



Cannabinoid CB₁-receptor mediated regulation of gastrointestinal motility in mice in a model of intestinal inflammation

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1 We have studied the effect of cannabinoid agonists (CP 55,940 and cannabinol) on intestinal motility in a model of intestinal inflammation (induced by oral croton oil in mice) and measured cannabinoid receptor expression, endocannabinoids (anandamide and 2-arachidonylglycerol) and anandamide amidohydrolase activity both in physiological and pathophysiological states.

2 CP 55,940 (0.03–10 nmol mouse⁻¹) and cannabinol (10–3000 nmol mouse⁻¹) were more active in delaying intestinal motility in croton oil-treated mice than in control mice. These inhibitory effects were counteracted by the selective cannabinoid CB₁ receptor antagonist SR141716A (16 nmol mouse⁻¹). SR141716A (1–300 nmol mouse⁻¹), administered alone, increased intestinal motility to the same extent in both control and croton oil-treated mice

3 Croton oil-induced intestinal inflammation was associated with an increased expression of CB₁ receptor, an unprecedented example of up-regulation of cannabinoid receptors during inflammation.

4 High levels of anandamide and 2-arachidonylglycerol were detected in the small intestine, although no differences were observed between control and croton oil-treated mice; by contrast anandamide amidohydrolase activity increased 2 fold in the inflamed small intestine.

5 It is concluded that inflammation of the gut increases the potency of cannabinoid agonists possibly by ‘up-regulating’ CB₁ receptor expression; in addition, endocannabinoids, whose turnover is increased in inflamed gut, might tonically inhibit intestinal motility.

British Journal of Pharmacology (2001) **134**, 563–570

Keywords: Anandamide; 2-arachidonylglycerol; endocannabinoids; cannabinoid receptors; intestine; intestinal motility; inflammatory bowel disease

Abbreviations: 2-AG, 2-arachidonylglycerol; CB, cannabinoids; DMSO, dimethyl sulphoxide

Introduction

Δ^9 -tetrahydrocannabinol, the major psychoactive principle of marijuana and hashish, produces a wide variety of biological effects, including inhibition of intestinal motility (Shook & Burks, 1989; Dewey, 1986). In recent years, it has been shown that Δ^9 -tetrahydrocannabinol binds to specific cannabinoid receptors, CB₁ receptors (expressed by central and peripheral neurones) and CB₂ receptors (that occur mainly in immune cells), both coupled to G-proteins (Matsuda *et al.*, 1990; Munro *et al.*, 1990; Di Marzo *et al.*, 2000; Izzo *et al.*, 2000a; Pertwee, 1999a, b). The discovery of cannabinoid receptors has led to the demonstration that there are endogenous agonists for these receptors, namely the ‘endocannabinoids’, anandamide and 2-arachidonylglycerol (2-AG) (Devane *et al.*, 1992; Mechoulam *et al.*, 1995; Sugiura *et al.*, 1995). Cannabinoid receptors and their endogenous ligands together are referred to as the ‘endogenous cannabinoid system’ (Di Marzo *et al.*, 2000).

Cannabinoid CB₁ receptors have been located in myenteric neurones and their activation can mediate the inhibition of excitatory transmission in the isolated guinea-pig (Izzo *et al.*, 1998; Pertwee *et al.*, 1996) and human ileum (Crocì *et al.*, 1998), inhibition of electrically-evoked acetylcholine release from guinea-pig myenteric nerves (Coutts & Pertwee, 1997) and reduction of peristalsis efficiency in the isolated guinea-pig ileum (Heinemann *et al.*, 1999; Izzo *et al.*, 2000c). *In vivo* studies have shown that cannabinoid receptor agonists decreased gastric and intestinal motility through activation of CB₁ receptor (Krowichi *et al.*, 1999; Izzo *et al.*, 1999a, b, c; Calignano *et al.*, 1997; Colombo *et al.*, 1998; Izzo *et al.*, 2000d), while SR141716A, a cannabinoid CB₁ receptor antagonist, increased intestinal motility in rodents (Calignano *et al.*, 1997; Colombo *et al.*, 1998; Izzo *et al.*, 1999a, c; Izzo *et al.*, 2000d). Both central and peripheral cannabinoid CB₁ receptors could modulate upper gastrointestinal transit, but the effect of systemic (intraperitoneally injected) cannabinoid drugs was mediated by peripheral receptors (Izzo *et al.*, 2000d).

In the present paper we have evaluated the role of the endogenous cannabinoid system in a model of intestinal inflammation induced by croton oil in mice; croton oil

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produces inflammation in the small intestine characterized by infiltration of lymphocytes in the submucosa (Puig & Pol, 1998). We have used the plant-derived cannabinoid agonist cannabinol (Petitet *et al.*, 1998), the synthetic cannabinoid receptor agonist CP 55,940 (D'ambra *et al.*, 1992), the cannabinoid CB₁ receptor antagonist SR141716A (Rinaldi-Carmona *et al.*, 1995) and the cannabinoid CB₂ receptor antagonist SR144528 (Rinaldi-Carmona *et al.*, 1998). In addition, the expression of cannabinoid receptors, the amounts of endocannabinoids and the activity of anandamide amidohydrolase (the enzyme responsible for the hydrolysis of anandamide and 2-AG) were assessed in both control and inflamed intestinal tissues.

Methods

Animals

Male ICR mice (Harlan Italy, Corezzana, MI, U.S.A.) (24–26 g) were used after 1 week of acclimation (temperature 23 ± 2°C; humidity 60%). Food was withheld 6 h before transit measurement and 18 h before the induction of chronic intestinal inflammation.

Intestinal inflammation

Inflammation was induced as previously described (Puig & Pol, 1998). Mice received orally two doses of croton oil (20 µl mouse⁻¹) on 2 consecutive days. Motility was measured 4 days after the first administration of croton oil. This time was selected on the basis of a previous work (Puig & Pol, 1998), which reported that maximal inflammatory response occurred 4 days after the first treatment.

