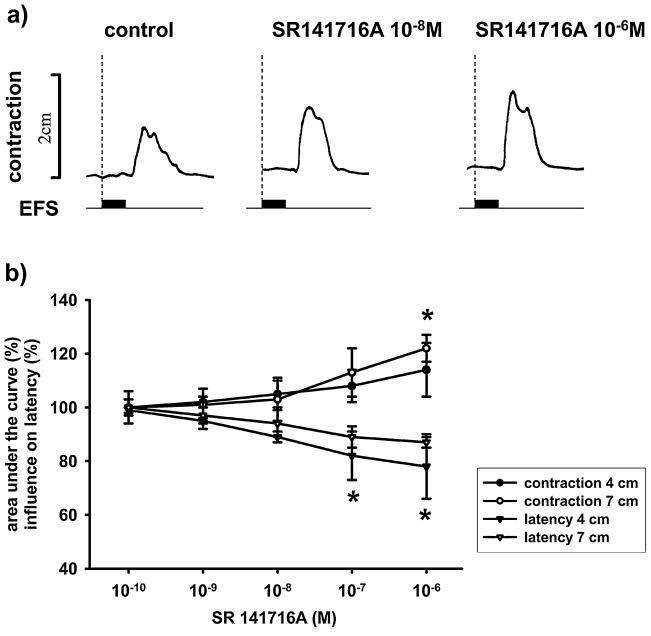

Fig. 4. Concentration response curves showing the effects of the CB1 receptor antagonist SR141716A (0.1–1000 nM) on the area under the curve (AUC) and the latency of the ascending myenteric part of the peristaltic reflex. AUC and latency were recorded 4 and 7 cm orally of the stimulation site ($n = 6$ each; *$p < 0.05$). (a) A representative tracing at 4 cm orally of the stimulation site and (b) shows the respective concentration response curves for recordings 4 and 7 cm orally of the stimulation site.

