British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2012.01879.x
www.brjpharmacol.org

RESEARCH PAPER

Inhibitory effect of cannabichromene, a major non-psychotropic cannabinoid extracted from *Cannabis sativa*, on inflammation-induced hypermotility in mice

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Keywords
2-arachydonoylglycerol; anandamide; cannabichromene; cannabinoids; cannabinoid receptors; gastrointestinal transit; ileum; intestinal motility; transient receptor potential (TRP) channels; TRPA1

Received
13 May 2011
Revised
20 December 2011
Accepted
14 January 2012

BACKGROUND AND PURPOSE
Cannabichromene (CBC) is a major non-psychotropic phytocannabinoid that inhibits endocannabinoid inactivation and activates the transient receptor potential ankyrin-1 (TRPA1). Both endocannabinoids and TRPA1 may modulate gastrointestinal motility. Here, we investigated the effect of CBC on mouse intestinal motility in physiological and pathological states.

EXPERIMENTAL APPROACH
Inflammation was induced in the mouse small intestine by croton oil. Endocannabinoid (anandamide and 2-arachidonoyl glycerol), palmitoylethanolamide and oleoylethanolamide levels were measured by liquid chromatography-mass spectrometry; TRPA1 and cannabinoid receptors were analysed by quantitative RT-PCR; upper gastrointestinal transit, colonic propulsion and whole gut transit were evaluated *in vivo*; contractility was evaluated *in vitro* by stimulating the isolated ileum, in an organ bath, with ACh or electrical field stimulation (EFS).

KEY RESULTS
Croton oil administration was associated with decreased levels of anandamide (but not 2-arachidonoyl glycerol) and palmitoylethanolamide, up-regulation of TRPA1 and CB_1 receptors and down-regulation of CB_2 receptors. *Ex vivo* CBC did not change endocannabinoid levels, but it altered the mRNA expression of TRPA1 and cannabinoid receptors. *In vivo*, CBC did not affect motility in control mice, but normalized croton oil-induced hypermotility. *In vitro*, CBC reduced preferentially EFS- versus ACh-induced contractions. Both *in vitro* and *in vivo*, the inhibitory effect of CBC was not modified by cannabinoid or TRPA1 receptor antagonists.

CONCLUSION AND IMPLICATIONS
CBC selectively reduces inflammation-induced hypermotility *in vivo* in a manner that is not dependent on cannabinoid receptors or TRPA1.

Abbreviations

2-AG, 2-arachydonoylglycerol; AP18, 4-(4-chlorophenyl)-3-methyl-3-buten-2-one oxime; CBC, cannabichromene; CPA, cyclopiazonic acid; DMSO, dimethyl sulfoxide; EFS, electrical field stimulation; EMT, endocannabinoid membrane transporter; GC, geometric centre; GDE1, glycerophosphodiester PDE 1; HC-030031, 2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7*H*-purin-7-yl)-*N*-(4-isopropylphenyl)acetamide; NAPE-PLD, N-acyl-phosphatidylethanolamine-selective phospholipase D, OEA, oleoylethanolamide; PEA, palmitoylethanolamide; 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; TRP, transient receptor potential; TRPA1, transient receptor potential of ankyrin type-1

Introduction

Cannabichromene (CBC) is, together with Δ^9-tetrahydrocannabinol, cannabidiol and cannabinol, the most abundant naturally occurring cannabinoid (Turner *et al.*, 1980; Russo, 2011). It is particularly abundant in freshly harvested dry-type *Cannabis* material and it is the second most abundant cannabinoid in some strains of marijuana growing in the USA (Brown and Harvey, 1990). A report covering 46 211 *Cannabis* preparations confiscated in the USA during 1993–2008 period showed that CBC represented 0.7 and 0.9% of the constituents from hashish or hash oil, respectively (Mehmedic *et al.*, 2010). Despite the relative abundance of this compound in *Cannabis* preparations, very little is known about its pharmacology (Izzo *et al.*, 2009a). Early reports showed that CBC prolonged hexobarbital hypnosis in mice (Hatoum *et al.*, 1981) exerted anti-inflammatory effects and modest analgesic activity in rodents (Wirth *et al.*, 1980; Turner and Elsohly, 1981; Davis and Hatoum, 1983), while showing no '*Cannabis* like' activity in the Rheseus monkey (Mechoulam *et al.*, 1970) and in human smoking experiments (Turner *et al.*, 1980). In more recent years, it has been shown that CBC exerts antimicrobial (Appendino *et al.*, 2008), anti-inflammatory (DeLong *et al.*, 2010; Tubaro *et al.*, 2010), analgesic (Maione *et al.*, 2011) and antidepressant-like activity in rodents (El-Alfy *et al.*, 2010). Pharmacodynamic studies have shown that CBC, like other plant natural products (Gertsch *et al.*, 2010), is an inhibitor of endocannabinoid cellular reuptake (Ligresti *et al.*, 2006) and a weak inhibitor of monoacylglycerol lipase (MAGL) (De Petrocellis *et al.*, 2011), but is also a potent activator of transient receptor potential (TRP) ankyrin 1-type (TRPA1) channels (De Petrocellis *et al.*, 2008; 2011). CBC was also recently found to stimulate the descending pathway of antinociception in the ventrolateral periaqueductal grey, probably through activation of TRPA1, inhibition of endocannabinoid inactivation and subsequent elevation of local endocannabinoid levels, and possibly via potentiation of adenosine signalling (Maione *et al.*, 2011).

Both endocannabinoids and TRPA1 are known to be involved in the control of intestinal motility. In brief, endocannabinoids [i.e. anandamide and 2-arachydonoylglycerol (2-AG)], are lipid mediators synthesized 'on demand' from membrane phospholipids by the concerted action of a number of enzymes including N-acyl-phosphatidylethanolamine-selective phospholipase D (NAPE-PLD) and glycerophosphodiester PDE 1 (GDE1) (involved in anandamide biosynthesis) and diacylglycerol lipase α (DAGLα) and DAGLβ (involved in 2-AG biosynthesis). Once synthesized, endocannabinoids, activate cannabinoid CB_1 and CB_2 receptors to elicit a biological response, after which they are inactivated through re-uptake (facilitated by the putative endocannabinoid membrane transporter (EMT)] and enzymatic degradation [anandamide is inactivated by fatty acid amide hydrolase (FAAH), and 2-AG mostly by MAGL] (Di Marzo, 2008; Pertwee, 2009). Cannabinoids, via enteric CB_1 receptor activation in physiological states and via CB_2 receptor activation in the inflamed gut, reduce excitatory enteric transmission *in vitro* and gastrointestinal motility *in vivo* (Izzo and Coutts, 2005; Sanger, 2007; Wright *et al.*, 2008; Izzo and Sharkey, 2010; Schicho and Storr, 2010). TRPA1, a member of the TRP family, is expressed by visceral (vagal, splanchnic and pelvic) afferents (Brierley *et al.*, 2009; Kondo *et al.*, 2009; Cattaruzza *et al.*, 2010; Yu *et al.*, 2010; Boesmans *et al.*, 2011; Holzer, 2011) and by cells of the intestinal mucosal (Purhonen *et al.*, 2008). TRPA1 agonists have been shown to evoke contractions of the guinea-pig isolated ileum and mouse colon (Penuelas *et al.*, 2007; Nozawa *et al.*, 2009) and to affect motility *in vivo* (Doihara *et al.*, 2009a,b).

In the present study we have evaluated the effect of CBC on intestinal motility in mice. CBC was evaluated on upper gastrointestinal transit (both in physiological and inflammatory conditions), colonic propulsion and whole gut transit *in vivo*. *In vitro*, we evaluated the effect of CBC on electrically or ACh-induced contractions in the ileum. A preliminary account of this work has been communicated to the 20th Annual Symposium of the International Cannabinoid Research Society (Romano *et al.*, 2010).

Methods

Animals

Male ICR mice (Harlan Laboratories, S. Pietro al Natisone, Italy) weighing 20–25 g were used after a 1 week acclimatization period (temperature 23 ± 2°C; humidity 60%, free access to water and standard food). All animal care and experimental procedures complied with the principles of laboratory animal care (NIH publication no.86-23, revised 1985) and the Italian D.L. no.116 of 27 January 1992 and associated guidelines in the European Communities Council Directive of 24 November 1986 (86/609/ECC).

Intestinal inflammation

Intestinal inflammation was induced as previously described (Pol and Puig, 1997; Capasso *et al.*, 2008a). Briefly, two doses of croton oil (20 μL per mouse) for two consecutive days were orally administered to mice and four days after the first administration of croton oil, upper gastrointestinal transit of mice was measured. This time was selected on the basis of

previous work (Pol and Puig, 1997), which reported that the maximal inflammatory response occurred 4 days after the first treatment.

Endocannabinoid extraction and measurement

The duodenum, jejunum and ileum from control and croton oil-treated mice (treated or not with CBC 15 mg·kg^{-1}, i.p., 30 min before croton oil) were removed (4 days after the first administration of croton oil), and tissue specimens were immediately weighed, immersed into liquid nitrogen, and stored at –80°C until extraction of endocannabinoids. Tissues were extracted, purified and analysed as described in detail elsewhere (Di Marzo *et al.*, 2008).

