Cannabinoid receptor type 1 modulates excitatory and inhibitory neurotransmission in mouse colon

M. Storr, A. Sibaev, G. Marsicano, B. Lutz, V. Schusdziarra, J.-P. Timmermans, and H. D. Allescher. Cannabinoid receptor type 1 modulates excitatory and inhibitory neurotransmission in mouse colon. Am J Physiol Gastrointest Liver Physiol 286: G110–G117, 2004. First published July 31, 2003; 10.1152/ajpgi.00148.2003.—The effects of cannabinoid receptor agonists and antagonists on smooth muscle resting membrane potentials and on membrane potentials following electrical neuronal stimulation in a myenteric neuron/smooth muscle preparation of wild-type and cannabinoid receptor type 1 (CB1)-deficient mice were investigated in vitro. Double staining for CB1 and nitric oxide synthase (neuronal) was performed to identify the myenteric CB1-expressing neurons. Focal electrical stimulation of the myenteric plexus induced a fast (f) excitatory junction potential (EJP) followed by a fast and a slow (s) inhibitory junction potential (IJP). Treatment of wild-type mice with the endogenous CB1 receptor agonist anandamide reduced EJP while not affecting IJP and sIJP. EJP was significantly higher in CB1-deficient mice than in wild-type littermate controls, and anandamide induced no effects in CB1-deficient mice. N-arachidonoyl ethanolamide (anandamide), R-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone, a synthetic CB1 receptor agonist, nearly abolished EJP and significantly reduced the IJP in wild-type mice. N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide (SR141716A), a CB1-specific receptor antagonist, was able to reverse the agonist effects induced in wild-type mice. SR141716A, when given alone, significantly increased EJP in wild-type mice without affecting IJP in wild-type and EJP in CB1-deficient mice. Interestingly, SR141716A reduced IJP in CB1-deficient mice. In the mouse colon, nitrergic myenteric neurons do not express CB1, implying that CB1 is expressed in cholinergic neurons, which is in line with the functional data. Finally, excitatory and inhibitory neurotransmission in the mouse colon is modulated by activation of CB1 receptors. The significant increase in EJP in CB1-deficient mice strongly suggests a physiological involvement of CB1 in excitatory cholinergic neurotransmission.

MATERIALS AND METHODS

Tissue preparation for electrophysiological experiments. Eight- to ten-week-old female CB1-deficient mice or wild-type littermates in

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C57BL/6N background (25%) were anesthetized by sodium phenobarbital and killed by cervical dislocation in accordance with the recommendations of the animal ethics committee at the Technical University of Munich. Colon was exposed through an abdominal midline incision. The complete large bowel was removed and placed into oxygenated Krebs solution I of the following composition (in mM): 120.35 NaCl, 5.9 KCl, 2.5 MgCl₂, 1.2 NaH₂PO₄, 15.5 NaHCO₃, 2.5 CaCl₂, and 11.5 glucose (pH 7.4). Colon was opened along the mesenteric border, washed off from remaining fecal material, and pinned out in a Sylgard-lined (Dow Corning; Midland, MI) dissecting dish containing oxygenated Krebs solution I. Proximal colon was separated, and the mucosa and submucosa were removed, resulting in sheets of tissue consisting of circular and longitudinal muscle layers together with the attached myenteric plexus.

Intracellular electrical recording. Sheets or strips of muscle (proximal colon) were pinned using ~150–200 micropins (15–25 mm in thickness), obtained from wolfram wire, to the Sylgard-based electrophysiological chamber, with the exposed circular muscle layer uppermost. The chamber was perfused (5 ml/min; Kwik Pump, World Precision Instruments; Sarasota, FL) with prewarmed (37°C) oxygenated (95% O₂-5% CO₂) Krebs solution I. Tissues were allowed to equilibrate for 90–120 min before the experiments were started. Nifedipine (1 μM) was present throughout all experiments. In experiments with drugs or stimulation, JPs and IJPs were further diluted in Krebs solution I to organ bath concentrations. Drugs were freshly dissolved in ethanol for anandamide, DMSO for WIN 55,212–2 (Biotrend; Köln, Germany), and N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide (SR141716A; gift from Sanofi Research, France). The drugs were freshly dissolved in ethanol for anandamide, DMSO for WIN 55,212–2 and SR141716A or saline for nifedipine, TTX, guanethidine, and atropine on the day of the experiment and further diluted in Krebs solution I to organ bath concentrations. Drugs were added to the perfusate, and experiments were controlled for the effects of the drug solvents. Neither ethanol nor DMSO nor saline in the final organ bath concentrations had a significant effect on the membrane resting potential or on evoked EJP and IJP, respectively.