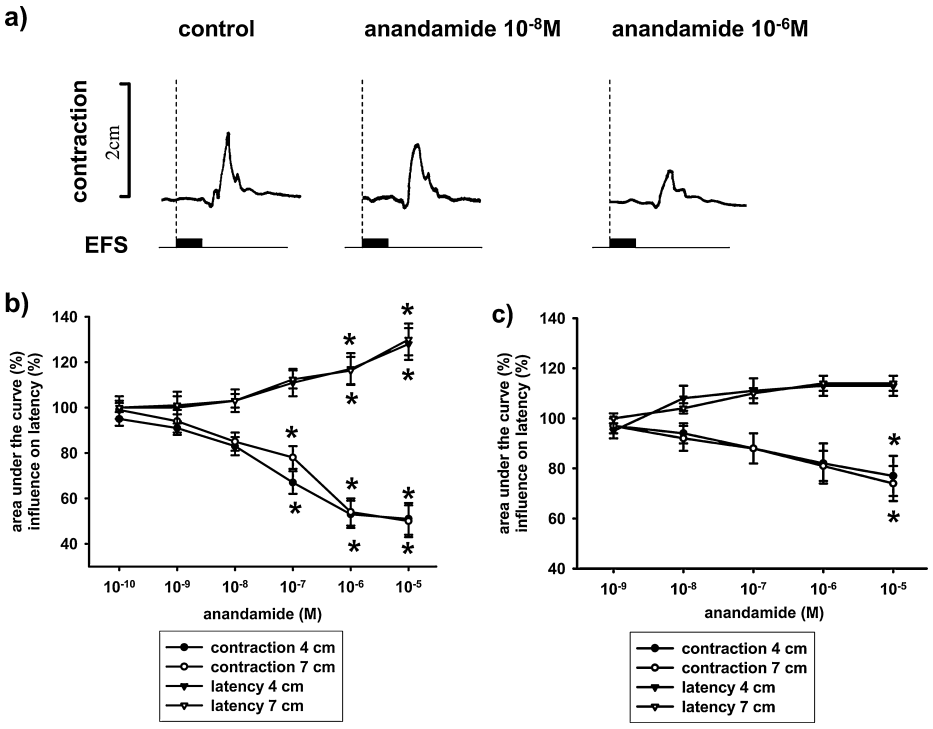

Fig. 5. (a) A representative tracing demonstrating the concentration dependent effect of anandamide on electrically induced reflex responses recorded 4 cm orally of the stimulation site. Concentration response curves showing the effects of (b) anandamide (0.1 nM–10 μM) and (c) SR141716A (1 μM) + anandamide on the area under the curve (AUC) and the latency of the ascending myenteric part of the peristaltic reflex. AUC and latency were measured 4 and 7 cm orally of the stimulation site. Note, in presence of SR141716A, anandamide at the highest concentration but not at the lower concentrations, had a significant effect on AUC or latency ($n = 6$ each; $^*p < 0.05$).

MP: control: -58.4 ± 2.6 mV, anandamide 10 μM: -60.8 ± 3.3 mV, SR141716A 10 μM: -58.8 ± 3.3 mV; amplitude: control: 21.2 ± 1.1 mV, anandamide 10 μM: 20.3 ± 0.8 mV, SR141716A 10 μM: 21.4 ± 1.3 mV; frequency: control: 28.5 ± 1.3 min^{-1}; anandamide 10 μM: 28.3 ± 1.2 min^{-1}, SR141716A 10 μM: 29.4 ± 1.3 min^{-1}; $n = 5$; Fig. 6a). Due to slow wave activity in the ileum, quantifiable recording of EJP or IJP was not possible in the ileum and therefore colon preparations were used to quantify EJP and IJP.

Cholinergic excitatory junction potentials are blocked by anandamide

Resting Membrane Potential (RMP) in the rat proximal colon was not changed by anandamide or SR141716A (RMP control: -55.2 ± 4.9 mV, anandamide 10 μM: -56.6 ± 6.5 mV, SR141716A 10 μM: -55.8 ± 5.9 mV; $n = 5$). In the presence of anandamide (1 nM–10 μM), the EJP was significantly reduced, an effect which was reversible following addition of SR141716A (1 μM) ($n = 4$; Fig. 7a and b). SR141716A (1 nM–1 μM) alone significantly increased EJP (Fig. 7b). IJP amplitudes were significantly reduced in presence of anandamide (Fig. 7c) but not changed in presence of SR141716A (Fig. 7c). The effects of anandamide on IJP were reversed by SR141716A ($n = 5$; Fig. 7c).

Discussion

The effect of cannabinoids on GI motility and the involvement of the endocannabinoid system (ECS) in the control of GI function is well known [1]. Little if anything is known on the distinct involvement of the endocannabinoids like anandamide in these mechanisms. Furthermore the exact sites of interaction within regulatory circuits involved in the control of GI motility are not yet fully understood. Thus we aimed to characterize the effects of the endocannabinoid anandamide on the control of GI motility and performed pharmacological and electrophysiological studies to identify the exact sites where the involved receptors are located.

The cannabinoid receptors like CB1 and CB2 are part of the ECS. The ECS comprises endogenous ligands, metabolizing enzymes and the cannabinoid receptors. From pharmacological studies it is known that cholinergic excitatory neurotransmission within the small intestine is reduced by CB1 receptor activation. The involved CB1 receptors are functionally located presynaptically where activation results in a decrease of acetylcholine release [34]. Still it is unknown whether this presynaptic site is a presynaptic neuro-neuronal site, a neuro-muscular site or both.

Using RT-PCR, we provide evidence of CB1 mRNA being present in the nerve plexus containing longitudinal muscle (LMMP) preparation as well as in the circular muscle layer but not in the mucosal layer. Though confirming evidence that CB1 is present at multiple sites, the PCR results are not able to identify the exact functional involvement of these CB1 receptors.