Upper gastrointestinal transit

Gastrointestinal transit was measured in control and in croton oil-treated mice. At this time, a black marker (10% charcoal suspension in 5% gum arabic, 0.1 ml per 10 g body weight) was administered orally to assess upper gastrointestinal transit as previously described (Puig & Pol, 1998). After 20 min the mice were killed by asphyxiation with CO₂ and the gastrointestinal tract removed. The distance travelled by the marker was measured and expressed as a percentage of the total length of the small intestine from pylorus to caecum. In some experiments, the inhibitory effect of cannabinoid agonists on upper gastrointestinal transit was expressed as a percentage of inhibition as follows:

$$\% \text{ inhibition} = (\% \text{ transit vehicle} - \% \text{ transit cannabinoid agonist}) / \% \text{ transit vehicle}$$

Drugs administration

The cannabinoid agonists CP 55,940 (0.03–10 nmol mouse⁻¹), cannabinol (10–3000 nmol mouse⁻¹), the CB₁ receptor antagonist SR141716A (1–300 nmol mouse⁻¹), the CB₂ receptor antagonist SR144528 (52 nmol mouse⁻¹) or vehicle (DMSO, 4–8 µl mouse⁻¹) were given intraperitoneally (i.p.) or intracerebroventricularly (i.c.v.) 20 min before charcoal administration. In some experiments SR141716A (16 nmol mouse⁻¹ = 0.3 mg kg⁻¹), SR144528

(52 nmol mouse⁻¹ = 1 mg kg⁻¹) or hexamethonium (69 nmol mouse⁻¹ = 1 mg kg⁻¹) were given (i.p.) 10 min before the cannabinoid agonists. These doses were selected on the basis of previous published work (Izzo *et al.*, 2000d).

Intracerebroventricular injections

Intracerebroventricular injections were performed as described by Haley & McCormich, (1957). Mice were briefly anaesthetized with enflurane and the drugs were delivered in a volume of 4 µl, using a Hamilton microlitre syringe fitted with 26-gauge needle. In preliminary experiments, the correct location of the intracerebroventricular injection has been verified by injecting 5 µl Evans blue dye (0.5%). The brains were then removed and examined to verify dye distribution.

Western blot analysis

Jejunum was homogenized in a buffer containing (mM): 100 HEPES, NaCl 1 M, EDTA 200, EGTA 100, dithiothreitol 1 M, phenylmethylsulphonyl fluoride 100, trypsin inhibitor 15 mg/ml⁻¹, pepstatin A 750 µ⁻¹/ml, benzamide 10, glycerol 20% v v⁻¹, MgCl₂ 100, Nonidet P40 1%, H₂O, using a glass homogenizer and a teflon pestle. Homogenates were centrifuged at 4°C at 13,000 r.p.m. for 15 min and the supernatants were separated from pellet and aliquoted and stored at –80°C. Protein concentration was determined before use by Bio-Rad protein assay kit. Immunoblotting analysis of CB₁ receptors was performed as previously described (Melek *et al.*, 2000). Equivalent amounts (100 µg) of proteins from intestinal homogenates were separated by SDS–PAGE polyacrilamide minigel and transferred onto a nitro-cellulose membrane. The blots were blocked with 10% non fat dry milk in phosphate buffered saline (PBS) and incubated overnight at 4°C with a polyclonal rabbit antiserum raised against human CB₁ receptor (dil. 1:800; Cayman Chemicals). After repeated washing with 1% Triton X-100 in PBS a peroxidase-conjugated goat anti-rabbit antibody (dil. 1:2000; Amersham) was added for 1 h at room temperature. Finally the immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham).

The relative expression of CB₁ receptors was quantified by densitometric scanning of the X-ray films with a GS 700 Imaging Densitometer (Bio-Rad) and a computer program (Molecular Analyst IBM).

Anandamide and 2-arachidonylglycerol assay

The small intestines of control and croton oil-treated mice were removed and tissue specimens were immediately weighed, immersed into liquid nitrogen, then stored at –70° until analysis. Tissue was then extracted with chloroform/methanol (2:1, by volume) containing 1 nmol each of d₈-anandamide and 2-AG, synthesized as described from the corresponding deuterated fatty acids and either ethanolamine or glycerol (Bisogno *et al.*, 1997). The lipid extracts were purified by silica column chromatography and normal phase high pressure liquid chromatography (NP-HPLC), carried out as described previously (Bisogno *et al.*, 1997), and the fractions corresponding to either anandamide (retention time 26–27 min) or 2-AG (retention time 18–22 min) were

derivatized and analysed by isotope dilution gas chromatography-mass spectrometry (GC-MS) carried out in the selected monitoring mode as described in detail elsewhere (Bisogno *et al.*, 1999). Results were expressed as pmol mg⁻¹ tissue. As during tissue extraction/purification both d₈- and native 2-AG are partly transformed into the 1(3)-isomers (which are eluted from the GC column 0.5 min later), and only little arachidonic acid is present on the sn-1(3) position of (phospho)glycerides, the amounts of 2-AG reported here represent the combined mono-arachidonoyl-glycerol peaks.

Anandamide amidohydrolase activity

To measure anandamide amidohydrolase activity [¹⁴C]-anandamide (5 mCi nmol⁻¹), synthesized as described previously from [¹⁴C]-ethanolamine and arachidonic acid (Bisogno *et al.*, 1997), was used as the radioligand at a 10 μM concentration. Membrane fractions prepared from small intestine of either control or croton oil-treated mice were assayed (Bisogno *et al.*, 1999). The assay was carried out in 50 mM Tris-HCl, pH=9, at 37°C for 30 min. [¹⁴C]-ethanolamine produced from the reaction was quantified as described previously (Bisogno *et al.*, 1997), and the activity was expressed as pmol of [¹⁴C]-ethanolamine produced min mg protein⁻¹.

Drugs

Drugs used were: CP 55,940 mesylate (Tocris Cookson, Bristol, U.K.), hexamethonium bromide and cannabinalol (SIGMA, Milan, Italy). SR141716A [(N-piperidin-1-yl)-5-(4-chlorophenyl)-1-2,4-dichlorophenyl]-4-methyl-1H-pyrazole-3-carboxamide hydrochloride and SR144528 (N-[1S-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide) were a gift from Dr Madaleine Mossè and Dr Francis Barth (SANOFI-Recherche, Montpellier, France). Cannabinoid drugs were dissolved in dimethyl sulphoxide (DMSO), while hexamethonium was dissolved in saline.