RESULTS

Recording of circular smooth muscle membrane potential and electrically induced junction potentials. In the absence of drugs or stimulation, circular smooth muscle cells of the proximal colon displayed stable resting membrane potentials (RMP) in wild-type (−52.8 ± 4.4; n = 6) and CB1-deficient mice (−56.7 ± 6.9; n = 6). Nifedipine, atropine, and guanethidine did not alter RMP of the smooth muscle cells of wild-type or CB1-deficient mice, respectively.

Electrical field stimulation (EFS) was used to stimulate enteric excitation and inhibitory neurons to demonstrate functional neuronal input to the circular smooth muscle. EFS of enteric nerves elicited TTX-sensitive junction potentials (JPs), as described in the mouse proximal colon by Shuttleworth et al. (33). In the proximal colon, single stimuli of EFS (15 V, 0.3 ms, single pulse) produced JPs consisting of three components: a fEJP followed by a fIJP and a sIJP (Fig. 1). The initial fast excitatory component was characterized by a rapid depolarization with an amplitude of 13.1 ± 4.8 vs. 27.2 ± 5.2 (CB1+/+ vs. CB1−−; P < 0.05) followed by an inhibitory “fast” 21.6 ± 8.1 vs. 23.7 ± 8.2 and “slow” 12.1 ± 3.3 vs. 13.5 ± 4.4 component (n = 6), which was characterized by a distinct and gradual recovery to RMP (n = 6). All components of the JPs
were blocked by $10^{-5}$ M TTX ($n = 5$, data not shown). Atropine (1 μM) abolished the EJP ($12.3 \pm 2.6$ vs. $0.9 \pm 0.5; n = 3; P < 0.01$) while leaving the fIJP ($25.9 \pm 4.9$ vs. $24.6 \pm 5.3; n = 3$) and the sIJP ($12.7 \pm 5.4$ vs. $11.8 \pm 3.6; n = 3$) unchanged (Fig. 1E). Hexamethonium did not significantly change the EJP ($13.3 \pm 1.2$ vs. $10.7 \pm 1.4; n = 3$), the fIJP ($26.7 \pm 6.2$ vs. $27.3 \pm 5.0; n = 3$), or the sIJP ($12.7 \pm 3.1$ vs. $11.7 \pm 1.2; n = 3$).

**Influence of anandamide, WIN 55,212–2, and SR141716A on junction potentials in wild-type mice.** Neither the endogenous CB1 receptor agonist anandamide [(1 mM) $-52.8 \pm 4.4$ vs. $-50.6 \pm 5.4$ (wild-type) and $-56.7 \pm 6.9$ vs. $-51.7 \pm 6.2$ (CB1$^{-/-}$)] nor the CB1-specific antagonist SR141716A [(1 mM) $-51.6 \pm 5.6$ vs. $-48.9 \pm 7.4$ (wild-type) and $-55.6 \pm 5.4$ vs. $-51.3 \pm 7.7$ (CB1$^{-/-}$)] nor the synthetic CB1 receptor agonist WIN 55,212–2 [(1 mM) $-52.3 \pm 6.7$ vs. $-54.4 \pm 3.7$ (wild-type) and $-55.1 \pm 4.6$ vs. $-52.1 \pm 8.1$ (CB1$^{-/-}$); Fig. 1 for WIN 55,212–2 and SR141716A)] influenced the resting membrane potential of smooth muscle cells in proximal colon.