The classical organ bath experiments confirmed that CB1 activation reduced cholinergic excitatory neurotransmission. The endogenous cannabinoid reduced cholinergic neurotransmission in a CB1 sensitive manner. In rats, comparable to previous reports from mice there is an endogenous, CB1 mediated, ongoing suppression of cholinergic neurotransmission, though this physiological tone is less pronounced when compared to reports from other species [15,17–19].

Since electrophysiological circuits like the ascending myenteric part of the peristaltic reflex are best studied in rats, we performed the present study in rats. A recent publication of our group found that the ascending myenteric part of the peristaltic reflex is modified by CB1 receptor active drugs [45]. Due to the organ bath used we were not able to distinguish between possible neuro-neuronal sites or neuro-muscular sites of action. Furthermore it remained unresolved whether endogenous cannabinoids were able to modify the ascending myenteric part of the peristaltic reflex

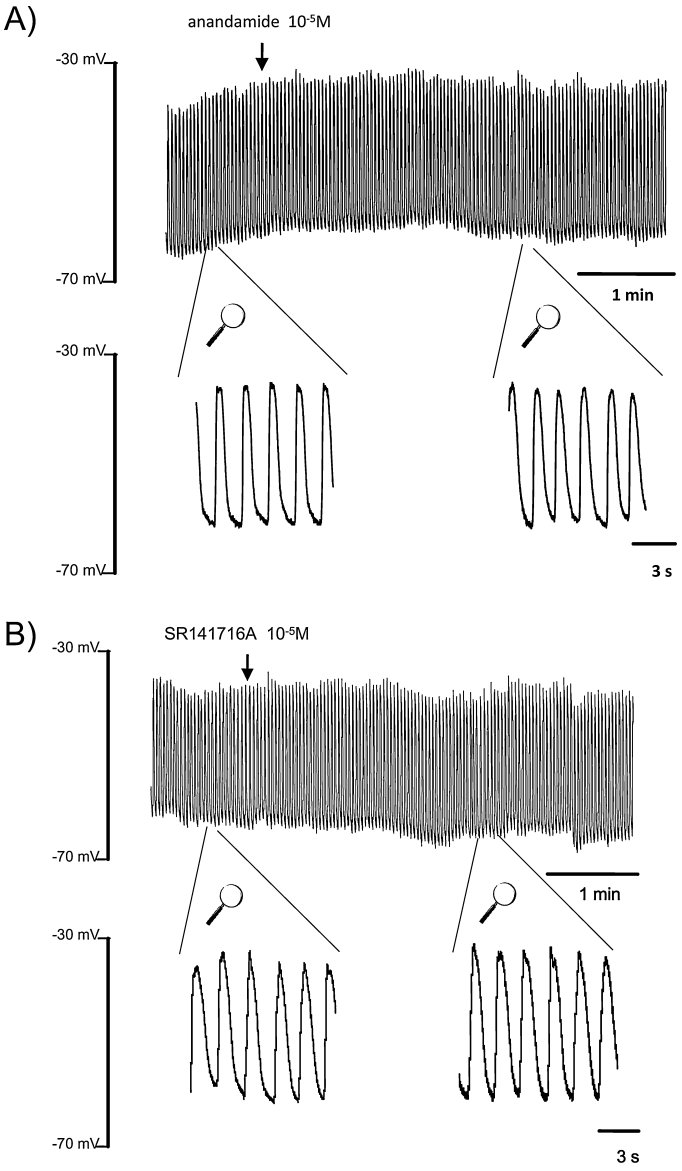

Fig. 6. Neither anandamide (A) (10 μM) nor SR141716A (B) (10 μM) had an effect on small intestinal slow wave activity.

and whether contractile force development, timing or both are under regulatory control of the CB1 receptor. Others also characterized effects of cannabinoids on electrical properties of myenteric neurons, especially in he light of different subsets of excitatory neurons and found that both, pre- and post-synaptic receptors are involved in the effects of cannabinoids on excitatory neurotransmission [23]. The pre-dominant effect of cannabinoids on excitatory neurotransmission is supported by immunohistochemical studies where CB1 receptors are essentially found on excitatory neurons co-labeling with ChAT [6]

In newly developed separated organ bath, developed as an extension of the initially reported Trendelenburg organ bath [40], we were able to investigate the exact sites involved. Our characterization experiments employing hexamethonium and atropine showed that drugs when given to chamber 1 target a neuro-neuronal site on interneurons. Such interactions were previously not reported since the classical organ bath pharmacology experiment focuses on neuro-muscular actions and cannot comment on possible neuro-neuronal actions.

