Evidence for a modulatory role of cannabinoids on the excitatory NANC neurotransmission in mouse colon

Flavia Mulè*, Antonella Amato, Sara Baldassano, Rosa Serio

Dipartimento di Biologia cellulare e dello Sviluppo, Università di Palermo, 90128 Palermo, Italia

Accepted 27 April 2007

Abstract

It is well accepted that endogenous cannabinoids and CB1 receptors are involved in the regulation of smooth muscle contractility and intestinal motility, through a mechanism mainly related to reduction of acetylcholine release from cholinergic nerve endings. Because, few data exist on a possible modulatory action of the cannabinoid agents on the non-adrenergic non-cholinergic (NANC) excitatory and inhibitory neurotransmission, the aim of the present study was to investigate the effects of cannabinoid drugs on the NANC responses elicited by electrical field stimulation (EFS) in the circular muscle of mouse proximal colon. Colonic contractions were monitored as changes in endoluminal pressure.

In NANC conditions, EFS evoked TTX-sensitive responses, characterized by a relaxation, nitricergic in origin, followed by a contraction. The EFS-evoked contraction was significantly reduced by SR48968, NK2 receptor antagonist, and abolished by co-administration of SR48968 and SR140333, NK1 receptor antagonist, suggesting that it was due to release of tachykinins. The cannabinoid receptor synthetic agonist, WIN55,212-2, the putative endogenous ligand, anandamide, the selective CB1 receptor agonist ACEA, but not the selective CB2 receptor agonist JWH-015, produced a concentration-dependent reduction of the NANC contractile responses, without affecting the NANC relaxation. ACEA or anandamide did not modify the contractions induced by exogenous [B-Ala8]-NKA(4–10), agonist of NK2 receptors. The selective antagonist of CB1 receptors, SR141716A, per se failed to affect the EFS-evoked responses, but antagonized the inhibitory effects of WIN55,212-2, anandamide and ACEA on NANC contractile responses. AM630, CB2 receptor antagonist, did not modify the inhibitory effects of WIN55,212-2 or anandamide. URB597, inhibitor of the fatty acid amid hydrolase, enzyme which catalyze the hydrolysis of anandamide, was without any effect on the NANC evoked responses. We conclude that the activation of prejunctional CB1 receptors produces inhibition of NANC contractile responses in mouse colonic preparations. However, endogenous ligands do not seem to modulate tonically the NANC transmission in mouse colon.

© 2007 Elsevier Ltd. All rights reserved.

Keywords: Cannabinoids; CB1 receptors; Intestinal motility; NANC contraction; NANC relaxation

1. Introduction

Cannabinoids have been shown to exert a broad variety of pharmacological actions, including central and peripheral effects, through receptor-mediated mechanisms [1]. Many of the reported pharmacological effects are mediated by two specific receptors, denoted CB1 and CB2 receptors, both coupled to G-proteins. CB1 receptors are found mainly in central and peripheral neurons, while CB2 receptors are located in peripheral tissues, particularly in immune cells [2]. Endogenous ligands for these receptors include arachidonylethanolamide (anandamide), 2-arachidonylglycerol (2-AG) and noladin ether. Inactivation of endocannabinoid signalling is dependent on cellular uptake, intracellular transport and enzymatic hydrolysis by the fatty acid amide hydrolase (FAAH) [3]. Therefore, the “endocannabinoid system” comprises the cannabinoid receptors, their endogenous ligands and the proteins participating in the inactivation of these latter compounds [4].

In the gastrointestinal tract CB1 receptors appear to be involved in several physiological process, including gastric secretion, intestinal motility, gastrointestinal transit and colonic propulsion [5–8]. In agreement with these observations, CB1 are present in the gastrointestinal tract and are localized in neurons of the myenteric and submucosal plexuses in a variety of species, co-expressed with cholinergic markers [9–13]. Endogenous ligands, anandamide and 2-AG have been detected also in the mouse small intestine [14]. In the mouse gastrointestinal tract the highest levels of CB1 receptors have been detected in...
stomach and colon and CB1 immunoreactivity is present in ganglia and in the smooth muscle layers of both the small and large intestine [15].