In wild-type mice, anandamide (1 mM) significantly reduced the EJP (basal: $13.1 \pm 4.8$; with anandamide: $7.0 \pm 2.2; n = 6; P < 0.05$) whereas fIJP and sIJP were not significantly affected (Fig. 2). WIN 55,212–2 (1 mM) significantly reduced the EJP (basal: $12.7 \pm 3.6$; with WIN 55,212–2: $3.3 \pm 3.4; n = 6; P < 0.05$) and the fIJP (basal: $22.7 \pm 2.3$; with WIN 55,212–2: $14.9 \pm 2.7; n = 6; P < 0.05$) but not sIJP (Fig. 3). SR141716A (1 mM) significantly increased the EJP (basal: $13.1 \pm 4.8$; with SR141716A: $20.7 \pm 7.6; n = 6; P < 0.05$), whereas fIJP and sIJP remained unchanged (Fig. 4). Addition of 1 mM SR141716A 20 min before anandamide (1 mM) application significantly reduced the anandamide effect on EJPs ($17.0 \pm 7.2; n = 6$ for SR141716A vs. $12.7 \pm 3.6; n = 6$ for SR141716A and anandamide; Fig. 5). SR141716A (1 mM) administered 20 min before WIN 55,212–2 (1 mM)
application significantly reduced the WIN 55,212–2 effect on EJPs and abolished the effect on fIJP (Fig. 6).

Influence of anandamide, WIN 55,212–2, and SR141716A on junction potentials in CB1-deficient mice. RMP in CB1-deficient mice was not significantly different from that in wild-type mice. CB1-deficient mice showed a significantly increased EJP, whereas fIJP and sIJP were not significantly changed compared with CB1+/− (Fig. 1). In CB1-deficient mice, anandamide (1 mM) or WIN 55,212–2 (1 mM; Figs. 1–3) did not significantly influence EJP, fIJP, or sIJP.

SR141716A (1 mM) did not significantly alter EJP nor sIJP, whereas fIJP, in the presence of SR141716A, was significantly reduced compared with wild-type mice (22.7 ± 2.6 vs. 15.8 ± 2.0 with SR141716A; n = 6; P < 0.05; Fig. 4). Addition of SR141716A (1 mM) 20 min before anandamide (1 mM) or WIN 55,212–2 (1 mM) application did not change EJP nor sIJP.

Fig. 2. Effect of anandamide on electrically induced changes of the membrane potential of mouse colonic circular muscle cells. Figure shows the amplitude of the respective junction potentials for wild-type and CB1-deficient mice in the presence or absence of anandamide (1 mM; *P < 0.05 for wild-type and anandamide vs. wild-type; §P < 0.05 for CB1−/− vs. wild-type).

Fig. 3. Effect of WIN on electrically induced changes of the membrane potential of mouse colonic circular muscle cells. Figure shows the amplitude of the respective junction potentials for wild-type and CB1-deficient mice in the presence or absence of WIN (1 mM; *P < 0.05 for wild-type and WIN vs. wild-type; §P < 0.05 for CB1−/− vs. wild-type).

Fig. 4. Effect of SR on electrically induced changes of the membrane potential of mouse colonic circular muscle cells. Figure shows the amplitude of the respective junction potentials for wild-type and CB1-deficient mice in the presence or absence of SR (1 mM; *P < 0.05 for wild-type and SR vs. wild-type; §P < 0.05 for CB1−/− vs. wild-type; #P < 0.05 for CB1−/− and SR vs. wild-type and SR).

Fig. 5. Effect of anandamide in the presence of SR on electrically induced changes of the membrane potential of mouse colonic circular muscle cells. The figure shows the amplitude of the respective junction potentials in the presence of SR (1 mM) for wild-type and CB1-deficient mice in the presence or absence of anandamide (1 mM; *P < 0.05 for wild-type and SR vs. wild-type; §P < 0.05 for CB1−/− vs. wild-type; #P < 0.05 for CB1−/− and SR vs. wild-type and SR).
sIJP, whereas fIJP s, in the presence of SR141716A and in the presence of a combination of SR141716A and anandamide/WIN 55,212–2, were reduced in a similar manner as with SR141716A alone (Figs. 5 and 6).