Statistics

Data are mean ± s.e.mean. To determine statistical significance, Student's *t*-test for unpaired data or one-way analysis of variance followed by Tukey-Kramer multiple comparisons test was used. A *P*-value less than 0.05 was considered significant. ED₅₀ (dose which produced a 50% variation of gastrointestinal transit) values were calculated using the computer program of Tallarida & Murray, 1986.

Results

Effect of cannabinoid agonists

Administration of croton oil produced a significant increase of upper gastrointestinal transit (per cent transit: control 46 ± 4; croton oil 66 ± 4, *n* = 12, *P* < 0.01). Both CP 55,940 and cannabinalol administration (i.p.) produced a dose-related inhibition of transit (Figure 1) and both agonists had a lower ED₅₀ value compared to the corresponding treatment (i.p.) in control mice (Table 1). CP 55,940 and cannabinalol were approximately equipotent in slowing gastrointestinal transit

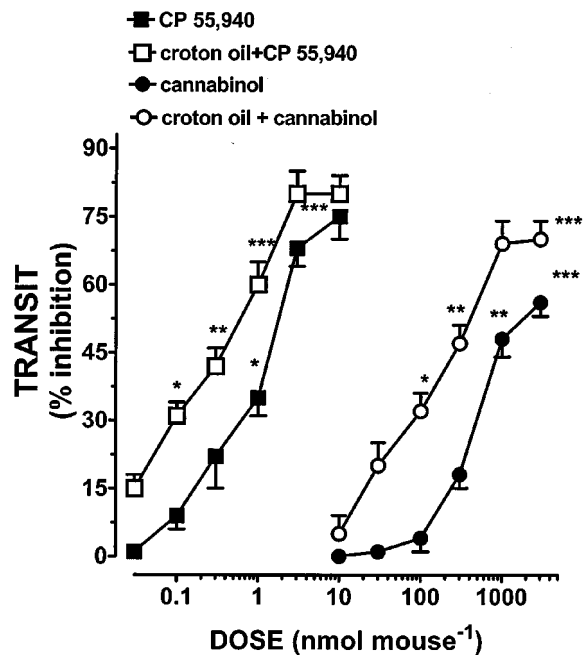


Figure 1 Dose related inhibition of upper gastrointestinal transit by CP 55,940 and cannabinalol (both given i.p.) in control mice or mice receiving croton oil. Each point represents the mean ± s.e.mean of 11–13 animals for each experimental group. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 vs corresponding control.

after both i.p. and i.c.v. routes of administration in croton oil-treated mice (Figure 2). The EC₅₀ values of i.c.v.-administered CP 55,940 and cannabinalol in croton oil-treated mice were 2.35 ± 0.31 nmol mouse⁻¹ and 1360 ± 154 nmol mouse⁻¹ respectively and were not statistically different from the corresponding i.p. treatment.

The cannabinoid CB₁ receptor antagonist SR141716A (16 nmol mouse⁻¹, i.p.), but not the cannabinoid CB₂ receptor antagonist SR144528 (52 nmol mouse⁻¹, i.p.) counteracted the inhibitory effect of CP 55,940 (0.3 nmol mouse⁻¹, i.p.) and cannabinalol (300 nmol mouse⁻¹, i.p.) in croton oil-treated mice (Figure 3). Hexamethonium (69 nmol mouse⁻¹, i.p.) did not modify significantly the effect of either (i.p.-injected) CP 55,940 (0.3 nmol mouse⁻¹, i.p.) or cannabinalol (300 nmol mouse⁻¹, i.p.) in croton oil-treated mice (Figure 3). Hexamethonium (69 nmol mouse⁻¹, i.p.), *per se*, did not significantly modify gastrointestinal transit in croton oil-treated mice (12 ± 6% increase, *n* = 10). DMSO (4 μl mouse⁻¹, i.c.v. or 4–8 μl mouse⁻¹, i.p.) had no effect on the response under study either in control or in croton oil-treated mice (data not shown).

Effect of the cannabinoid CB₁ receptor antagonist SR141716A alone

In absence of any drugs, the cannabinoid CB₁ receptor antagonist SR141716A (i.p.) increased gastrointestinal transit both in control and in croton oil-treated mice with the same potency (Figure 4 and Table 1). SR141716A (i.c.v.) also increased gastrointestinal transit, but only at doses which were also active when injected i.p. (data not shown). Hexamethonium (69 nmol mouse⁻¹, i.p.) did not modify significantly the inhibitory effect of SR141716A (100 nmol

Table 1 ED₅₀ (nmol mouse⁻¹ ± s.e.mean) of cannabinoid drugs after i.p. administration in control mice and in mice with chronic inflammation (croton oil-treated mice)

Drug	Control	Croton oil-treated mice
CP 55,940	4.75 ± 0.38	2.15 ± 0.32**
Cannabinol	2280 ± 187	1295 ± 112**
SR14176A	280 ± 20	266 ± 24

Data are means ± s.e.mean of 9–13 animals, ***P* < 0.01 vs control mice.

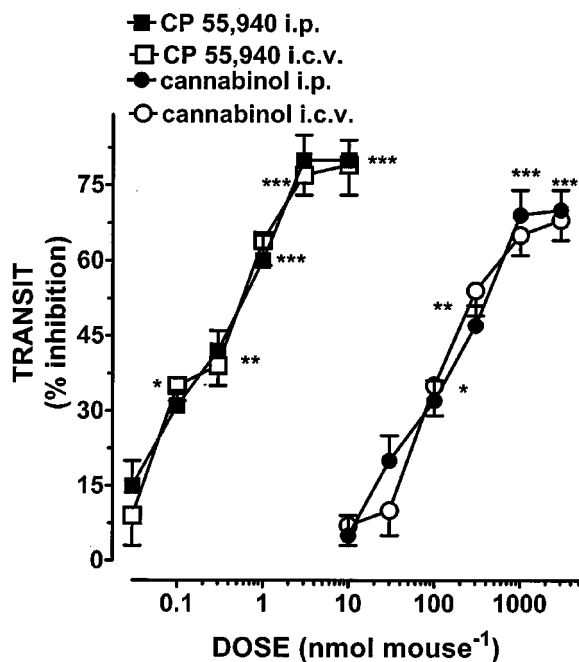


Figure 2 Effect of CP 55,940 and cannabinal on upper gastrointestinal transit after i.p. or i.c.v. administration in mice treated with croton oil. Results are mean ± s.e.mean of 8–11 animals for each experimental group. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 vs corresponding control.

mouse⁻¹ i.p., data not shown) in croton oil-treated mice. SR144528 (52 nmol mouse⁻¹, i.p.) did not modify significantly intestinal motility either in control (per cent transit: control 46 ± 6%; SR144528 48 ± 5, *n* = 6 for each experimental group) or croton oil-treated mice (per cent transit: control 65 ± 4, SR144528 66 ± 5, *n* = 6 for each experimental group).