Quantitative (real-time) RT-PCR analysis

The duodenum, jejunum and ileum from control and croton oil-treated mice (treated or not with CBC 15 mg·kg^{-1}, i.p., 30 min before croton oil) were removed (4 days after the first administration of croton oil) and collected in RNA later (Invitrogen, Carlsbad, CA, USA) and homogenized by a rotor-stator homogenizer in 1.5 mL of Trizol® (Invitrogen). Total RNA was extracted according to the manufacturer's recommendations, dissolved in RNAase-free water, and further purified by spin cartridge by the Micro-to-Midi total RNA purification system (Invitrogen). Total RNA was dissolved in RNA storage solution (Ambion, Austin, TX, USA), UV-quantified by a Bio-Photometer® (Eppendorf, Santa Clara, CA, USA), and stored at –80°C until use. RNA aliquots (6 µg) were digested by RNAse-free DNAse I (Ambion DNA-free™ kit) in a 20 µL final volume reaction mixture to remove residual contaminating genomic DNA. After DNAse digestion, concentration and purity of RNA samples were evaluated by the RNA-6000-Nano® microchip assay using a 2100 Bioanalyzer® equipped with a 2100 Expert Software® (Agilent, Santa Clara, CA, USA) following the manufacturer's instructions.

For all samples tested, the RNA integrity number was greater than 8 relative to a 0–10 scale. One microgram of total RNA, as evaluated by the 2100 Bioanalyzer, was reverse-transcribed in cDNA by the SuperScript III SuperMix (Invitrogen). The reaction mixture was incubated in a termocycler iCycler-iQ5® (Bio-Rad, Hercules, CA, USA) for a 5 min at 60°C step, followed by a rapid chilling for 2 min at 4°C. The protocol was stopped at this step and the reverse transcriptase was added to the samples, except the negative controls (–RT). The incubation was resumed with two thermal steps: 10 min at 25°C followed by 40 min at 50°C. Finally, the reaction was terminated by heating at 95°C for 10 min. Quantitative real-time PCR was performed by an iCycler-iQ5® in a 20µL reaction mixture containing 1 × SsoFast EVAGreen supermix (Bio-Rad), 10 ng of cDNA (calculated on the basis of the retro-transcribed RNA) and 330 nM for each primer. The amplification profile consisted of an initial denaturation of 2 min at 94°C and 40 cycles of 30 s at 94°C, annealing for 30 s at TaOpt (optimum annealing temperature, see following discussion) and elongation for 45 s at 68°C. Fluorescence data were collected during the elongation step. A final extension of 7 min was carried out at 72°C, followed by melt-curve data analysis. Assays were performed in quadruplicate (maximum ΔCt of replicate samples <0.5), and a standard curve from consecutive fivefold dilutions (100 to 0.16 ng) of a cDNA pool representative of all samples was included for PCR efficiency determination. Optimized primers for SYBR-green analysis and optimum annealing temperatures were designed by the Allele-Id software version 7.0 (Biosoft International, Palo Alto, CA, USA) and were synthesized (HPLC-purification grade) by MWG-Biotech (NAPE-PLD accession NM_178728, F: CGCTGATGGTG GAAATGG, R: GTGGTTGTGACTGATGAGG; CB_1 accession NM_007726, F: CTACCTGATGTTCTGGAT, R: GTGTGAAT GATGATGCTT; CB_2 accession, F: ATCTCCTCTCACTCACT TATCTG, R: GGTTTCTTGCTCTCACACTTT; TRPA1 accession NM_177781, F: GGAGATATGTGTAGATTAGAAGAC, R: TCG GAGGTTTGGATTTGC; GDE1 accession NM_019580.4, F: ATAACACAGTAGATAGGACAACA, R: AGCAGCAGAAGC CATATC; FAAH accession NM_010173, F: GCCTCAAGGAAT GCTTCA, R: AGTCACTCTCCGATGTCA).

Relative expression calculation – to correct for PCR efficiency and normalized with respect to reference gene β-actin (accession: NM_007393; F: CCAGGCATTGCTGACAGG; R: TGGAAGGTGGACAGTGAGG) and HPRT (accession: NM_013556; F: TTGACACTGGTAAAACAATGC; R: GCCTG TATCCAACACTTCG) – was performed by iQ5 software. Results are expressed as fold expression, compared with control (=1) (Izzo *et al.*, 2008).

Upper gastrointestinal transit in vivo

Transit was measured by evaluating the intestinal location of rhodamine-B-labelled dextran (Izzo *et al.*, 2009b). Animals were given fluorescent-labelled dextran (100 µL of 25 mg·mL^{-1} stock solution) via a gastric tube into the stomach. At 20 min after administration, the animals were killed by asphyxiation with CO_2 and the entire small intestine with its contents was divided into 10 equal parts.

The intestinal contents of each bowel segment were vigorously mixed with 2 mL of saline solution to obtain a supernatant containing the rhodamine. The supernatant was centrifuged at 35 x *g* to precipitate the intestinal chyme. The fluorescence in duplicate aliquots of the cleared supernatant was read in a multi-well fluorescence plate reader (LS55 Luminescence spectrometer, Perkin-Elmer Instruments, Waltham, MA, USA; excitation 530 ± 5 nm and emission 590 ± 10 nm) for quantification of the fluorescent signal in each intestinal segment. From the distribution of the fluorescent marker along the intestine, we calculated the geometric centre (GC) of small intestinal transit as follows: $GC^1/_4S$ (fraction of fluorescence per segment·segment number^{-1}) GC ranged from 1 (minimal motility) to 10 (maximal motility).

CBC (1–20 mg·kg^{-1}), or vehicle was given (i.p.) 30 min before the oral administration of the fluorescent marker, both to control mice and to mice with intestinal inflammation induced by croton oil. In croton oil-treated animals, the effect of CBC (10 mg·kg^{-1}) was evaluated in animals pretreated (i.p., 10 min before CBC) with the CB_1 receptor antagonist rimonabant (0.1 mg·kg^{-1}), the CB_2 receptor antagonist SR144528 (1 mg·kg^{-1}) or the selective TRPA1 antagonists HC-030031 (30 mg·kg^{-1}) and AP18 (100 mg·kg^{-1}). The doses of the cannabinoid receptor antagonists used have been previously shown in our laboratory to counteract the effect of selective cannabinoid receptor agonists on croton-oil- induced

hypermotility in mice (Capasso *et al.*, 2008b). The dose of HC-030031 (30 mg·kg^{-1}) was selected based on previous work (McNamara *et al.*, 2007), in which it was shown that this antagonist, given i.p., attenuated TRPA1-mediated pain in mice. Higher doses of HC-030031 were not used because they tend to increase, given alone, upper gastrointestinal transit (data not shown). On the other hand, AP18, even at the high dose of 100 mg·kg^{-1}, given alone, did not affect transit.

Colonic propulsion in vivo

Distal colonic propulsion was measured as previously described (Broccardo *et al.*, 1998; Borrelli *et al.*, 2006). A single 3 mm glass bead was inserted 2 cm into the distal colon of each mouse with the aid of a catheter and the time to expulsion of the glass bead was determined for each animal. CBC (10 and 20 mg·kg^{-1}), WIN 55,212-2 (1 mg·kg^{-1}, used as a positive control) or vehicle was given (i.p.) 30 min before glass bead insertion.

Whole gut transit time in vivo

Mice were housed in individual cages 72 h before the experiment. On the day of the experiment, they were acclimatized to an empty cage (devoid of bedding) for 1 h before drug treatment. Thirty minutes after i.p. administration of CBC (10 and 20 mg·kg^{-1}), vehicle or the cannabinoid receptor agonist WIN 55,212-2 (1 mg·kg^{-1}, used as a positive control), mice received by gastric gavage 0.2 mL of 6% carmine red suspension in 0.5% carboxymethylcellulose. The time to the first red bowel movement was measured in min and constituted the whole gut transit time (Storr *et al.*, 2010).

Electrically (and agonists)-induced contractions in the isolated ileum

Mice were killed by asphyxiation with carbon dioxide and the ileum was removed, flushed of luminal contents, and placed in Krebs solution (composition: NaCl 119 mM, KCl 4.75 mM, KH2PO4 1.2 mM, NaHCO3 25 mM, $MgSO_4$ 1.5 mM, $CaCl_2$ 2.5 mM, and glucose 11 mM). Segments of 1.0–1.5 cm were cut from the distal ileum and placed in 20 mL thermostatically controlled (37°C) organ bath containing Krebs solution gassed with 95% O_2 and 5% CO_2. The tissues were connected to an isometric transducer (tension: 5 mN) in such a way as to record contractions from the longitudinal axis. Mechanical activity was digitized on an analogue-to-digital converter, visualized, recorded and analysed on a personal computer using the PowerLab/400 system (Ugo Basile, Comerio, Italy). All experiments started after a minimal 1 h equilibration period.

Contractions to electrical field stimulation (EFS; 8 Hz for 10 s, 400 mA, 1 ms pulse duration) were obtained by a pair of electrodes placed around the ileal tissue derived from both control and croton oil-treated animals; the interval between each contraction was 20 min. EFS-induced contractions were performed in the presence of the acetylcholinesterase inhibitor neostigmine (1 μM), to potentiate cholinergic neurotransmission (Baldassano *et al.*, 2009). After stable control contractions evoked by EFS had been recorded, the contractile responses were observed in the presence of increasing cumulative concentrations of CBC (10^{-8}–10^{-4} M). The contact time for each concentration was 20 min. Preliminary experiments showed that this contact time was sufficient for CBC to achieve maximal pharmacological effect. The effect of CBC on EFS-induced contractions was also evaluated after the administration in the bath (contact time ≥30 min) of the non-selective channel-blocker ruthenium red (3×10^{-6} M), the selective TRPA1 HC-030031 (10^{-5} M), the cannabinoid CB_1 receptor antagonist rimonabant (3×10^{-8} M), the CB_2 receptor antagonist SR144528 (10^{-7} M), L-NAME (3×10^{-4} M) plus apamin (10^{-7} M) (alone or in combination), ω-conotoxin (10^{-8} M), the non-selective PDE inhibitor IBMX (10^{-7} M), the cAMP-selective PDE inhibitor rolipram (10^{-6} M) or the cell-permeable activator of AC, forskolin (10^{-7} M). The concentration of rimonabant (3×10^{-8} M) was able to counteract the inhibitory effect of the cannabinoid receptor agonist WIN55,212-2 on EFS-induced contractions (data not shown). The concentrations of HC-030031 (10^{-5} M) and ruthenium red (3×10^{-6} M) were approximately two-three fold higher than the IC_{50} value calculated for these compounds as TRPA1 antagonists (McNamara *et al.*, 2007; Alexander *et al.*, 2011). Higher concentrations of the two TRPA1 antagonists were not used because they inhibited, *per se*, the EFS-induced-induced contractions. The other concentrations used in the present study were selected on the basis of previous work (Coutts and Pertwee, 1998; Nocerino *et al.*, 2002; Capasso *et al.*, 2008b; Borrelli *et al.*, 2011). In a separate set of experiments, the effect of the selective cannabinoid agonist WIN5555,212-2 (10^{-9}–10^{-6} M, contact time for each concentration: 20 min) on EFS-induced contractions was also evaluated [alone or in the presence of ω-conotoxin (10^{-8} M), IBMX (10^{-7} M), rolipram (10^{-6} M) or forskolin (10^{-7} M)].