Changes in latency of the ascending myenteric part of the peristaltic reflex involve interneurons, as suggested by the sensitivity to hexamethonium. A CB1 receptor location on presynaptic sites is accepted from many studies, but an attribution to motor-neuronal sites as shown by the muscle strip experiments and inter-neuronal sites as shown by the reflex-setup was recently not shown. Others induced peristaltic activity by intraluminal perfusion showing that CB1 receptor activation reduced contractility in a SR141716A sensitive manner [13]. Both studies discuss influences on motor neurons but an additional involvement of sensory neurons, which seems unlikely in our setup, cannot be ruled out. In our experiments activation of CB1 receptors reduced ascending peristaltic activity by reducing ascending contractile force development and by increasing latency of these contractile responses. This suggests that the site of action is a neuro-neuronal site on interneurons. Anandamide, the endogenous cannabinoid, is able to elicit these effects.

The CB1 receptor selective antagonist SR141716A increased contractile force and reduced the latency of reflex responses, strongly suggesting an augmentation of the ascending myenteric part of the peristaltic reflex. This is the first evidence that complex physiological circuits are under control of a permanently active ECS, an observation that may be relevant for future therapeutic developments.

In additional electrophysiological experiments we were interested whether CB1 activation is involved in the control of other intestinal circuits. For small intestinal slow waves, an electrophysiological pattern that is under control of the interstitial cells of Cajal (ICC), we did not find evidence for such an involvement. We show that neither frequency nor amplitude of the small intestinal slow wave activity nor the RMP are altered by anandamide or SR141716A, making an involvement of the ECS in the regulation of ICC generated intestinal slow wave activity unlikely. This finding is supported by immunohistochemical studies, where ckit-ICC cells did not stain positive for CB1 [6,7,39].

Electrophysiology is the gold standard to record neuronal elicited EJP in smooth muscle cells, which allows further characterization of the involved mechanisms. Due to slow wave activity in the ileum, EJP, which are cholinergically mediated, have to be recorded in the colon. The here reported EJP reduction by anandamide, which was reversible by SR141716A and the augmentation of the EJP in presence of SR141716A alone further stresses that it is the cholinergic enteric neurotransmission that is controlled by the ECS. The additionally reported IJP reduction by anandamide, which is reversible by SR141716A indicates that CB1 additionally controls inhibitory neurotransmission and further studies are needed to characterize this in full extent. It is furthermore in agreement with a recent study from our group, where anandamide was shown to increase the release of the inhibitory neurotransmitter NO from enteric neuronal cell preparations [21].

In conclusion, we show that CB1 receptors are involved in the control of GI peristaltic activity by modulating activity of motor neurons and interneurons. Anandamide an endogenous cannabinoid can elicit the observed effects and may be the compound physiologically involved. Using a newly designed separated organ bath we discover that one crucial underlying mechanism is reducing the ascending peristaltic reflex pathway, and CB1 receptors located on both, motor-neurons and inter-neurons are involved. Other GI circuits like the ICC generated slow wave activity are not altered by CB1 receptors. The here reported findings suggest the ECS being an important puzzlestone in the regulation of GI motility and implies that by activation or deactivation of the CB1 receptor, motility can be either accelerated or decelerated. This suggests the CB1 receptor being a promising target for the future treatment of GI motility disorders.