**Immunocytochemical identification of CB1 receptor-expressing myenteric neurons.** To characterize a possible colocalization of CB1 and nNOS, as a marker of nitrergic inhibitory neurons, we performed double-staining experiments. CB1 immunoreactivity was demonstrated in myenteric neurons of whole mounts of mouse colon by using an antibody directed toward a fusion protein containing the first 77 amino acid residues of the rat CB1 receptor. Both large- and small-sized somata of neurons were found to display CB1 immunoreactivity. Double staining with antibodies raised against neuronal NOS revealed that the nitrergic myenteric population is clearly distinct from the neuronal population containing CB1 (Fig. 7). In addition, some non-neuronal CB1 immunoreactive cells were observed at the level of the outer muscle layer, but their nature remains to be determined.

**DISCUSSION**

It is well accepted that endogenous cannabinoids and CB1 are involved in the regulation of smooth muscle contractibility and intestinal motility. This has been demonstrated in different regions of the gastrointestinal tract including colon in a variety of species including humans (22, 23, 27, 29). Endogenous cannabinoids in the gastrointestinal tract act via CB1, and recently, interactions between cannabinoid and other receptor systems, such as vanilloid receptors (VR1) and adenosine A1 receptors have been suggested (2, 24, 27). The mechanisms by which CB1 activation influences gastrointestinal neurotransmission mainly relate to reduction of acetylcholine release from cholinergic nerve endings, although other mechanisms, such as reduction of contractile and relaxant nonadrenergic, noncholinergic neuronal activity have also been proposed (13, 34).

**CB1 involvement in colonic neurotransmission in wild-type and CB1-deficient mice.** The present study demonstrates that CB1 is involved in neurotransmission in the mouse colon. Electrically induced EJPs are significantly reduced by the CB1 agonist anandamide and, to a greater extent, by the synthetic agonist WIN 55,212–2. The effects of anandamide and WIN 55,212–2 are antagonized in the presence of the CB1 antagonist SR141716A. Interestingly, the presence of SR141716A alone increases EJP, suggesting a permanent basal activation of CB1 by endogenous cannabinoids.

The antagonistic effects of SR141716A in different tissues are still the subject of much debate. It is concluded in a large number of pharmacological investigations that activation of CB1 reduces cholinergic neurotransmission by reducing acetylcholine release (24, 28). However, other studies (28) using...
SR141716A obtained contrasting results, i.e., either effects or no effects induced by SR141716A were observed, which are sometimes explained by antagonism to endogenous CB1 activation and sometimes by inverse agonist activity of SR141716A. Moreover, these different results were obtained using different tissues and different species, implying that at present, no clear distinction can be made between a pure antagonistic or an inverse agonist effect. Our data suggest that the SR141716A effect also involves a CB1-independent mode of action, because this drug also reduced fIJP in the CB1-deficient mice. Apart from CB1-mediated effects of anandamide described in the literature, several recent publications (2, 24, 28, 34) report effects of this endocannabinoid, which cannot be ascribed to CB1 activation but which likely involved the activation of VR1 and/or non-CB1/non-CB2 receptors.