In some experiments, the effect of CBC (10^{-8}–10^{-4} M) was also evaluated (contact time 20 min) on the contractions produced by exogenous ACh (10^{-6} M) or KCl (10^{-2} M). ACh or KCl was left in contact with the tissue for 60 and 90 s, respectively, and then washed out. In one set of experiments, the effect of CBC on ACh-induced contractions was evaluated in the presence (contact time ≥30 min) of cyclopiazonic acid (CPA; 10^{-5} M, a sarcoplasmic reticulum Ca^{2+} inhibitor), verapamil (10^{-6} M) (a L-type Ca^{2+} blocker) or ω-conotoxin (10^{-8} M). In this set of experiments, we also evaluated the effect of eugenol (10^{-7}–3×10^{-4} M, contact time for each concentration: 20 min) on ACh-induced contractions.

Contractions are expressed as % of contractions produced by 10^{-3} M ACh; this concentration of ACh produced a maximal contractile response (100% contraction).

Statistics

Data are expressed as the mean ± SEM of experiments in *n* mice. To determine statistical significance, Student's *t*-test was used for comparing a single treatment mean with a control mean, and a one-way ANOVA followed by a Tukey–Kramer multiple comparisons test was used for analysis of multiple treatment means. *P*-values < 0.05 were considered significant.

Materials

CBC (purity by HPLC: 97.3) was kindly supplied by GW Pharmaceuticals (Porton Down, Wiltshire, UK). ACh hydrochloride, atropine sulphate, N^G-nitro-L-arginine methyl ester (L-NAME) hydrochloride, apamin, ruthenium red, tetrodot-

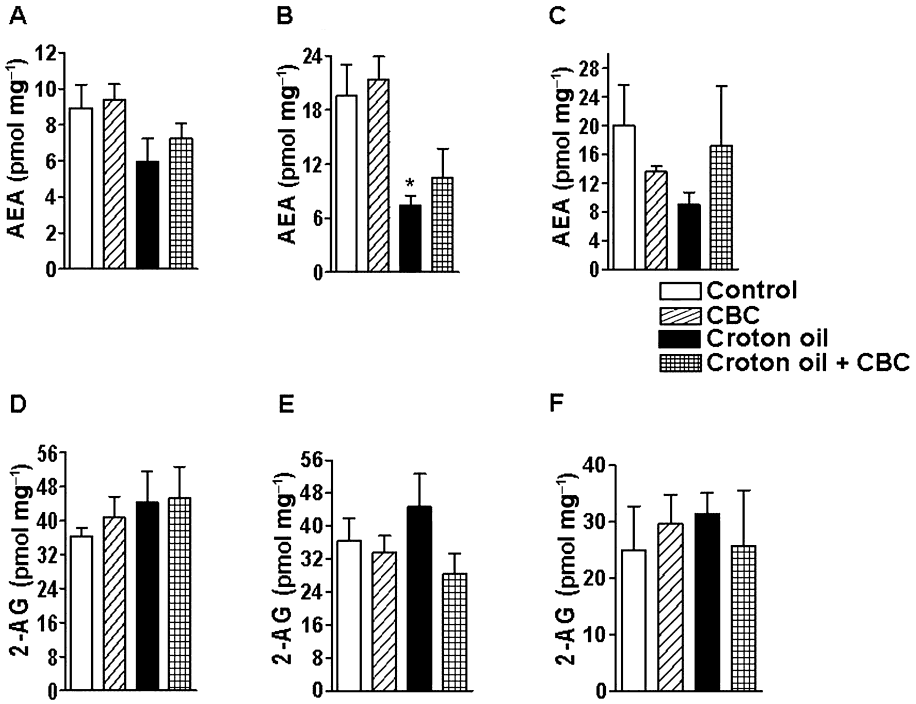

Figure 1

Anandamide (AEA) and 2-AG levels in the duodenum (A,D), jejunum (B,E) and ileum (C,F) of mice treated or not with croton oil. Some mice treated with croton oil were also treated with CBC (15 mg·kg^{-1}, i.p.). Data are mean ± SEM of four mice. *P < 0.05 versus control.

oxin, IBMX, rolipram, forskolin, eugenol, CPA, verapamil hydrochloride were purchased from (Sigma, Milan, Italy). WIN 55,212-2 mesylate, ω-conotoxin GVIA, AP18 and HC-030031 were was purchased from Tocris Cookson (Bristol, UK). Rimonabant and SR144528 were a kind gift from Sanofi-Aventis (Montpellier, France).

Rimonabant, SR144528, WIN55,212-2, HC-030031, AP18, IBMX, rolipram, forskolin, eugenol and CPA were dissolved in dimethyl sulfoxide (DMSO), CBC in ethanol (stock solution at 10^{-2} M; subsequent dilutions in distilled water), whereas the other drugs were dissolved in saline.

The drug vehicles (ethanol or DMSO, 4 μL per mouse *in vivo*; DMSO < 0.01% or ethanol <0.02% *in vitro*) had no significant effect on the responses under study.

The drug/molecular target nomenclature conforms to the *BJP*'s Guide to Receptors and Channels (Alexander *et al.*, 2011).

Results

Endocannabinoid, PEA and oleoylethanolamide (OEA) levels in control and croton oil-treated mice: effect of CBC

Assays were performed in the duodenum, jejunum and ileum, both in control and in croton oil-treated animals. Compared with control mice, croton oil administration caused a significant reduction in anandamide (but not 2-AG) levels in the jejunum, but not in the duodenum or ileum (Figure 1). In addition, although a conventional statistical significance was not fully achieved, PEA levels were also reduced by croton oil in the ileum (P < 0.06), but not in the duodenum or jejunum (Figure 2). No significant differences between control and croton oil-treated animals were observed in OEA levels in the duodenum, jejunum and ileum (Figure 2). CBC (15 mg·kg^{-1}) did not modify significantly endocannabinoid (anandamide and 2-AG), PEA and OEA levels either in control or in croton oil-treated mice (Figures 1 and 2).

Messenger RNA expression of enzymes involved in anandamide biosynthesis and degradation in the jejunum of control and croton oil-treated mice: effect of CBC

A significant decrease of anandamide was observed in the jejunum (but not in the duodenum or ileum) of croton oil-treated mice, therefore, we measured the mRNA expression of enzymes involved in anandamide biosynthesis (i.e. NAPE-PLD, GDE1) and degradation (i.e. FAAH) in this tissue. Croton oil administration was associated, in the mouse jejunum, with a significant up-regulation of the anandamide biosynthetic enzyme GDE1 (NAPE-PLD showed a strong trend towards an increase) (Figure 3) and a down-regulation of the degrading enzyme FAAH (Figure 3).

CBC (15 mg·kg^{-1}) did not modify the expression in control mice, but partially down-regulated croton oil-induced GDE1 hyper-expression and reduced FAAH expression further in tissues from croton oil-treated mice (Figure 3).

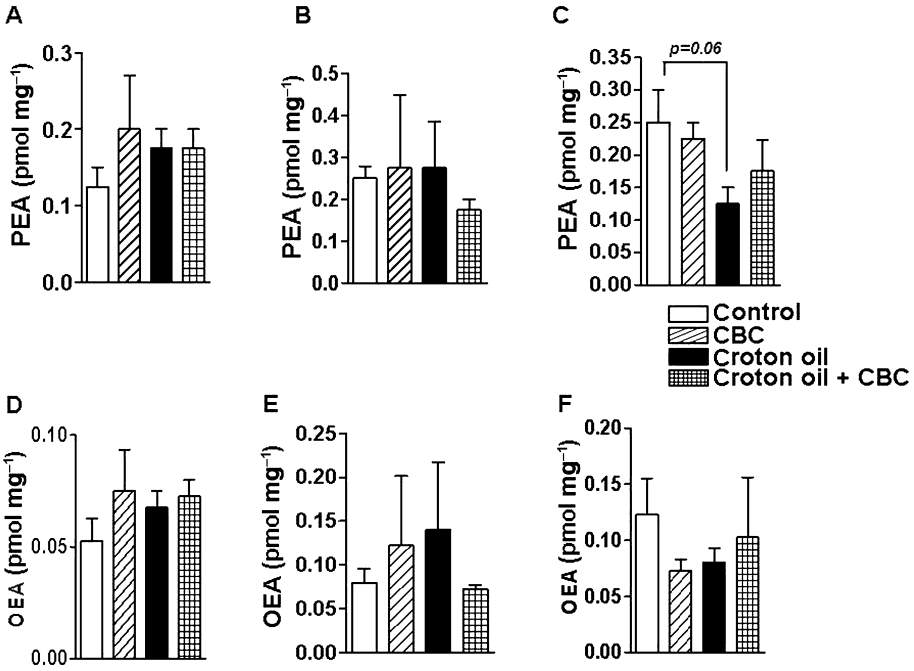

Figure 2

PEA and OEA levels in the duodenum (A,D), jejunum (B,E) and ileum (C,F) of mice treated or not with croton oil. Some mice treated with croton oil were also treated with CBC (15 mg·kg^{-1}, i.p.). Data are mean ± SEM of four mice.