It is well accepted that the mechanism by which the activation of CB1 regulates smooth muscle contractility and intestinal motility is related to reduction of acetylcholine release from cholinergic nerve endings [16,17]. However, other mechanisms, such as activation of vanilloid receptor [18,19], modulation of adenosine release [20], stimulation of myenteric cholinergic neurons through lipoxygenase metabolites [21] have been also proposed.

In the last decade, non-adrenergic non-cholinergic (NANC) excitatory transmission has been object of vigorous research because it has become clear that it contributes to the intestinal motor function and its role becomes even more important in a pathophysiological setting. In particular, tachykinins, acting as co-transmitters in the neuromuscular transmission, function as a backup system during peristalsis and a reduction in the ability of tachykinin receptor agonists to contract the intestinal smooth muscle has been reported in various disorders of the gastrointestinal tract [22]. Inhibition of NANC excitatory responses by cannabinoid agonists has been reported just in guinea-pig circular muscle [17]. Moreover, few studies have investigated the effects of CB ligands on the intestinal NANC inhibitory neurotransmission. However, those that have appear not to be concordant, because facilitation in guinea-pig ileum [23], inhibition in rat gastric fundus [24] or no effect in human colon [25] have been reported.

Therefore, the purpose of the present study was to verify whether cannabinoid drugs could influence the NANC excitatory and inhibitory neural transmission in mouse proximal colon. For this aim, we used the exogenous and endogenous non-selective CB receptor agonists, respectively, WIN 55,212-2 and anandamide; the selective CB1 agonist ACEA; the selective CB2 agonist JWH-015; the selective CB1 and CB2 antagonists, respectively SR141716A and AM630. Data from the study were presented at the 1st Joint International Society Meeting in Neurorastrenterology and Motility [26].

2. Materials and methods

Experimental procedures were in accordance with the European Communities Council Directive of 24 November 1986 and were approved by Ministero della Sanità (Rome, Italy). Adult mice (C57BL/10) were killed by cervical dislocation. The abdomen was immediately opened and the proximal intestine was ligated with a silk thread and preloaded to 0.5 g. Mechanical activity was detected as changes in intraluminal pressure, which are mainly generated by circular muscle, and recorded on ink-writer polygraph (Grass model 7D). This experimental set-up was chosen in order to analyse the muscle function under conditions where the influence of external factors is removed, but the muscle itself performs in a manner analogous to its in vivo capacity. To provide electrical field stimulation (EFS), a pair of platinum plates was placed in parallel on either side of the colonic segment. EFS was applied by an S88 square-wave pulse generator (Grass Medical Instruments, Quincy, MA, USA) coupled via a stimulus isolation unit (Grass SIU5) to the electrodes. Preparations were allowed to equilibrate for 60 min before starting the experiment.

2.1. Experimental protocol

After the equilibration time, EFS (0.5 ms duration, supramaximal voltage, in trains of 5 s, 2–32 Hz) was applied to the tissue at intervals of 5 min. In these conditions, stable and reproducible responses were observed for 6 h. At first, the effect of tetrodotoxin (TTX) (0.1 μM), SR48968 (0.1 μM), SR140333 (0.1 μM), or t-NAME (300 μM) was evaluated on electrically induced responses. These compounds were added to the perfusing solution and left in contact with the preparation for 30 min.