Therefore, we additionally performed the experiments in CB1-deficient mice, allowing us to monitor unambiguously which effects of the respective agonists or antagonists can be truly attributed to CB1. In the CB1-deficient mice, EJP was found to be significantly increased compared with wild-type littermate controls. This finding might be explained by the possibility that in wild-type animals, a permanent suppressant activity on cholinergic mechanisms, e.g., acetylcholine release, via permanently activated CB1 is present, whereas this mechanism is lacking in CB1-deficient mice. This notion is supported by other experiments on CB1-deficient mice, e.g., reporting an increased acetylcholine release in hippocampal slices in these mutants (17) and further reinforced by the effect of SR141716A on EJP in wild-type animals and by the effect of SR141716A in other investigations, where the CB1 antagonist significantly increased acetylcholine-mediated smooth muscle responses and where SR141716A increased acetylcholine release (7). The finding that EJPs in our tissue preparations were abolished by the presence of either atropine or TTX led us to conclude that CB1 receptors are tonically activated in the mouse proximal colon and exert a suppressant effect on cholinergic excitatory neurotransmission. Additional activation of CB1 results in a reduction and, in the case of the synthetic agonist WIN 55,212–2, nearly an abolition of the cholinergic excitatory postsynaptic potentials, providing evidence that CB receptors are not only involved in neuromuscular but also in neuromuscular cholinergic transmission in the gut (15). However, the question of whether the presence of cannabinoid receptors is confined to one or more functional classes of neurons has remained unanswered to the present. Although these findings on neuronal stimulation are in agreement with our findings, it has to be kept in mind that in our setup, recordings were made from the smooth muscle cell and not from the neurons directly. According to our findings in the hexamethonium experiments, the most likely location of the involved CB1 receptors is varicosities of the motor nerve terminals. However, our data do not suggest that CB1 receptors within the myenteric plexus are exclusively located on varicosities of the motor nerve terminals. Additional locations within the myenteric plexus as suggested by others (20) seem likely.

Apart from established localization of CB1 on non-nitrergic myenteric neurons, both our functional and immunocytochemical data do not exclude another non-neuronal location of this receptor. In agreement with earlier immunocytochemical reports (6, 18, 28), no direct immunocytochemical evidence could be provided for the expression of CB1 on smooth muscle cells. From our present results, both locations of CB1 seem to be possible. Besides different peripheral receptor locations, the literature suggests that additionally, central CB1 receptors might contribute to the regulation of colonic motility in analogy to the finding that both central and peripheral CB1 receptors are involved in the regulation of intestinal motility (15).

With the rat stomach, CB1 immunoreactivity was detected on neuronal elements innervating blood vessels of mucosa and submucosa as well as on smooth muscle (1). Within the enteric nervous system, CB1 immunoreactivity was found to be colocalized with immunoreactivity to choline acetyltransferase, a marker of cholinergic neurons (6, 18, 29). This colocalization is in agreement with functional experiments demonstrating a suppressant effect of CB1 activation on neurally mediated...
cholinergic mechanisms or on neuronal acetylcholine release (24). Our finding that CB1 and NOS immunoreactivity are not colocalized in the same myenteric neurons is in line with our electrophysiological recordings showing no change in sIJP after treatment with cannabinoids.

Regarding the suppressant effect on the excitatory postsynaptic potential, our findings are in good agreement with other studies. In the guinea pig ileum, activation of CB1 by WIN 55,212–2 or the synthetic CB1 agonist CP 55,940 suppressed excitatory postsynaptic potentials by 37–46% in concentration- and CB1-dependent manners (20). SR141716A antagonized the effect of WIN 55,212–2 but, when given alone, caused a 40–50% reduction of excitatory postsynaptic potentials similar to the reduction caused by WIN 55,212–2.

In summary, the present investigation for the first time provides evidence that in the murine proximal colon, cannabinoids are involved in both excitatory cholinergic and inhibitory nonadrenergic, noncholinergic neurotransmission and that these effects are mediated via the CB1 receptor. The specific involvement of CB1 in the suppressant effect on cholinergically mediated EJP of CB1 agonists has clearly been shown, because the effects induced by anandamide and WIN 55,212–2 were reversed by the selective CB1 antagonist SR141716A and because anandamide and WIN 55,212–2 were not able to induce effects in CB1-deficient mice. The physiological impact of the reported modulation of colonic neurotransmission via peripheral CB1 receptors has yet to be established, because modulators of enteric neurotransmission might serve as therapeutic tools in a variety of functional gastrointestinal disorders (9).

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REFERENCES