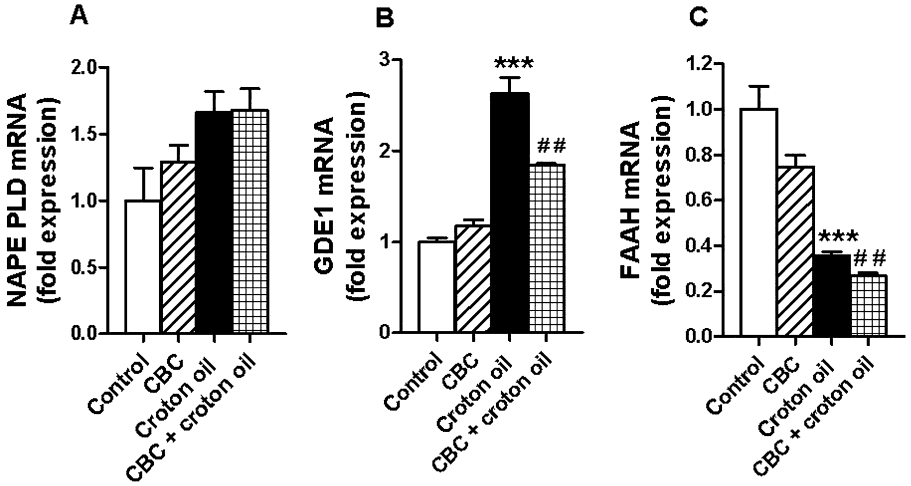

Figure 3

Relative expression of NAPE PLD (A), GDE1 (B) and FAAH (C) mRNA in the jejunum of animals treated or not with croton oil. Some mice treated with croton oil were also treated with CBC (15 mg·kg^{-1}, i.p.). Total RNA extracted from the intestine of control and croton-oil-treated mice was subjected to quantitative (real-time) RT-PCR analysis as described in Methods. Data were analysed by GENEX software for groupwise comparisons and statistical analysis. The expression in control tissues for each target was considered as 1. Results are means ± SEM of four experiments. ***$P < 0.001$ versus control and $^{\#\#}P < 0.01$ versus croton oil.

Messenger RNA expression of cannabinoid receptors in control and croton oil-treated mice: effect of CBC

In control animals (i.e. not given croton oil), CBC (15 mg·kg^{-1}) up-regulated CB_1 receptors in the jejunum only (Figure 4A) and down-regulated CB_2 receptors in the duodenum and ileum, but not in the jejunum (Figure 4B).

Croton oil administration was associated with a significant up-regulation of CB_1 receptor (in the jejunum only) mRNA expression as well as with a down regulation of CB_2 receptor mRNA expression (in the duodenum, jejunum and ileum) (Figure 4A,B). In croton oil-treated mice, CBC (15 mg·kg^{-1}) reduced the expression of both CB_1 and CB_2 receptors mRNA (in the jejunum, but not in the ileum)

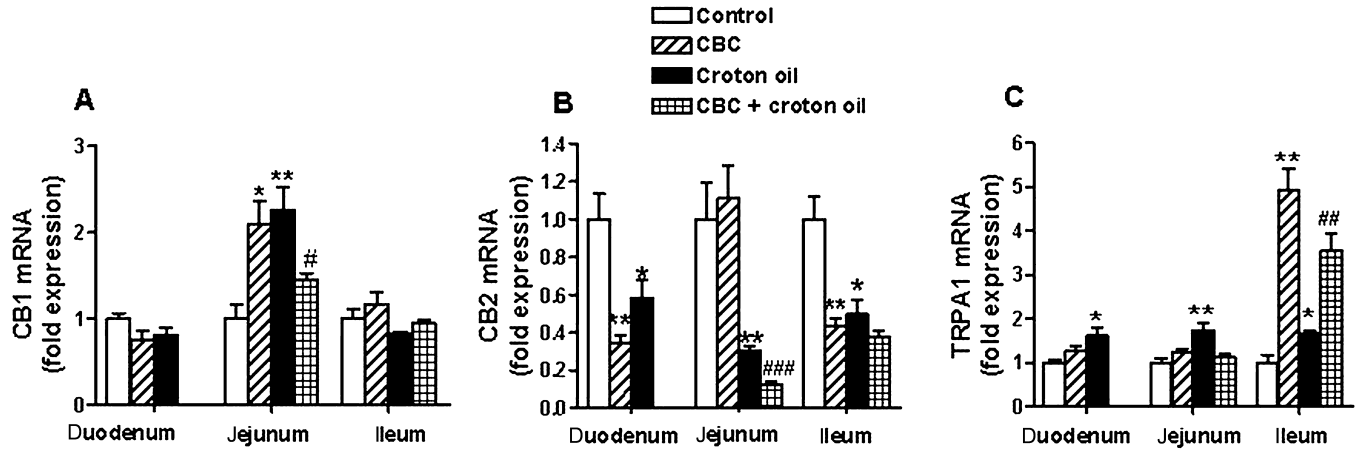

Figure 4

Relative expression of cannabinoid (CB_1 and CB_2) receptors (A, B) and TRPA1 (C) mRNA in the duodenum, jejunum and ileum of animals treated or not with croton oil. Some mice treated with croton oil were also treated with CBC (15 mg·kg^{-1}, i.p.). Note that the effect of CBC was evaluated in the jejunum and ileum only (mRNA in the samples of the duodenum was degraded). Total RNA extracted from the intestine of control and croton-oil-treated mice was subjected to quantitative (real-time) RT-PCR analysis as described in Methods. Data were analysed by GENEX software for groupwise comparisons and statistical analysis. The expression in control tissues (duodenum, jejunum and ileum) for each target was considered as 1. Results are means ± SEM of four experiments. *$P < 0.05$ and **$P < 0.01$ versus control; $^{\#}P < 0.05$, $^{\#}P < 0.01$ and $^{\#\#\#}P < 0.01$ versus croton oil.

(Figure 4A,B). No data are available for the effect of CBC in the duodenum of croton oil-treated mice (mRNA in the samples of the duodenum was degraded).

TRPA1 mRNA expression in control and croton oil-treated mice: effect of CBC

In control mice (i.e. not given croton oil), CBC (15 mg·kg^{-1}, i.p.) significantly increased TRPA1 expression in ileum, but not in the duodenum or jejunum (Figure 4C).

Compared with control mice, croton oil administration caused a significant up-regulation of TRPA1 in the duodenum, jejunum and ileum (Figure 4C). CBC counteracted the up-regulation of TRPA1 caused by this inflammatory stimulus in the jejunum, while it further increased the up-regulated TRPA1 in the ileum (Figure 4C). No data are available for the effect of CBC in the duodenum of croton oil treated mice (mRNA in the samples of the duodenum was degraded).

Upper gastrointestinal transit, colonic propulsion and whole gut transit time in control mice

CBC (10 and 20 mg·kg^{-1}) did not affect upper gastrointestinal transit (Figure 5A), colonic propulsion (Figure 5B) or whole gut transit (Figure 5C). In contrast, the psychotropic cannabinoid receptor agonist WIN 55,212-2 (1 mg·kg^{-1}), used as a reference drug, inhibited upper gastrointestinal transit, increased the time of expulsion of a glass bead inserted into the distal colon colonic propulsion (thus indicating an inhibitory effect on colonic propulsion) and increased the time of expulsion of an orally given red marker (which indicates an inhibitory effect on whole gut transit).

Upper gastrointestinal transit in the inflamed intestine

Oral administration of croton oil produced a significant increase in intestinal transit, shown as an increased value of the GC (Figure 6). Administration of CBC, i.p., caused a reduction in intestinal motility in croton oil-treated animals, which was statistically significant at doses of 10 and 20 mg·kg^{-1} (Figure 6). The inhibitory effect of CBC 10 mg·kg^{-1} was not significantly modified by the cannabinoid CB_1 receptor antagonist rimonabant (0.1 mg·kg^{-1}), the CB_2 receptor antagonist SR144528 (1 mg·kg^{-1}) or the TRPA1 antagonists HC-030031 (30 mg·kg^{-1}) and AP18 (100 mg·kg^{-1}) (Figure 7). At the doses used, these antagonists, did not affect, *per se*, upper gastrointestinal transit (in either control or in croton oil-treated mice).

Electrically (and agonists)-induced contractions in the isolated ileum

The contractile responses of mouse ileum to EFS reached 59 ± 7% in control mice, and 69 ± 8% in croton oil treated mice, of the maximal contraction produced by ACh 10^{-3} M ($n = 8$). Both in control mice and in croton oil-treated mice, EFS of the mouse ileum evoked contractions that were abolished by tetrodotoxin (3×10^{-8} M) or atropine (10^{-6} M) and strongly reduced (63 ± 4% inhibition, $n = 7$) by ω-conotoxin (10^{-8} M), thus indicating that these contractions were due to the release of ACh from enteric nerves and that N-type Ca^{2+} channels have a major role in ACh release. The ω-conotoxin-resistant contractions were abolished by tetrodotoxin.