EFS was also performed in the presence of increasing cumulative concentrations of WIN55,212-2 (1–1000 nM), anandamide (10–100 μM), ACEA (0.01–1 μM), JWH-015 (0.1 μM), SR141716A (0.1–3 μM) or URB597 (0.01–1 μM). The contact time for each concentration was 20 min. In a different series of experiments, the cannabinoid agonists were tested also 30 min after SR141716A (0.1 μM), selective antagonist of CB1 receptors, or AM630 (0.1 μM), selective antagonist of CB2 receptors. In another series of experiments, the myogenic contractions produced by β-Ala₈-NKA(4–10) (10 nM to 10 μM), NK2 receptor agonist, were evaluated in the presence of ACEA (0.1 μM) or anandamide (10 μM) to examine if the two ligands were able to alter the smooth muscle response.

2.2. Data analysis and statistical tests

All data are expressed as mean values ± S.E.M. The letter n indicates the number of experimental animals. The contractile response to EFS was expressed as a percentage of the maximal response produced by EFS, which was obtained at 16 or 32 Hz depending on the preparations. The concentration (EC₅₀) with 95% confidence limits (c.l.) producing half maximum response of β-Ala₈-NKA(4–10) was calculated using Prism 4.0, GraphPad (San Diego, CA, USA). Comparison between two sets of data were made by Student’s t-test for paired data. When mul-
tiple comparisons against a single control were made, analysis of variance was performed, followed by Bonferroni t-test. A probability value of less than 0.05 was regarded as significant.

2.3. Drugs

The following drugs were used and stock solutions were prepared using distilled water or as indicated below. The working solutions were prepared fresh the day of the experiments by diluting the stock solutions in Krebs, Arthopine sulphate, guanethidine monosulphate, tetrodotoxin (TTX), Nω-nitro-l-arginine methyl ester (l-NAME), AM630, R-[2,3-dihydro-5-methyl-3(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoazin-6-yl]-1-naphthalenylmethanone mesylate (WIN 55,212-2), (2-methyl-1-propyl-1H-indol-3-yl)-1-naphthalenylmethanone (JWH-015), cyclohexyl carbamic acid [2,3-dihydro-5-methyl-3(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoazin-6-yl]-1-naphthalenylmethanone mesylate (JWH-015), cyclohexyl carbamic acid [2,3-dihydro-5-methyl-3(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoazin-6-yl]-1-naphthalenylmethanone mesylate (JWH-015), and SR141716A (0.1 μM). The inhibitory actions of WIN55,212-2 were prevented by SR141716A (0.1 μM) and AM630 (0.1 μM). However, the inhibitory actions of WIN55,212-2 were abolished by SR48968 (0.1 μM), a selective NK2 receptor antagonist, and abolished by co-administration of SR48968 and SR140333 (0.1 μM), a selective NK1 receptor antagonist suggesting that they were due to release of tachykinins.

3.2. Effect of cannabinoid drugs on NANC evoked responses

Neither the cannabinoid receptor agonists, WIN55,212-2 (1–1000 nM), and SR140333 (0.1–1 μM) nor the cannabinoid receptor antagonists SR141716A (0.1 μM) and AM630 (0.1 μM), had any influence on the basal tone or spontaneous activity (amplitude of contractions, contractile frequency) of mouse colonic preparations.

WIN55,212-2 (1 nM to 1 μM), non-selective synthetic cannabinoid receptor agonist, and anandamide (1–100 μM), endogenous cannabinoid receptor agonist, produced a concentration-dependent reduction of the NANC contractile responses (Figs. 1 and 2). WIN55,212-2 at a concentration of 1 μM abolished NANC contractions evoked by all stimulations frequencies. Indeed, anandamide was less potent than WIN55,212-2, being effective at higher concentrations than WIN55,212-2. The inhibitory actions of WIN55,212-2 (0.1 μM) and anandamide (10 μM) were antagonized by the selective cannabinoid CB1 antagonist, SR141716A (0.1 μM), but not by the selective cannabinoid CB2 antagonist, AM630 (0.1 μM), suggesting CB1 receptor involvement (Fig. 3).