Western blot analysis

Densitometric analysis of immunoreactive bands showed a significant (*P* < 0.01) increase of CB₁ expression in the inflamed jejunum compared to control tissues (Figure 5).

Endocannabinoid and amidohydrolase activity

Table 2 shows that anandamide and 2-AG were detected in control tissues and that this production was not significantly

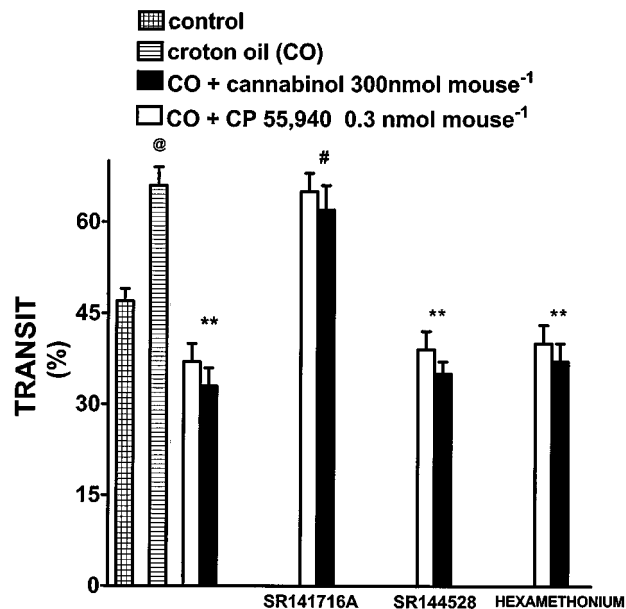


Figure 3 Croton oil-treated mice: effect of CP 55,940 (0.3 nmol mouse⁻¹, i.p.) and cannabinal (300 nmol mouse⁻¹, i.p.) on upper gastrointestinal transit alone or in mice treated with SR14176A (16 nmol mouse⁻¹, i.p.) or SR144528 (52 nmol mouse⁻¹, i.p.) or hexamethonium (69 nmol mouse⁻¹, i.p.). Results are mean ± s.e.mean of 10–11 animals for each experimental group @*P* < 0.01 vs control ***P* < 0.01 vs croton oil and #*P* < 0.01 vs CP 55,940 (or cannabinal).

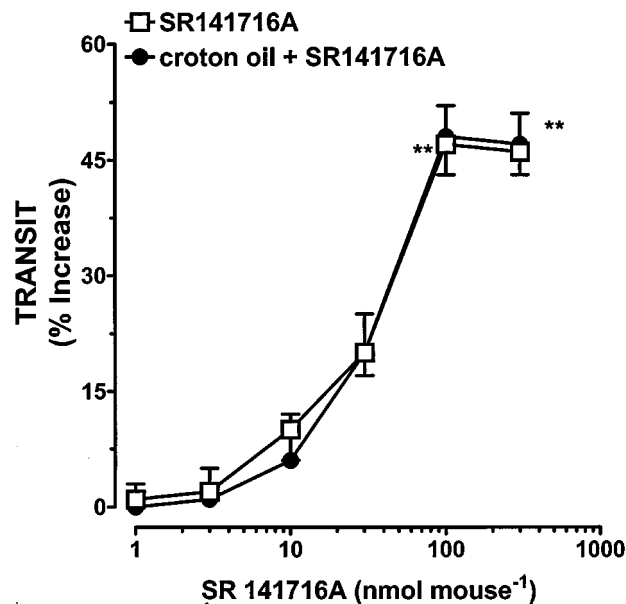


Figure 4 Dose-related increase of upper gastrointestinal transit by SR14176A (i.p.) in control mice or mice treated with croton oil. Results are mean ± s.e.mean of 8–10 animals for each experimental group. ***P* < 0.01 vs corresponding control.

modified in the small intestine of croton oil-treated mice. By contrast, a significant increase in anandamide amidohydrolase activity was observed in croton oil-treated mice.

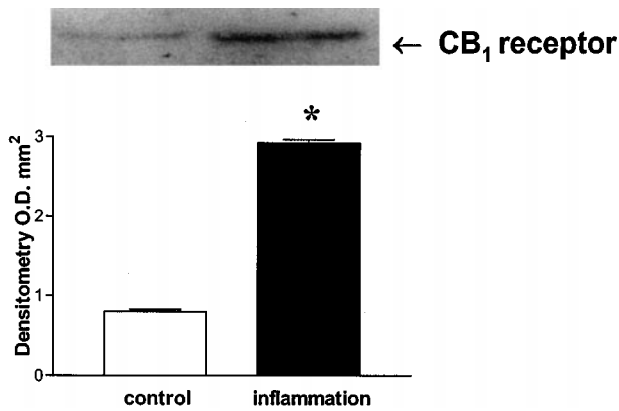


Figure 5 Western blot analysis, carried out with an antibody against CB₁, of proteins of the jejunum of control and croton oil-treated mice. The Figure is representative of three separate experiments.

Table 2 Levels of anandamide, 2-arachidonylglycerol and anandamide amidohydrolase activity in the mouse small intestine during control and croton oil-induced gut inflammation conditions

	Control	Croton oil
Anandamide (pmol g tissue ⁻¹)	36.4 ± 6.1	29.2 ± 1.6
2-Arachidonylglycerol (nmol g tissue ⁻¹)	44.1 ± 4.0	35.6 ± 2.5
Anandamide amidohydrolase (pmol mg protein ⁻¹ min ⁻¹)	0.400 ± 0.043	0.833 ± 0.148*

Results are means ± s.e. mean of 6–9 animals. **P* < 0.05 vs control. On average 1 g tissue yields 50–100 mg protein.