Apamin (10^{-7} M), L-NAME (3×10^{-4} M) ruthenium red (3×10^{-6} M), HC-030031 (10^{-5} M), rimonabant (3×10^{-8} M), SR144528 (10^{-7} nM), IBMX (10^{-7} M), rolipram (10^{-6} M), for-

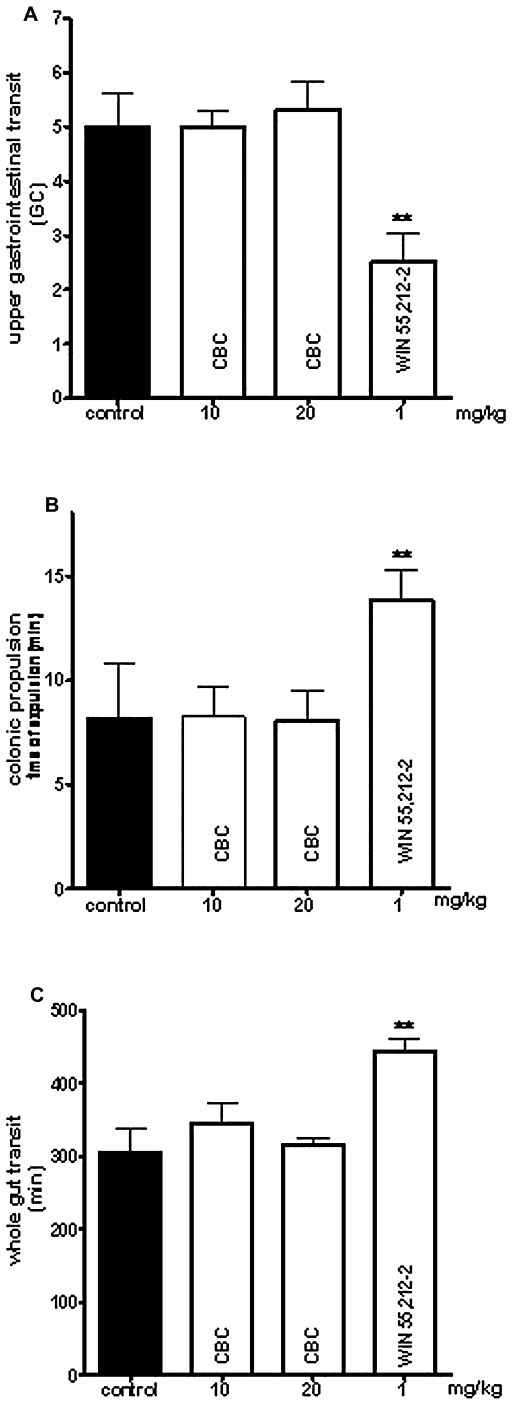

Figure 5

Effect of i.p. injected CBC (10 and 20 mg·kg^{-1}) and of the psychotropic cannabinoid receptor agonist WIN 55,212-2 (1 mg·kg^{-1}) on upper gastrointestinal transit (A), colonic propulsion (B) and whole gut transit in mice (C) (see Methods for details concerning the measurement of motility). Columns represent the mean ± SEM of 6–11 mice for each experimental group $^{**}P < 0.01$ versus control. Note that a decreased transit is indicated by a decreased value of GC (A), by an increased value of 'time of expulsion (min)' (B) or by an increased time of 'whole gut transit (min)' (C).

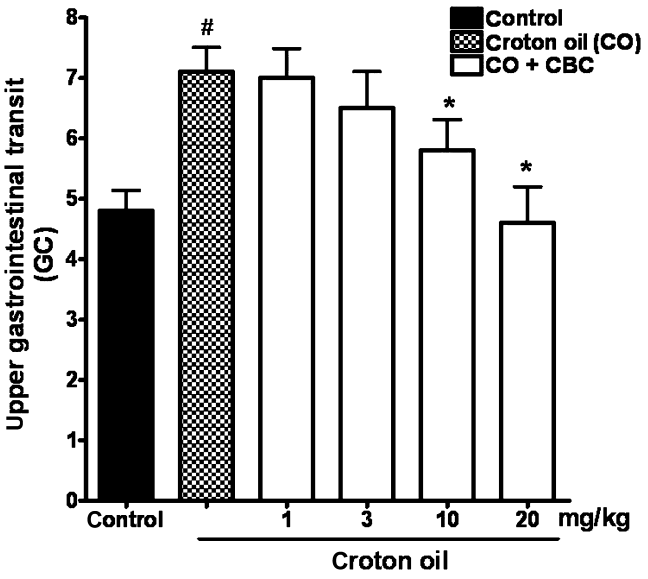

Figure 6

Inhibitory effect of i.p.-injected CBC (1–20 mg·kg^{-1}) on intestinal transit in croton oil-treated mice *in vivo*. Transit was expressed as the GC of the distribution of a fluorescent marker along the small intestine. GC ranged from 1 (minimal motility) to 10 (maximal motility) (see Methods section). Columns represent the mean ± SEM of 10–12 mice for each experimental group. $^{\#}P < 0.05$ versus control and $^{*}P < 0.05$ versus croton oil. Note that CBC (10 and 20 mg·kg^{-1}) did not affect transit in control mice (see Figure 5).

skolin (10^{-7} M), at the concentration used, did not modify significantly EFS-induced contractions (data not shown, see also Methods).

Tetrodotoxin did not modify the contractions induced by ACh (10^{-6} M), which were similar in amplitude to those evoked by EFS (data not shown). ACh-induced contractions were strongly reduced by verapamil (10^{-6} M, 53 ± 3% inhibition, $n = 8$) and CPA (10^{-5} M, 59 ± 4% inhibition, $n = 9$), but left unchanged by ω-conotoxin (10^{-8} M).

CBC (10^{-8}–10^{-4} M) significantly and in a concentration-dependent manner, inhibited the contractions induced by ACh or by EFS, both in control mice and in croton oil-treated mice (Figure 8). CBC was significantly more potent and effective at inhibiting the contractions induced by EFS than those induced by ACh. Both in control and in croton oil-treated animals (Figure 9), the inhibitory effect of CBC on EFS was not significantly modified by the CB_1 receptor antagonist rimonabant (3×10^{-8} M) or the CB_2 receptor antagonist SR144528 (10^{-7} M) (Figure 9A,B), nor by the non-selective TRP channel blocker ruthenium red (3×10^{-6} M) or the selective TRPA1 antagonist HC-030031 (10^{-5} M) (Figure 9C,D). L-NAME (3×10^{-4} M) and apamin (10^{-7} M) (alone or in combination) were also ineffective at antagonizing the effects of CBC (Figure 9E,F). In contrast, ω-conotoxin (10^{-8} M), but not IBMX (10^{-7} M), rolipram (10^{-6} M) or forskolin (10^{-7} M), significantly reduced the inhibitory effect of CBC, both in control and in croton oil-treated animals (Figure 10). Under the same experimental conditions, IBMX, rolipram and forskolin significantly

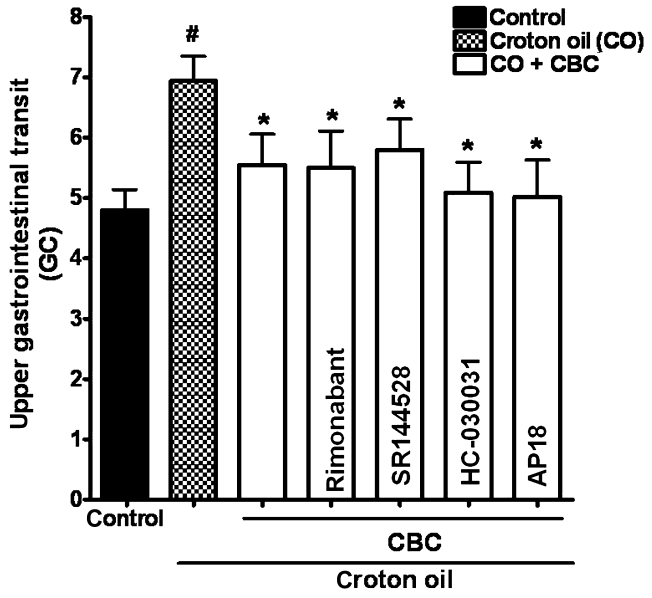

Figure 7

Croton oil-treated mice: effect of CBC (10 mg·kg^{-1}, i.p.) alone or in the presence of the cannabinoid CB_1 receptor antagonist rimonabant (0.1 mg·kg^{-1}, i.p.), the CB_2 receptor antagonist SR144528 (1 mg·kg^{-1}, i.p.) or the TRPA1 antagonists HC-030031 (30 mg·kg^{-1}, i.p.) and AP18 (100 mg kg^{-1}, i.p.) on upper gastrointestinal transit *in vivo*. Transit was expressed as the GC of the distribution of a fluorescent marker along the small intestine. GC ranged from 1 (minimal motility) to 10 (maximal motility) (see Methods section). Columns represent the mean ± SEM of 8–11 mice for each experimental group. $^{\#}P < 0.05$ versus control, $^{*}P < 0.05$ versus croton oil.

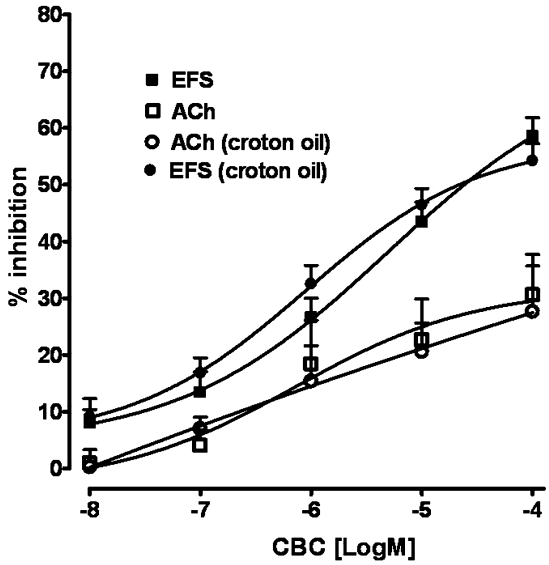

Figure 8

Inhibitory effect of CBC (10^{-8}–10^{-4} M) on the contractions induced by ACh (10^{-6} M) or EFS in the isolated mouse ileum of control and croton-oil-treated mice. Each point represents mean ± SEM of 7–8 experiments. Both in control mice and in croton oil-treated mice the curve representing the inhibitory effect of CBC on ACh-induced contractions was statistically different ($P < 0.001$) from the curve representing the inhibitory effect of CBC on EFS-induced contractions.

reduced the inhibitory response to WIN55,212-2 on EFS-induced contractions in control mice (see insert to Figure 10C).