To confirm the subtype of CB receptor responsible of the observed effects, we tested the NANC evoked responses in the presence of ACEA, selective CB1 receptor agonist or JWH-015, selective CB2 receptor agonist. ACEA (0.01–1 μM), but not JWH-015 (up to 0.1 μM), produced a concentration-dependent reduction of NANC contractile responses to EFS (Figs. 4 and 5). ACEA effects were prevented by SR141716A (0.1 μM) (Fig. 4).

None of the cannabinoid drugs used had a significant influence on the relaxant responses evoked by EFS (Fig. 6).

Fig. 1. Typical tracings showing the effects of different concentrations of the non-selective synthetic cannabinoid receptor agonist, WIN 55,212-2, on the NANC responses evoked by EFS (0.5 ms duration, supramaximal voltage, in trains of 5 s, 16 Hz) in mouse proximal colon. The inhibitory effects were prevented by the CB1 cannabinoid receptor antagonist, SR141716A (0.1 μM), but not by the CB2 cannabinoid receptor antagonist, AM630 (0.1 μM).
Fig. 2. Effects of different concentrations of WIN55,212-2, exogenous non-selective CB receptor agonist, or anandamide, endogenous non-selective CB receptor agonist, on NANC contractile responses evoked by EFS. The ordinates show the amplitude of the evoked contraction expressed as a percentage of the maximal response produced by EFS. Data are means ± S.E.M. (n = 5 for each series of experiments). *P < 0.05 compared to control.

Fig. 3. Effects of WIN55,212-2 or anandamide, alone or in combination with SR141716A, CB1 receptor antagonist, AM630, CB2 receptor antagonist, or URB597, inhibitor of FAAH, on NANC contractile responses evoked by EFS. The ordinates show the amplitude of the evoked contraction expressed as a percentage of the maximal response produced by EFS. Data are means ± S.E.M. (n = 5 for each series of experiments). *P < 0.05 compared to WIN55,212-2 (0.1 μM) or anandamide (10 μM), respectively.

Fig. 4. Effects of different concentrations of ACEA, selective CB1 receptor agonist, alone or in combination with SR141716A, CB1 receptor antagonist, on NANC contractile responses evoked by EFS. The ordinates show the amplitude of the evoked contraction expressed as a percentage of the maximal response produced by EFS. Data are means ± S.E.M. (n = 5 for each series of experiments). *P < 0.05 compared to control. #P < 0.05 compared to ACEA (0.1 μM).
Fig. 5. Effects of JWH-015 (0.1 µM), CB2 receptor agonist, on NANC contractile responses evoked by EFS. The ordinate shows the amplitude of the evoked contraction expressed as a percentage of the maximal response produced by EFS. Data are means ± S.E.M. (n = 4).

The EFS-evoked responses were not significantly modified by SR141716A (0.1–3 µM). Also URB597, inhibitor of FAAH, up to a concentration of 1 µM, per se did not modify the EFS-induced responses (Fig. 7). However, URB597 (0.1 µM) significantly increased the inhibitory effects caused by exogenous application of anandamide (10 µM) (Fig. 3).

3.3. Effect of ACEA on exogenously evoked NK2 mediated contractions

[β-Ala⁸]-NKA(4–10) (10 nM to 10 µM), NK2 receptor agonist, induced a concentration-dependent TTX-insensitive contractile response, that was maintained for the entire application time. ACEA (0.1 µM) or anandamide (10 µM) failed to affect the contractions evoked by [β-Ala⁸]-NKA(4–10) and the EC₅₀ value [EC₅₀ (95% c.l.) control: 67 (42–106) nM; ACEA: 76 (45–128) nM (n = 4); anandamide: 60 (31–117) (n = 4)] (Fig. 8).

Fig. 6. Effects of the cannabinoid agonists, WIN55,212-2, anandamide and ACEA (0.1 µM), on the relaxation evoked by EFS. Data are means ± S.E.M. (n = 5 for each treatment).