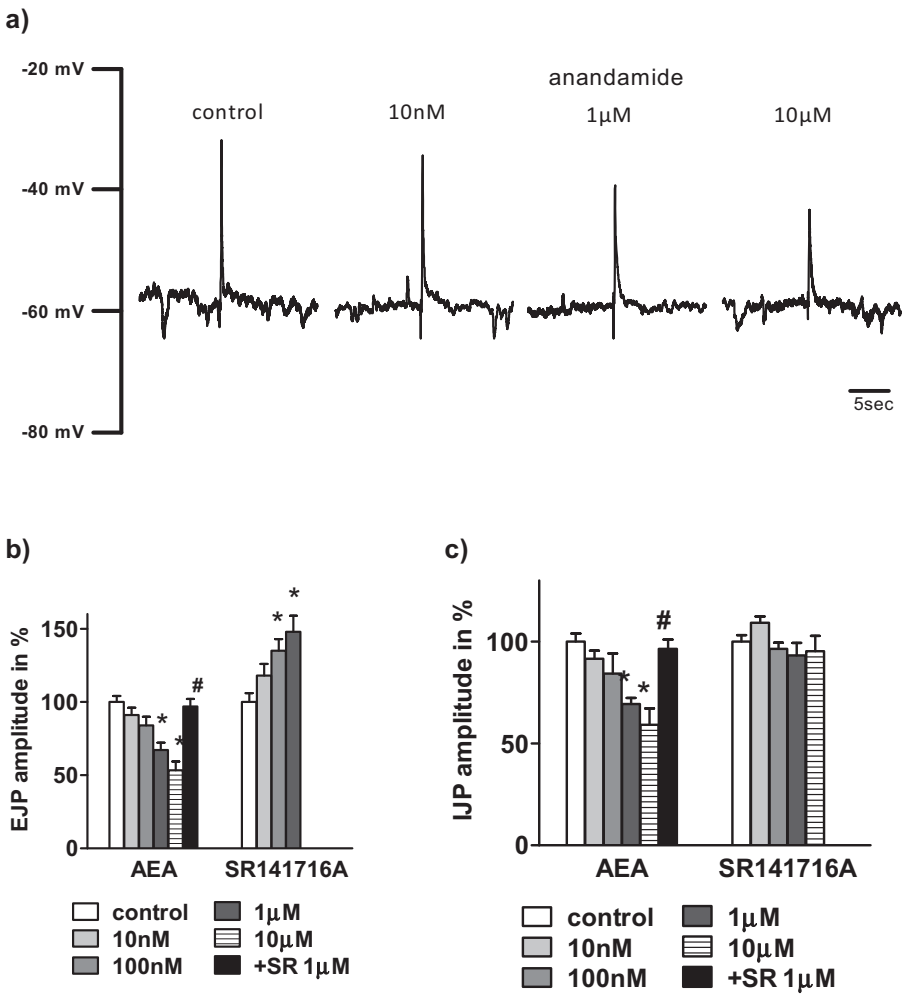

Fig. 7. (a) Representative recording of colonic excitatory junction potentials (EJP) evoked by electrical stimulation in presence of increasing concentrations of anandamide (10 nM–10 μM). (b) Effect of anandamide and SR141716A (10 nM–10 μM) on EJP amplitude. The last bar of the left group shows the antagonizing effect of SR141716A (1 μM) on anandamide effects. Note SR141716A addition in increasing concentrations (10 nM–1 μM) resulted in significant increased EJP amplitudes. (c) Effects of anandamide and SR141716A (10 nM–10 μM) on IJP amplitudes. The last bar of the left group shows the antagonizing effect of SR141716A (1 μM) on anandamide effects. Note SR141716A (10 nM–1 μM) did not alter IJP amplitudes ($^{*}p < 0.05$ *vs.* control; $^{\#}p < 0.05$ for anandamide (10 μM) + SR141716A (1 μM) *vs.* anandamide (10 μM) alone).

Conflict of interest

No conflict of interest to disclose.

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