Both in control (Figure 11A) and in croton oil-treated animals (Figure 11B), the inhibitory effect of CBC on ACh-induced contractions was significantly reduced by the L-type Ca^{2+} channel blocker verapamil (10^{-6} M), but not by CPA (10^{-5} M), an inhibitor of the sarcoplasmatic reticulum Ca^{2+}ATPase or by ω-conotoxin. In the presence of verapamil, CBC, at the lowest concentrations tested (10^{-8} M, 10^{-7} M and 10^{-6} M) slightly (less than 10%) increased ACh-induced contractions (Figure 11).

Finally, CBC (10^{-8}–10^{-4} M) also inhibited the contractions evoked by KCl in control mice (% inhibition: 10^{-8} M CBC 2.4; 10^{-7} M CBC 5 ± 4; 10^{-6} M CBC 8.0 ± 2.1; 10^{-5} M CBC 15.8 ± 3.9; 10^{-4} M CBC 29.0 ± 1.9, $n = 5$).

Discussion

In the present study we evaluated the effect of CBC, a major non-psychotropic ingredient of the marijuana plant *Cannabis sativa*, on intestinal motility, both in physiological states and in a model of intestinal ileitis induced by croton oil. Intestinal inflammation induced by croton oil is characterized by disruption of the mucosa and an infiltration of lymphocytes into the submucosa (Pol and Puig, 1997), with increased activity of myeloperoxidase (an index of neutrophil infiltration) (Pol *et al.*, 2005) and vascular permeability (Jiménez *et al.*, 2006). Such changes are associated with the induction of iNOS (Pol *et al.*, 2005) and up-regulation of cannabinoid CB_1, opioid (κ and δ) receptors and α_2-adrenoceptors (Pol *et al.*, 1996; Puig and Pol, 1998; Izzo *et al.*, 2001).

Adaptive changes of the endogenous cannabinoid system and of TRPA1 in the croton oil model of intestinal inflammation

The first step in the present study was to measure endocannabinoid levels in the duodenum, jejunum and ileum of control and croton oil-treated animals. In a previous paper, we found that the levels of anandamide slightly decreased in the whole small intestine of croton oil-treated mice, although the difference did not reach statistical significance (Izzo *et al.*, 2001). Here, by measuring endocannabinoid levels in the different portions of the small intestine, we detected a significant decrease in anandamide levels in the jejunum (but not in the duodenum or ileum) of mice treated with croton oil. These changes, which constitute an unprecedented example of down regulation of anandamide in an experimental model of gut dysfunction, could be not explained by corresponding changes in the mRNA expression of anandamide metabolic enzymes (i.e. NAPE-PLD and GDE1, involved in anandamide synthesis, as well as FAAH, involved in anandamide degradation). It is possible that other enzymes or the

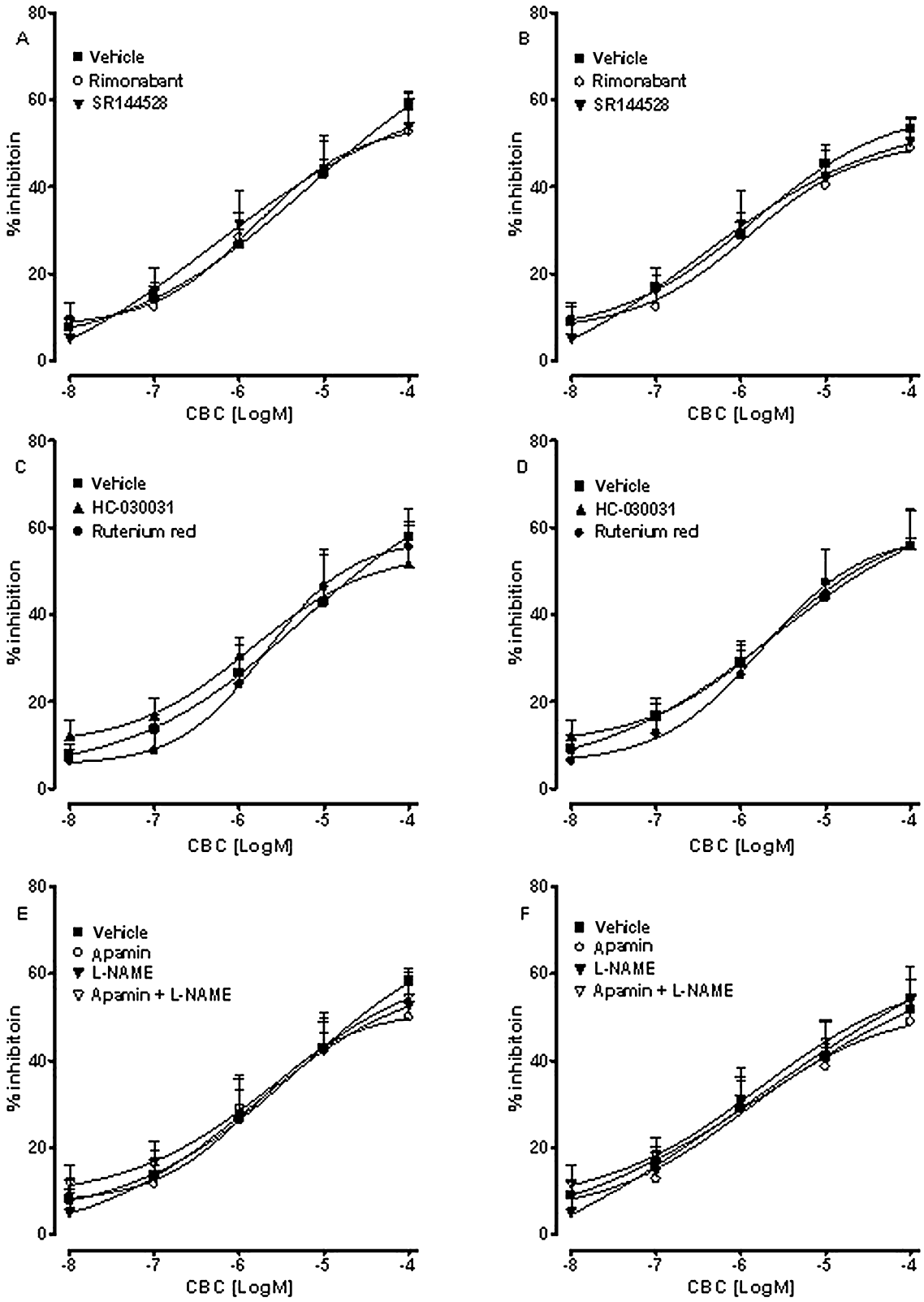

Figure 9

Electrically-induced contractions in the isolated mouse ileum of (A,C,E) control and (B,D,F) croton oil-treated mice: inhibitory effect of CBC (10^{-8}–10^{-4} M) alone (vehicle) or in the presence of rimonabant (3×10^{-8} M) and SR144528 (10^{-7} M) (A,B), HC-030031 (10^{-5} M) and ruthenium red (3×10^{-6} M) (C,D) or L-NAME (3×10^{-4} M) and apamin (10^{-7} M) (alone or in combination (E,F). Each point represents mean ± SEM of 7–8 experiments. No significant differences among the curves were observed.