Discussion

We have shown that the synthetic cannabinoid agonist CP 55,940 and the natural cannabinoid receptor agonist cannabidiol reduce upper gastrointestinal motility both in control and in croton oil-treated mice. However, the anti-transit effects of both agonists were augmented during intestinal inflammation. The relative potencies of CP 55,940 and cannabidiol reflected their affinity for CB₁ receptors (Pertwee, 1999b). More importantly, the inhibitory effects of the two cannabinoid agonists were counteracted by SR141716A, a cannabinoid CB₁ receptor antagonist, but not by SR144528, a cannabinoid CB₂ receptor antagonist, thus indicating an involvement of cannabinoid CB₁, but not cannabinoid CB₂ receptors. The present results are consistent with previous studies carried out in control animals, in which it has been shown that cannabinoid agonists reduce intestinal motility by activating cannabinoid CB₁ receptors, either *in vitro* (Izzo *et al.*, 1998; 2000c; Pertwee *et al.*, 1996; Croci *et al.*, 1998; Coutts & Pertwee, 1997; Heinemann *et al.*, 1999) or *in vivo* (Krowicki *et al.*, 1999; Izzo *et al.*, 1999a, c; 2000d; Calignano *et al.*, 1997; Colombo *et al.*, 1998). In addition, acute diarrhoea enhances the potency of cannabinoid agonists in reducing intestinal motility (Izzo *et al.*, 2000d).

The central nervous system has a significant role in the initiation and co-ordination of gastrointestinal motor activity

(Krowicki & Hornby, 1995); thus, we were interested in the extent to which the inhibitory effect of cannabinoid agonists is mediated *via* a central or peripheral site of action. This is because the cannabinoid CB₁ receptor is located within both the central (Matsuda *et al.*, 1990; Izzo *et al.*, 2000a; Pertwee, 1999a) and the enteric nervous system (Griffin *et al.*, 1997; Izzo *et al.*, 2000b). Cannabinoid agonists are lipophilic molecules which can easily cross blood brain barrier (Petitet *et al.*, 1999). In a previous work, carried out in control mice, we have shown that cannabinoid agonists were significantly more active in reducing motility in mice when administered *i.c.v.* than when administered *i.p.* The higher central-to-peripheral potency of cannabinoid agonists suggested a central site of action (Izzo *et al.*, 2000d). However, central CB₁ receptors probably contribute little to the effects of peripherally administered cannabinoid as the effect of *i.p.*-injected cannabinoid agonists was not modified by the ganglionic blocker hexamethonium (Izzo *et al.*, 2000d). In the present study, the anti-transit effect likely involves peripheral (enteric) CB₁ receptors since: (i) cannabinoid agonists, injected *i.c.v.* inhibited transit, but only at doses which were also active when injected *i.p.* (i.e. cannabinoid agonists were equally active after *i.p.* or *i.c.v.* administration) and (ii) the ganglion blocker hexamethonium, at a dose previously shown to antagonize the effect of *i.c.v.*-injected cannabinoid drugs (Izzo *et al.*, 2000d), did not alter the inhibitory effect of *i.p.*-injected cannabinoids. However, we cannot definitely rule out the possibility that also central CB₁ receptors may have a role in the control of intestinal motility.

Cannabinoid CB₁ receptors are located on peripheral neurones, including the myenteric plexus (Griffin *et al.*, 1997; Izzo *et al.*, 2000b; Kulkarni-Narla & Brown, 2000). The myenteric plexus of the guinea-pig small intestine contains CB₁, but not CB₂, cannabinoid receptor mRNA (Griffin *et al.*, 1997). In the porcine gut, cannabinoid CB₁ receptors have been immunohistochemically localized on enteric cholinergic neurones (Kulkarni-Narla & Brown, 2000). Using Western blot analysis, we have detected the presence of CB₁ receptors in the mouse jejunum and its increased expression in the inflamed gut. This is, to the best of our knowledge, the first example of up-regulation of CB₁ cannabinoid receptors during an inflammatory state. This up-regulation explains the increased potency of exogenous cannabinoid agonists during inflammation. Others have shown that croton oil-induced inflammation increases the effect of μ -opioid receptor agonists, suggesting a sensitization of peripheral μ -opioid receptors (Puig & Pol, 1998; Valle *et al.*, 2000).

There is evidence in the literature that intestinal motility can be tonically inhibited by the endogenous cannabinoid system (Izzo *et al.*, 2000b). Indeed SR141716A increased electrically-induced contractions in the isolated guinea-pig ileum (Izzo *et al.*, 1998; Pertwee *et al.*, 1996) as well as intestinal motility and defecation in rodents (Colombo *et al.*, 1998; Izzo *et al.*, 1999a, c; Izzo *et al.*, 2000d). The observation that SR141716A, *per se*, increased intestinal motility does not necessarily imply that endogenous cannabinoid agonists are involved in the control of intestinal motility as SR141716A behaves as inverse agonist at the human CB₁ and CB₂ cannabinoid receptors (Landsman *et al.*, 1997; MacLennan *et al.*, 1998). In view of the increased CB₁ expression in the inflamed small intestine observed in the present study, one may have expected that, if SR141716A behaved as an inverse

agonist in this context, this compound would have exerted a higher enhancement of intestinal transit in croton oil-treated than in vehicle-treated mice. However, this hypothesis was not confirmed in the present study, in which we have shown that SR141716A increased motility in control and inflamed mice with exactly the same potency. This finding would suggest, on the contrary, that SR141716A exerts an antagonistic action on a possible tonic anti-transit effect of endocannabinoids, and that, during inflammation, increased expression of CB₁ receptors does not result in an increased tonic activity of these receptors, possibly due to the lack of increased concentrations of endogenous ligands.