Fig. 7. Effects of SR141716A, CB1 receptor antagonist, or URB597, inhibitor of FAAH, on NANC contractile responses evoked by EFS. The ordinate shows the amplitude of the evoked contraction expressed as a percentage of the maximal response produced by EFS. Data are means ± S.E.M. (n = 10 for SR141716A; n = 5 for URB597).

Fig. 8. (A) Contractions evoked by [β-Ala⁸]-NKA(4–10), selective agonist of NK2 receptors, before and after ACEA (0.1 µM) or anandamide (10 µM). The arrows indicate the application of [β-Ala⁸]-NKA(4–10) (3 µM). (B) Concentration–response curves for the contractile effects induced by [β-Ala⁸]-NKA(4–10), in control conditions and after pre-treatment with ACEA or anandamide. Contractile response is expressed as percent of the maximal response. Data are means ± S.E.M. (n = 4 for each treatment).

4. Discussion

The results of the present study suggest that activation of prejunctional cannabinoid CB1 receptors is able to modulate the
Several immunohistochemical and pharmacological studies have shown the role of CB1 receptors in the inhibition of motility in the mouse small and large intestine [13,15,29–31] and in the electrically evoked peristaltic activity of the mouse isolated distal colon [32]. The mechanism by which CB1 activation influences mouse colonic propulsion mainly relates to reduction of acetylcholine release from cholinergic nerve endings, as proposed in an electrophysiological study [33]. Although inhibition of cholinergic and NANC excitatory responses has been reported in guinea-pig circular muscle [17], little is known about cannabinoid action on NANC excitatory and inhibitory neurotransmission in the gut.

We have shown that WIN55,212-2, a non-selective cannabinoid agonist and, anandamide, an endogenous ligand of these receptors, can produce concentration-related inhibition of the electrically evoked NANC contractile responses in mouse colonic smooth muscle. WIN55,212-2 was more active than anandamide in inhibiting electrically evoked contractions suggesting that WIN55,212-2 is more potent than anandamide at the CB1 receptors, as reported in other studies [7,17]. Alternatively anandamide could be more rapidly degraded. The inhibitory actions of both WIN55,212-2 and anandamide were antagonized by SR141716A, but not by AM630, suggesting an involvement of CB1 receptors. The concentration of SR141716A we used has been established to be specific for cannabinoid receptors, as it is devoid of any influence on other receptors involved in the regulation of intestinal motor activity [16,17,34]. In addition, the hypothesis that CB1 receptor activation is able to depress the NANC excitatory neurotransmission is strengthened by the observations that ACEA, selective CB1 agonist, but not JWH-015, selective CB2 agonist, produced a concentration-dependent reduction of NANC evoked contractile responses.

Our results appear to rule out a potential function of CB2 receptors in the modulation of the NANC responses, at least in our experimental conditions. This observation would agree with other studies reporting that CB2 receptors failed to affect rat intestinal motility in normal conditions, but they can contribute to attenuation of the gastrointestinal transit after inflammatory stimulus [35]. However, further experiments are required to confirm our conclusion because mRNA for CB2 receptors has been isolated from rat fundus and guinea-pig whole gut and CB2 receptors have been shown to exert inhibitory effects in these preparations [24,36].