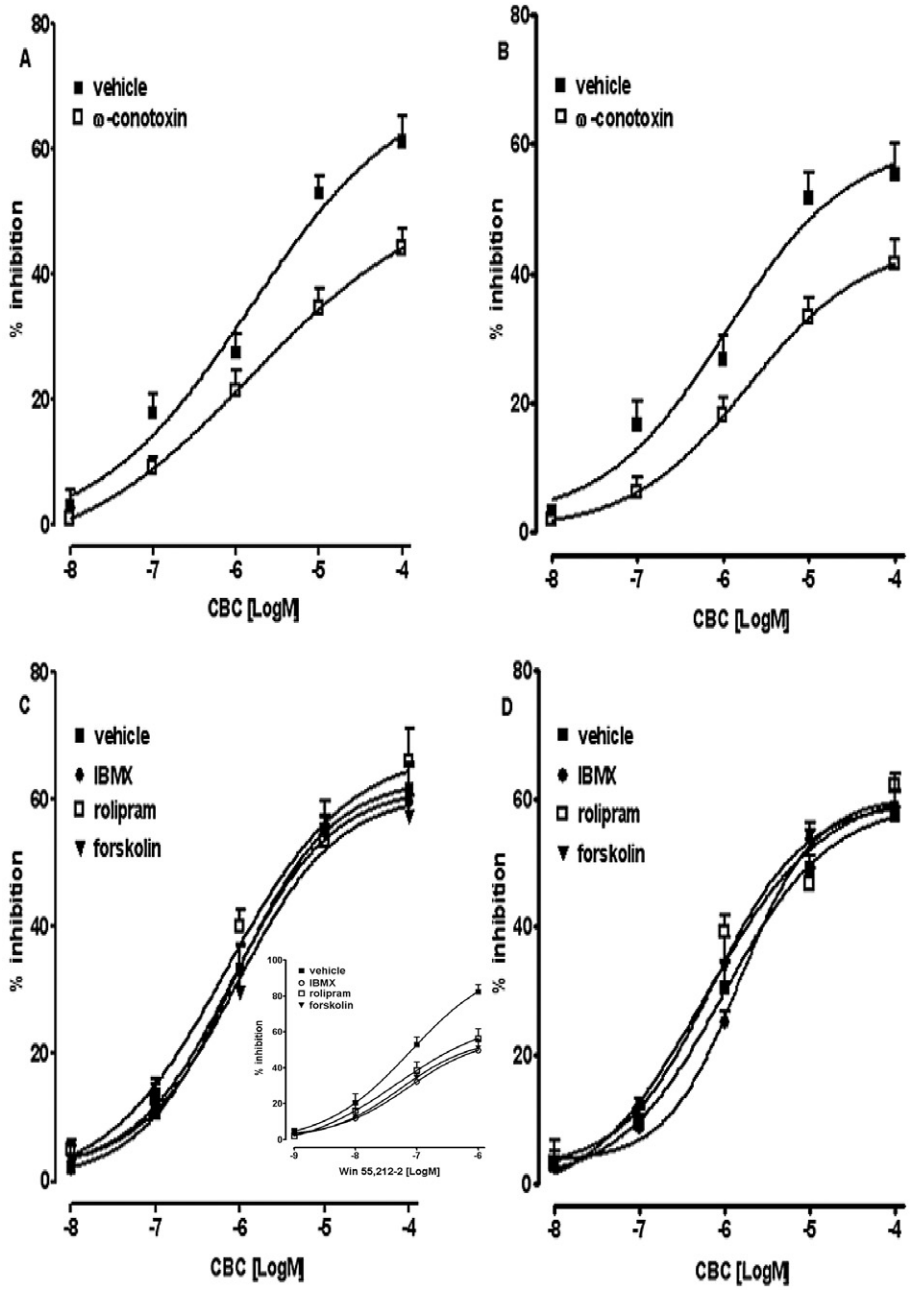

Figure 10

Electrically-induced contractions in the mouse isolated ileum of (A,C) control and (B,D) croton oil-treated mice: inhibitory effect of CBC (10^{-8}–10^{-4} M) alone (vehicle) or in the presence of ω-conotoxin (10^{-8} M) (A,B), IBMX (10^{-7} M), rolipram (10^{-6} M) or forskolin (10^{-7} M) (C,D). Each point represents mean ± SEM of 7–8 experiments. Both in control mice (A) and in croton oil-treated mice (B), ω-conotoxin (but not IBMX, rolipram or forskolin) significantly ($P < 0.01$) reduced the inhibitory effect of CBC (significant difference between curves). The insert in (C) shows that IBMX (10^{-7} M), rolipram (10^{-6} M) and forskolin (10^{-7} M) significantly ($P < 0.01$) reduced the inhibitory effect of WIN55,212-2 (10^{-9}–10^{-6} M) on electrically induced contractions in the ileum of control mice ($n = 5$).

availability of phospholipid biosynthetic precursors (Hansen and Diep, 2009) underlie changes in the levels of this endocannabinoid in the mouse small intestine evoked during croton oil-induced inflammation. We also measured the levels of PEA and OEA, two acylethanolamides chemically related to anandamide, which reduce gastric and intestinal motility (Capasso *et al.*, 2001; 2005; Aviello *et al.*, 2008; Cluny *et al.*, 2009) and the levels of which are known to change in response to noxious stimuli (Darmani *et al.*, 2005; Borrelli and Izzo, 2009; Hansen and Diep, 2009). We found that PEA, but not OEA, decreased in the intestine of croton oil-treated animals (although a full statistical difference was

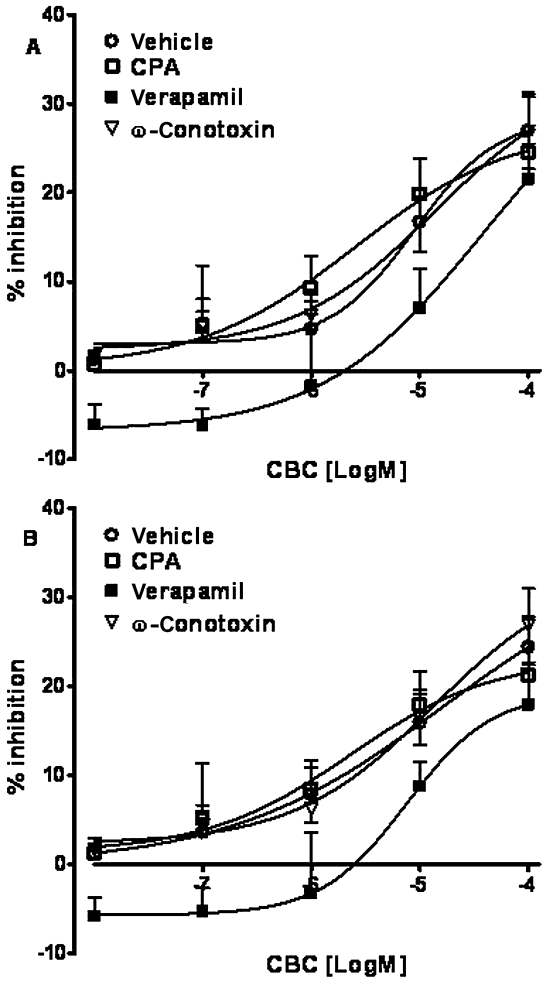

Figure 11

ACh (10^{-6} M)-induced contractions in the isolated mouse ileum of (A) control and (B) croton oil-treated mice: inhibitory effect of CBC (10^{-8}–10^{-4} M) alone (vehicle) or in the presence of ω-conotoxin (10^{-8} M), verapamil (10^{-6} M) or CPA (10^{-5} M). Each point represents mean ± SEM of 8–9 experiments. Both in control mice (A) and in croton oil-treated mice (B), verapamil (but not CPA or ω-conotoxin) significantly ($P < 0.01$) reduced the inhibitory effect of CBC (significant difference between curves).

not achieved, the P value being less than 0.06 but more than 0.05), a finding that is in line with our previous work (Capasso *et al.*, 2001).

When we analysed the expression of cannabinoid receptors, we found that croton oil administration was associated with an up-regulation of CB_1 mRNA receptor expression in the mouse jejunum. This result is in line with our previous data showing an increase in CB_1 protein expression in this model of intestinal inflammation (Izzo *et al.*, 2001) as well as with other studies showing increased CB_1 receptor expression in the inflamed intestine (Massa *et al.*, 2004; Wright *et al.*, 2008; Izzo and Camilleri, 2009). However, we also found, perhaps quite surprisingly, a down-regulation of CB_2 mRNA receptor expression in the duodenum, jejunum and ileum of mice treated with croton oil. This result is at odds with previous immunohistochemical studies showing an increase in CB_2 expression in the mustard oil model of inflammatory bowel disease (Kimball *et al.*, 2006) as well as in patients with ulcerative colitis or Crohn's disease (Wright *et al.*, 2005). On the other hand, others have shown no changes in CB_2 receptor mRNA, in both experimental models of inflammation (Duncan *et al.*, 2008) and in the colon of patients with inflammatory bowel disease (D'Argenio *et al.*, 2006; Stintzing *et al.*, 2011).

A further step in our study was the evaluation of the mRNA expression of TRPA1, a channel that can be activated by CBC. It is well known that TRPA1 is involved in inflammatory visceral pain (Kimball *et al.*, 2006; Yang *et al.*, 2008; Brierley *et al.*, 2009; Cattaruzza *et al.*, 2010; Mitrovic *et al.*, 2010); in addition, intestinal inflammatory stimuli may cause up-regulation of TRPA1 in colonic afferent dorsal root ganglia (Yang *et al.*, 2008). In agreement with previous studies, we found TRPA1 mRNA to be expressed in the mouse small intestine of healthy mice. However, we found, for the first time, that the mRNA encoding for this channel is up-regulated in the gut wall in our experimental model of intestinal inflammation. The TRPA1 channels were found to be up-regulated in all of the small intestine (i.e. duodenum, jejunum and ileum). During the review process of this paper, it was also shown that TRPA1 mediates colitis in mice (Engel *et al.*, 2011).

Effect of CBC on endocannabinoid levels and on the mRNA expression of cannabinoid receptors, TRPA1 and anandamide metabolizing enzymes in the intestine of control and croton oil-treated mice

We found that CBC did not modify endocannabinoid levels in croton oil-treated animals whereas, in these animals, but not in control mice, CBC down-regulated the mRNA expression of both GDE1 and FAAH, which are involved in the biosynthesis and degradation of anandamide respectively. These findings leave open the possibility that, unlike croton oil, the inhibitory effects of CBC on both anandamide synthesizing and degrading enzymes, or lack thereof, in the mouse small intestine might underlie the lack of effect of CBC on anandamide levels in this tissue. We also found that CBC, in croton oil treated animals, decreased the expression of the mRNA encoding for both CB_1 and CB_2 receptors in the jejunum, while exerting no effect in the ileum. In summary, the inhibitory effects of CBC on motility observed here (see the following) are unlikely to be due to changes in endocannabinoid signaling, as treatment with the phytocannabinoid caused a decrease in cannabinoid receptor expression and no changes in endocannabinoid levels, which, taken together, if anything should have resulted in increased motility. Accordingly, selective cannabinoid receptor antagonists failed to modify CBC-induced changes on motility (see pharmacological experiments discussed in the following). In addition, CBC also changed the mRNA expression of cannabinoid receptors in control mice, making it unlikely that these effects of CBC are specific for intestinal inflammation.