In order to investigate the mechanism of action of SR141716A we measured the levels and the metabolism of endogenous cannabinoids in normal and inflamed small intestine under the same conditions used to study the effect of the cannabinoid receptor agonists and the expression of CB₁ receptors. Anandamide amidohydrolase is responsible for the hydrolysis of anandamide amide bond, and was shown to recognize as substrates also palmitoylethanolamide (a saturated acylethanolamide co-released with anandamide) and 2-AG (Mechoulam *et al.*, 1998). The presence of this enzyme in the rat small intestine has been previously demonstrated and it was shown that, due to the presence of endogenous inhibitors, low activity, similar to those found here in mice, is found in the intestine under normal conditions (Katayama *et al.*, 1997). Nevertheless, this activity would still ensure the hydrolysis of 40 pmol of anandamide per min and per g of tissue, which reflects the amounts of anandamide that we found in the tissue (Table 2). In fact, while Mechoulam *et al.* (1995) detected 2-AG, but not anandamide, in the canine small intestine, in the present study we have revealed, in mice, amounts of anandamide and, particularly, 2-AG compatible with a tonic activation of CB₁ receptors. In fact, these amounts are about three (for anandamide) or 10 (for 2-AG) times higher than those measured in the rat brain (Sugiura *et al.*, 1995), and are very likely to yield tissue concentrations similar to or higher than the K_i values for the activation of CB₁ cannabinoid receptors (Devane *et al.*, 1992; Facci *et al.*, 1995; Calignano *et al.*, 1998). Anandamide is known to reduce intestinal motility in mice *via* activation of CB₁ receptors (Fride, 1995; Calignano *et al.*, 1997; Izzo *et al.*, 2001a) and thus, the displacement from these receptors of the endocannabinoid by SR141716A could explain, at least in part, the prokinetic action of the antagonist in the small intestine (see above). A relatively low basal rate of endocannabinoid inactivation in normal intestine might explain these high levels. In fact, as mentioned above, anandamide amidohydrolase activity in the intestine of rodents is relatively low under normal conditions (Katayama *et al.*, 1997) [and our present data], whereas Calignano *et al.* (1997) previously suggested the lack, in this tissue, of a functional transport mechanism limiting anandamide action.

In the present study 2-AG levels in the mouse small intestine were more than 1000 fold higher than those of anandamide. There are no data in the literature concerning the pharmacological effects of 2-AG on intestinal motility. However, in the vas deferens assay, which, like the model used here, involves peripheral CB₁ receptors, 2-AG was about 100 times less potent than anandamide as a depressant of electrically-induced contractions (Mechoulam *et al.*, 1995).

Thus, collectively, these results tend to suggest a physiological role for both anandamide and 2-AG as physiological modulators of intestinal motility, and indicate that counteraction of their action at CB₁ receptors underlies at least in part the effect of SR141716A on intestinal motility in normal small intestine.

Beaulieu *et al.* (2000) have recently observed that the rat paw skin concentration of anandamide and 2-AG did not differ between control and inflamed tissues. Similarly, we did not find significant difference between inflamed and non-inflamed intestinal tissues. However, the fact that we could not observe an increase in endogenous cannabinoid levels could be due to up-regulation of anandamide amidohydrolase, since we have showed here that the activity of this enzyme markedly increased in the inflamed intestine, whereas no change in the activity of this enzyme was observed in rat skin after formalin injection (Beaulieu *et al.*, 2000). At any rate, the finding of no change in endocannabinoid levels following croton oil treatment may suggest that, despite the over-expression of CB₁ receptors under these conditions, the overall anti-transit cannabinoid tone in the small intestine does not increase during inflammation, in agreement with the observation that SR141716A exerts the same effects in normal as well as inflamed small intestine and *via* counteraction of endocannabinoid actions rather than as an inverse agonist.

In conclusion, some major issues emerged from this study: we have shown that inflammation enhances the potency of cannabinoid receptor agonists probably by up-regulating CB₁ expression in the small intestine, thus raising the possibility of a clinical use of selective, non-psychotropic cannabinoid CB₁ receptor agonists for the treatment of the motility disorders associated with intestinal inflammation. The presence of high amounts of endogenous cannabinoids in the small intestine strongly suggests a physiological role in the regulation of intestinal motility. The increased activity of the enzyme anandamide amidohydrolase, in consideration of the lack of decrease of endocannabinoids in the inflamed small intestine of croton oil-treated mice, suggests a more rapid turnover (biosynthesis plus degradation) of endogenous cannabinoids in the inflamed gut. These substances might be produced in order to better counteract the effects of inflammation in the gut by activating the up-regulated CB₁ receptors in this tissue, but would be immediately inactivated by an increased amidohydrolase activity. We also found that the CB₁ receptor antagonist SR141716A stimulates intestinal transit to the same extent in control and inflamed small intestine. This finding: (i) argues against a direct, inverse agonist effect of this compound on CB₁ receptors (which being over-expressed in inflamed tissue should have led to a higher effect of the antagonist under these conditions), and (ii) supports the existence of an endocannabinoid tone that counteracts intestinal motility during both physiological and pathological states.

This work was supported by *Cofinanziamento Murst* and Enrico and Enrica Sovena Foundation (Roma). SR141716A and SR144528 were a kind gift from SANOFI (Montpellier, France).