Indirect evidence suggests that endogenous cannabinoids can exert a tonic influence on intestinal motility [6,13,32]. The observation that in our experiments SR141716A, even at concentrations higher than those sufficient to inhibit the effects caused by CB1 agonists, failed to affect the evoked NANC contractions suggests that the CB1 receptors modulating the NANC excitatory pathways are not tonically activated. However, previous investigations have shown that in the mouse, as in other preparations [17,34], the CB1 receptor antagonist SR141716A, administered alone, can enhance motility in small intestine [29,30] and increase colonic propulsion [12,32]. The apparent discrepancy could be explained assuming that the tonic action of cannabinoid system influences only the cholinergic transmission, which is blocked in our experimental conditions, although it has been shown that cholinergic transmission to the human ileum and colon [37–39] and gastric emptying in rats in vivo were unaffected by SR141716A [39]. Indeed, also in guinea-pig ileum SR141716A increased the amplitude of the electrically evoked NANC contractions [17]. Reasons for this discrepancy remain unclear although species, as well as regional, differences are possible contributing factors. On the other hand, increases in the intestinal transit or in the evoked contractions by CB1 receptor antagonist have been observed, respectively, in vivo conditions [29,30] or in vitro using both circular or longitudinal strips [16,17]. Different experimental preparations (strips versus entire segments with recording of intraluminal pressure) could represent another likely explanation for our inability to detect any action of SR141716A on its own. In fact, Mancinelli et al. [32], using colonic entire segments, did not observe any increase in the pressure waves, when SR141716A was added alone to the organ bath, but only enhancement of tonic and phasic activity of the longitudinal smooth muscle.

To further verify if in mouse colon there is a cannabinoid tone we used URB597, a potent and selective inhibitor of the FAAH [40]. FAAH activity has been detected in the rodent intestine [14] and this enzyme has been proposed as a regulator of the intestinal motility [41]. In our preparation URB597 did not affect the evoked NANC responses suggesting that there is not a release of endogenous cannabinoid (likely anandamide) sufficient to reduce the NANC contractions. Moreover in the present study we have shown that URB597 is able to increase the inhibitory effect of anandamide confirming that the FAAH enzyme may metabolize the amine.

ACEA or anandamide failed to affect the contractions induced by the NK2 agonist [β-Ala³]–NKA(4–10). Similarly, the potency of [β-Ala³]–NKA(4–10) in evoking 50% of the maximal contraction was unaffected by ACEA or anandamide. As the agonist of NK2 receptors evokes contraction by activating receptors directly on the smooth muscle [28], the results indicate that CB1 receptor activation does not directly depress smooth muscle activity and that the inhibitory action of ACEA and of the endogenous cannabinoid on excitatory NANC transmission is achieved primarily by acting at prejunctional receptors. Consistent with this hypothesis, a high degree of co-localization of CB1 receptors with a population of tachykinin immunoreactive neurons has been reported in guinea-pig, rat and porcine enteric nervous system [9,10,12].

Methanandamide, a derivative of anandamide that is metabolically more stable than the parent compound, has been shown to depress intestinal peristalsis in guinea-pig via activation of CB1 receptors on enteric neurons, which results in the blockade of excitatory motor pathways and in the facilitation of inhibitory pathways [23]. Moreover, a modest but not significant potentiation of EFS-evoked relaxations has been also observed in human colonic circular muscle [25]. Therefore, we also analysed the effect of cannabinoid drugs on the NANC evoked...
relaxation. In our experiments, none of the cannabinoid agonists used had a significant influence on the relaxant responses evoked by EFS, ruling out a modulatory role for cannabinoid on the inhibitory neurotransmission. On the other hand, the present and our previous studies have shown that the relaxation is due to nitricergic pathways [27] and several studies have reported that in mouse colon myenteric plexus there is no overlapping between nitric oxide synthase (NOS) and CB1 immunoreactivity [13,33]. These observations are also in agreement with previous findings reporting that cannabinoids have no actions on inhibitory junction potentials mediated by electrical stimulation of nitricergic nerves [33].

In conclusion, our study provides evidence for a modulatory role of cannabinoids on NANC excitatory neurotransmission through prejunctional CB1 receptors in mouse colon. This mechanism could contribute to the delay of the colonic propulsion reported in in vivo studies in the mouse.

Acknowledgements

This work was supported by a grant from Ministero dell’Università e della Ricerca Scientifica (MIUR), Italy. We thank Sanofi Recherche (Montpellier Cédex, France) for supplying SR141716A, SR140333 and SR48968.

References