It has been recently demonstrated that CBC can alter the intestinal TRP channels of vanilloid types 1–4 mRNA expression after a pharmacological *in vivo* treatment as short as

30 min (as in the present study), thus providing another potential mechanism – in addition to direct activation – through which this phytocannabinoid can exert pharmacological actions (De Petrocellis *et al.*, 2012). In the present study we showed that, while CBC inhibited TRPA1 expression in the jejunum of croton oil-treated animals, it elevated the expression of this channel in the ileum, thus pointing to a possible overall null net effect on TRPA1-mediated modulation of intestinal motility. In future studies, changes in cannabinoid receptor and TRPA1 mRNA expression caused by CBC both in the healthy and in the inflamed intestine could help reveal the anti-inflammatory and/or analgesic effect of CBC in the gut.

Pharmacological experiments in vivo

Little is known about the effects of non-psychotropic phytocannabinoids in the gut. While cannabidiol, the most studied among the non-psychotropic phytocannabinoids, was shown to exert a protective effect in the inflamed gut (Capasso *et al.*, 2008a; Borrelli *et al.*, 2009; Jamontt *et al.*, 2010; Alhamoruni *et al.*, 2012), there are no data in the literature concerning the effect of CBC in the digestive tract. In this study, we found that this phytocannabinoid did not affect upper gastrointestinal transit, colonic propulsion or whole gut transit in healthy mice *in vivo*. However, CBC reduced croton-oil-induced intestinal hypermotility (upper gastrointestinal transit). In order to measure upper gastrointestinal transit, we used a method that reflects a combination of gastric emptying and small intestinal transit; even if our results do not establish a distinct site (gastric or intestinal) of action for CBC, but a combination of both, they clearly show that CBC is pharmacologically active *in vivo* only when intestinal homoeostasis is perturbed by an inflammatory stimulus. The observation that CBC administration is not associated with constipation under physiological conditions is relevant as one of the major side effects associated with opiate administration (the most known agent able to reduce intestinal motility) is constipation (Jafri and Pasricha, 2001). To investigate the mechanism of action of CBC-induced delay in motility *in vivo*, we examined the possible involvement of cannabinoid receptors as well as TRPA1 channels. We found that the inhibitory effect of CBC on croton oil-induced intestinal hypermotility was not modified by the CB_1 receptor antagonist rimonabant, nor by the CB_2 receptor antagonist SR144528. These antagonists, at the doses used in the present paper, were previously shown to counteract the inhibitory effect of selective CB_1 and CB_2 receptor agonists on croton oil-induced intestinal hypermotility (Capasso *et al.*, 2008b). Furthermore, the inhibitory effect of CBC on motility was not significantly modified by the selective TRPA1 antagonist HC-030031, given at a dose (30 $mg \cdot kg^{-1}$) and a route of administration (i.p.) previously shown to attenuate TRPA1-mediated pain in mice (McNamara *et al.*, 2007) nor by AP18 (100 $mg \cdot kg^{-1}$), another selective TRPA1 antagonist.

Pharmacological experiments in vitro

In order to obtain more information on the site of action of CBC, we have performed *in vitro* experiments on the isolated ileum from control and croton oil-treated mice. Our data showed that CBC preferentially inhibits the contractile response elicited by EFS (which is mediated by the release of ACh form enteric nerves) rather than that induced by exogenously administered ACh (which contracts the ileum through direct activation of muscarinic receptors located on smooth muscles). These results indicate that CBC exerts its inhibitory effect mainly by acting at prejunctional sites; a direct inhibitory effect on smooth muscle was observed only at higher concentrations of CBC. It is unlikely that the inhibitory effect of CBC was due to antimuscarinic actions, because it also inhibited the contractions induced by KCl. In contrast to the *in vivo* results, CBC inhibited ACh- and EFS-induced contractions both in the healthy and in the inflamed intestine (with no significant differences in potency or efficacy). Differences between *in vitro* and *in vivo* actions of cannabinoids have been previously documented in the digestive tract (Coruzzi *et al.*, 2006; Sanger, 2007; Capasso *et al.*, 2008a).

Because CBC preferentially inhibited EFS-induced contractions, we performed further studies by evaluating the effect of cannabinoid receptor antagonists and TRPA1 antagonists on CBC-induced inhibition of EFS-evoked contractions. Similar to the *in vivo* studies, we found that the inhibitory effect of CBC on EFS-induced contractions was not significantly modified by cannabinoid receptor antagonists (rimonabant and SR144528) or by TRPA1 blockers [i.e. ruthenium red (a pan-TRP blocker) and HC-030031 (a selective TRPA1 antagonist)]. The concentrations of TRPA1 antagonists used were approximately two-threefold higher than the IC_{50} values of these antagonists previously reported (McNamara *et al.*, 2007; Alexander *et al.*, 2011). Higher concentrations of the two TRPA1 antagonists were not used because they inhibited *per se* the EFS-induced-induced contractions. Importantly, in a recent study, we also showed that the effect of the prototypical TRPA1 agonist allyl isothiocyanate on intestinal motility, both *in vitro* and *in vivo*, was not modified by a number of selective and non-selective TRPA1 antagonists, including HC-030031 (Capasso *et al.*, 2012). In addition, during the review process of this paper, it was demonstrated that genetic ablation of the TRPA1 channel does not affect upper gastrointestinal transit in mice and that TRPA1 activation inhibits spontaneous neurogenic contractions and transit in the mouse proximal colon (Poole *et al.*, 2011).

In order to gain further insights into CBC-induced inhibition of EFS, we investigated the possible involvement of N-type Ca^{2+} channels and cAMP, both involved in the regulation of neurotransmitter(s) release from myenteric nerves. We found that the inhibitory effect of CBC was strongly reduced in the presence of the N-type Ca^{2+} channel blocker ω-conotoxin, but left unchanged by drugs that are expected to increase intracellular cAMP levels either by stimulating its production (forskolin) or by inhibiting its catabolism (rolipram, IBMX). Under the same experimental conditions, and as previously reported for the guinea-pig ileum (Coutts and Pertwee, 1998), forskolin, rolipram and IBMX significantly reduced the inhibitory effect WIN55,212-2, a cannabinoid receptor agonist that is known to inhibit EFS in the mouse ileum via activation of prejunctional CB_1 receptors (Bashashati *et al.*, 2012). Additionally, ω-conotoxin did not modify the inhibitory effect of CBC on ACh-induced contractions, indicating that it is unlikely ω-conotoxin reduces the inhibi-

tory effect of CBC by acting postjunctionally. Collectively these results suggest that CBC inhibits EFS-induced contraction, at least in part, by limiting the availability of intraneuronal Ca^{2+} via inhibition of N-type Ca^{2+} channels, without the intermediacy of the AC/cAMP/PDE system.

We also excluded the possibility that CBC inhibited EFS-induced contractions by activating enteric inhibitory nerves (at least, the inhibitory component mediated by NO and ATP), as a combination of apamin (a blocker of Ca^{2+} activated K^+ channels that blocks the enteric inhibitory component mediated by ATP or related purine (Crist *et al.*, 2002)] and L-NAME (an inhibitor of NO synthase), a combination that is known to block enteric inhibitory nerves (Waterman and Costa, 1994), did not modify the inhibitory effect of CBC on twitch responses.

Finally, we also evaluated the effect of CBC on ACh-induced contractions (i.e. the small portion of the inhibitory effect of CBC, which is exerted at the postjunctional level) in the presence of drugs that influence Ca^{2+} levels in smooth muscles. We found that the inhibitory effect of CBC on ACh-induced contractions was reduced by verapamil (a L-type Ca^{2+} channel antagonist), but not by CPA, which is known to deplete internal Ca^{2+} stores by inhibiting the sarcoplasmatic reticulum Ca^{2+}-ATPase pump (Alexander *et al.*, 2011). The effect of verapamil was specific for CBC as this L-type Ca^{2+} channel antagonist did not modify the inhibitory effect of eugenol, another plant compound. On the whole, these results suggest that CBC inhibits, at least in part, ACh-induced contractions by a mechanism involving L-type Ca^{2+} channels rather than by influencing Ca^{2+} release from sarcoplasmic stores. The ability of eugenol to exert antispasmodic actions via a mechanism independent of extracellular Ca^{2+} influx has been previously documented in the rat small intestine (Leal-Cardoso *et al.*, 2002).

Conclusions

We showed that CBC, a major non-psychotropic component of the marijuana plant, normalizes *in vivo* intestinal motility in an experimental model of intestinal inflammation, but does not slow the rate of transit in control animals. These protective effects of CBC were accompanied by intestinal changes in cannabinoid and TRPA1 expression, but not of endocannabinoid levels. *In vitro* results on intestinal ileal segments showed that this phytocannabinoid preferentially reduces EFS-induced contractions – rather than ACh-induced contractions – by a mechanism involving N-type Ca^{2+} channels. The inhibitory effect of CBC, both *in vitro* and *in vivo*, does not involve cannabinoid receptors or TRPA1 channels. Although the precise mechanism of the inhibitory effect of CBC requires further studies, the present results are of potential clinical interest because intestinal dysmotility in inflammatory diseases is a well-recognized and clinically accepted phenomenon (Ohama *et al.*, 2007), in which the only drugs currently available to counteract it are often associated with constipation (Jafri and Pasricha, 2001). In addition, by revealing that CBC affects the expression of both cannabinoid receptor and TRPA1 mRNA, the present results suggest that the effects of this safe plant compound should be investigated in other pathophysiological conditions (e.g. intestinal secretion, mucosal inflammation, visceral pain and intestinal cancer) in which these receptors are potentially involved.

Acknowledgements

This study was partly funded by GW Pharma, UK. GA and BR are grateful to Enrico and Enrica Sovena foundation (Rome, Italy).

Conflict of interests

VDM and AAI are recipients of research grants from GW Pharma, Ltd.

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