References

- BEAULIEU, P., BISOGNO, T., PUNWAR, S., FARQUHAR-SMITH, P., AMBROSINO, G., DI MARZO, V. & RICE, A.S.C. (2000). Role of the endogenous cannabinoid system in the formalin test of persistent pain in the rat. *Eur. J. Pharmacol.*, **396**, 85–92.
- BISOGNO, T., BERRENDERO, F., AMBROSINO, G., CEBEIRA, M., RAMOS, J.A., FERNANDEZ-RUIZ, J.J. & DI MARZO, V. (1999). Brain regional distribution of endocannabinoids: implications for their biosynthesis and biological function. *Biochem. Biophys. Res. Commun.*, **256**, 377–380.
- BISOGNO, T., MAURELLI, S., MELCK, D., DE PETROCELLI, L. & DI MARZO, V. (1997). Biosynthesis, uptake, and degradation of anandamide and palmitoylethanolamide in leukocytes. *J. Biol. Chem.*, **272**, 3315–3323.
- CALIGNANO, A., LA RANA, G., GIUFFRIDA, A. & PIOMELLI, D. (1998). Control of pain initiation by endogenous cannabinoids. *Nature*, **394**, 277–281.
- CALIGNANO, A., LA RANA, G., MAKRYANNIS, A., LIN, S.Y., BELTRAMO, M. & PIOMELLI, D. (1997). Inhibition of intestinal motility by anandamide, an endogenous cannabinoid. *Eur. J. Pharmacol.*, **340**, R7–R8.
- COLOMBO, G.R., AGABIO, C., LOBINA, R., REALI, R. & GESSA, G.L. (1998). Cannabinoid modulation of intestinal propulsion in mice. *Eur. J. Pharmacol.*, **344**, 67–69.
- COUTTS, A.A. & PERTWEE, R.G. (1997). Inhibition by cannabinoid receptor agonists of acetylcholine release from the guinea-pig myenteric plexus. *Br. J. Pharmacol.*, **121**, 1557–1566.
- CROCI, T., MANARA, L., AUREGGI, G., GUAGNIN, F., RINALDI-CARMONA, M., MAFFRAND, J.P., LE FUR, G., MUKENGE, S. & FERLA, G. (1998). *In vitro* functional evidence of neuronal cannabinoid CB₁ receptors in human ileum. *Br. J. Pharmacol.*, **125**, 1393–1396.
- D'AMBRA, T.E., ESTEP, K.G., BELL, M.R., EISSENSTAT, M.A., JOSEF, K.A., WARD, S.J., HAYCOCK, D.A., BAIZMAN, E.R., CASIANO, F.M. & BEGLIN, N.C. (1992). Conformationally restrained analogues of pravadoline: nanomolar potent, enantioselective, (aminoalkyl)indole agonists of the cannabinoid receptor. *J. Med. Chem.*, **35**, 124–135.
- DEVANE, W.A., HANUS, L., BREUER, A., PERTWEE, R.G., STEVENSON, L.A., GRIFFIN, G., GIBSON, D., MANDELBAUM, A., ETINGER, A. & MECHOULAM, R. (1992). Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science*, **258**, 1946–1949.
- DEWEY, W.L. (1986). Cannabinoid Pharmacology. *Pharmacol. Rev.*, **38**, 151–178.
- DI MARZO, V., BISOGNO, T. & DE PETROCELLI, L. (2000). Endocannabinoids: new targets for drug development. *Curr. Pharm. Design.*, **6**, 1361–1380.
- FACCI, L., DAL TOSO, R., ROMANELLO, S., BURIANI, A., SKAPER, S.D. & LEON, A. (1995). Mast cells express a peripheral cannabinoid receptor with differential sensitivity to anandamide and palmitoylethanolamide. *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 3376–3380.
- FRIDE, E. (1995). Anandamides: tolerance and cross-tolerance to Δ^9 -tetrahydrocannabinol. *Brain Res.*, **697**, 83–90.
- GRIFFIN, G., FERNANDO, S.R., ROSS, R.A., MCKAY, N.G., ASHFORD, M.L.J., SHIRE, D., HUFFMAN, J.W., YU, S., LANTON, J.A.H. & PERTWEE, R.G. (1997). Evidence for the presence of CB₂-like cannabinoid receptors on peripheral nerve terminals. *Eur. J. Pharmacol.*, **339**, 53–61.
- HALEY, T.J. & McCROMICK, W.G. (1957). Pharmacological effects produced by intracerebral injection of drugs in the conscious mouse. *Br. J. Pharmacol.*, **12**, 12–15.
- HEINEMANN, A., SHAHBAZIAN, A. & HOLZER, P. (1999). Cannabinoid inhibition of guineapig intestinal peristalsis via inhibition of excitatory and activation of inhibitory neural pathways. *Neuropharmacology*, **38**, 1289–1297.
- IZZO, A.A., CAPASSO, R., PINTO, L., DI CARLO, G., MASCOLO, N. & CAPASSO, F. (2001a). Effect of vanilloid drugs on gastrointestinal transit in mice. *Br. J. Pharmacol.*, **133**, 44P (Abstract).
- IZZO, A.A., CAPASSO, R., PINTO, L., IUVONE, T., ESPOSITO, G., MASCOLO, N. & CAPASSO, F. (2001b). Effect of cannabinoid agonists on intestinal motility in a chronic model of intestinal inflammation. *Br. J. Pharmacol.*, **133**, 44P (Abstract).
- IZZO, A.A., MASCOLO, N., BORRELLI, F. & CAPASSO, F. (1998). Excitatory transmission to the circular muscle of the guinea-pig ileum: evidence for the involvement of cannabinoid CB₁ receptor. *Br. J. Pharmacol.*, **124**, 1363–1368.
- IZZO, A.A., MASCOLO, N., BORRELLI, F. & CAPASSO, F. (1999a). Defaecation, intestinal fluid accumulation and motility in rodents: implications of cannabinoid CB₁ receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **359**, 65–70.
- IZZO, A.A., MASCOLO, N. & CAPASSO, F. (2000b). Forgotten target for Marijuana: the endocannabinoid system in the gut. *Trends. Pharmacol. Sci.*, **21**, 372.
- IZZO, A.A., MASCOLO, N. & CAPASSO, F. (2000a). Marijuana in the new millennium: perspectives for cannabinoid research. *Trends. Pharmacol. Sci.*, **21**, 81–82.
- IZZO, A.A., MASCOLO, N., CAPASSO, R., GERMANÒ, M.P., DE PASQUALE, R. & CAPASSO, F. (1999b). Inhibitory effect of cannabinoid agonists on gastric emptying in the rat. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **360**, 221–223.
- IZZO, A.A., MASCOLO, N., PINTO, L., CAPASSO, R. & CAPASSO, F. (1999c). The role of cannabinoid receptors in intestinal motility, defaecation and diarrhoea in rats. *Eur. J. Pharmacol.*, **384**, 37–42.
- IZZO, A.A., MASCOLO, N., TONINI, M. & CAPASSO, F. (2000c). Modulation of peristalsis by cannabinoid CB₁ ligands in the isolated guinea pig ileum. *Br. J. Pharmacol.*, **129**, 984–990.
- IZZO, A.A., PINTO, L., BORRELLI, F., CAPASSO, R., MASCOLO, N. & CAPASSO, F. (2000d). Central and peripheral cannabinoid modulation of gastrointestinal transit in physiological states or during the diarrhoea induced by croton oil. *Br. J. Pharmacol.*, **129**, 1627–1632.
- KATAYAMA, K., UEDA, N., KURAHASHI, Y., SUZUKU, H., YAMAMOTO, S. & KATO, I. (1997). Distribution of anandamide amidohydrolase in rat tissues with special reference to small intestine. *Biochim. Biophys. Acta.*, **1347**, 212–218.
- KROWICKI, Z.K. & HORNBY, P.J. (1995). Hindbrain neuroactive substances controlling gastrointestinal functions. In *Regulatory mechanisms in gastrointestinal function*. ed. Gaginella T.S. pp. 277–319. New York: CRC Press.
- KROWICHI, Z.K., MOERSCHBAECHER, J.M., WINSAUER, P.J., DIVAGALLI, S.V. & HORNBY, P.J. (1999). Δ^9 -tetrahydrocannabinol inhibits gastric motility in the rat through cannabinoid CB₁ receptors. *Eur. J. Pharmacol.*, **371**, 187–196.
- KULKARNI-NARLA, A. & BROWN, D.R. (2000). Localization of CB₁-cannabinoid receptor immunoreactivity in the porcine enteric nervous system. *Cell. Tissue Res.*, **302**, 73–80.
- LANDSMAN, R.S., BURKEY, T.H., CONSROE, P. & ROESKE, W.R. (1997). SR14171A is an inverse agonist at the human cannabinoid CB₁ receptors. *Eur. J. Pharmacol.*, **334**, R1–R2.
- MACLENNAN, S.J., REYNEN, P.H. & BONHAUS, D.W. (1998). Evidence for inverse agonism of SR141716A at human recombinant cannabinoid CB₁ and CB₂ receptors. *Br. J. Pharmacol.*, **24**, 619–622.
- MATSUDA, L.A., LOLAIT, S.J., BROWNSTEIN, B.J., YOUNG, A.C. & BONNER, T.L. (1990). Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature*, **346**, 561–564.
- MECHOULAM, R., BEN-SHABAT, S., HANUS, L., LIGUMSKY, M., KAMINSKI, N.E., SCHATZ, A.R., GOPHER, A., ALMOG, S., MARTIN, B.R., COMPTON, D.R., PERTWEE, R.G., GRIFFIN, G., BAYEWITCH, M., BARG, J. & VOGEL, Z. (1995). Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to a cannabinoid receptor. *Biochem. Pharmacol.*, **50**, 83–90.
- MECHOULAM, R., FRIDE, E. & DI MARZO, V. (1998). Endocannabinoids. *Eur. J. Pharmacol.*, **359**, 1–18.
- MELEK, D., DE PETROCELLI, L., ORLANDO, P., BISOGNO, T., LAEZZA, C., BIFULCO, M. & DI MARZO, V. (2000). Suppression of nerve growth factor Trk receptors and prolactin receptors by endocannabinoids leads to inhibition of human breast and prostate cancer cell proliferation. *Endocrinology*, **141**, 118–126.
- MUNRO, S., THOMAS, K.L. & SHAAR, M. (1990). Molecular characterization of a peripheral receptor for cannabinoids. *Nature*, **365**, 61–65.

- PERTWEE, R.G. (1999a). Evidence for the presence of CB₁ cannabinoid receptors on peripheral neurones and for the existence of neuronal non-CB₁ cannabinoid receptors. *Life Sci.*, **65**, 597–605.
- PERTWEE, R.G. (1999b). Pharmacology of cannabinoid receptor ligands. *Curr. Med. Chem.*, **6**, 635–664.
- PERTWEE, R.G., FERNANDO, S.R., NASH, J.E. & COUTTS, A.A. (1996). Further evidence for the presence of cannabinoid CB₁ receptors in guinea-pig small intestine. *Br. J. Pharmacol.*, **118**, 2199–2205.
- PETITET, F., JEANTAUD, B., BERTRAND, P. & IMPERATO, A. (1999). Cannabinoid penetration into mouse brain as determined by ex vivo binding. *Eur. J. Pharmacol.*, **374**, 417–721.
- PETITET, F., JEANTAUD, B., REIBAUD, M., IMPERATO, A. & DUBROEUCQ, M.C. (1998). Complex pharmacology of natural cannabinoids: evidence for partial agonist activity of Δ⁹-tetrahydrocannabinol and antagonist activity of cannabidiol on rat brain cannabinoid receptors. *Life Sci.*, **63**, PL1–PL6.
- PUIG, M.M. & POL, O. (1998). Peripheral effects of opioids in a model of chronic intestinal inflammation in mice. *J. Pharmacol. Exp. Ther.*, **287**, 1068–1075.
- RINALDI-CARMONA, M., BARTH, F., HEAULME, M., ALFONSO, R., SHIRE, D., CONGY, C., SOBRIÈ, P., BRELIÈRE, J.C. & LE FUR, G. (1995). Biochemical and pharmacological characterization of SR141716A, the first potent and selective brain cannabinoid receptor antagonist. *Life Sci.*, **56**, 1941–1947.
- RINALDI-CARMONA, M., BARTH, F., MILLAN, J., DEROCQ, J.M., CASELLAS, P., CONGY, C., SOBRIÈ, P., BRELIÈRE, J.C. & LE FUR, G. (1998). SR144528, the first potent and selective antagonist of the CB₂ cannabinoid receptors. *J. Pharmacol. Exp. Ther.*, **284**, 644–650.
- SHOOK, J.E. & BURKS, T.F. (1989). Psychoactive cannabinoids reduce gastrointestinal propulsion and motility in rodents. *J. Pharmacol. Exp. Ther.*, **249**, 444–449.
- SUGIURA, T., KONDO, S., SUKAGAWA, A., NAKANE, S., SHINODA, A., ITOH, K., YAMASHITA, A. & WAKU, K. (1995). 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. *Biochem. Biophys. Res. Commun.*, **215**, 89–97.
- TALLARIDA, R.J. & MURRAY, R.B. (1986). *Manual of Pharmacological Calculations with computer programs*. New York: Springer-Verlag.
- VALLE, L., PUIG, M.M. & POL, O. (2000). Effects of mu-opioid receptor agonists on intestinal secretion and permeability during acute intestinal inflammation in mice. *Eur. J. Pharmacol.*, **389**, 235–242.

(Received May 1, 2001
Revised July 5, 2001
Accepted July 18, 2001)