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RESEARCH PAPER

Inhibitory effect of salvinorin A, from *Salvia divinorum*, on ileitis-induced hypermotility: cross-talk between κ-opioid and cannabinoid CB_1 receptors

R Capasso[1,4], F Borrelli[1,4], MG Cascio[2,4], G Aviello[1,4], K Huben[3], JK Zjawiony[3,5], P Marini[2,4], B Romano[1,4], V Di Marzo[2,4], F Capasso[1,4] and AA Izzo[1,4]

[1]*Department of Experimental Pharmacology, University of Naples Federico II, Naples, Italy;* [2]*Institute of Biomolecular Chemistry, National Research Council, Pozzuoli (NA), Italy;* [3]*Department of Pharmacognosy, School of Pharmacy, University of Mississippi, University, MS, USA;* [4]*Endocannabinoid Research Group, Italy and* [5]*National Center for Natural Products Research, Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi, University, MS, USA*

Background and purpose: Salvinorin A, the active component of the hallucinogenic herb *Salvia divinorum*, inhibits intestinal motility through activation of κ-opioid receptors (KORs). However, this compound may have target(s) other than the KORs in the inflamed gut. Because intestinal inflammation upregulates cannabinoid receptors and endogenous cannabinoids, in the present study we investigated the possible involvement of the endogenous cannabinoid system in salvinorin A-induced delay in motility in the inflamed gut.
Experimental approach: Motility *in vivo* was measured by evaluating the distribution of a fluorescent marker along the small intestine; intestinal inflammation was induced by the irritant croton oil; direct or indirect activity at cannabinoid receptors was evaluated by means of binding, enzymic and cellular uptake assays.
Key results: Salvinorin A as well as the KOR agonist U-50488 reduced motility in croton oil treated mice. The inhibitory effect of both salvinorin A and U-50488 was counteracted by the KOR antagonist nor-binaltorphimine and by the cannabinoid CB_1 receptor antagonist rimonabant. Rimonabant, however, did not counteract the inhibitory effect of salvinorin A on motility in control mice. Binding experiments showed very weak affinity of salvinorin A for cannabinoid CB_1 and CB_2 and no inhibitory effect on 2-arachidonoylglycerol and anandamide hydrolysis and cellular uptake.
Conclusions and implications: The inhibitory effect of salvinorin A on motility reveals a functional interaction between cannabinoid CB_1 receptors and KORs in the inflamed—but not in the normal—gut *in vivo*.
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Keywords: cannabinoid receptors; fatty acid amide hydrolase; endocannabinoid transport; inflammatory bowel disease; intestinal motility; κ-opioid receptors (KORs); myenteric plexus; *Salvia divinorum*; salvinorin A

Abbreviations: ACEA, arachidonyl-2-chloroethylamide; [^{3}H]-CP-55,940, [^{3}H]-(−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)-phenyl]-*trans*-4-(3-hydroxy-propyl)-cyclohexanol|EFS, electrical field stimulation; EMT, endocannabinoid membrane transporter; FAAH, fatty acid amide hydrolase; GC, geometric centre; JWH 015, (2-methyl-1-propyl-1*H*-indol-3-yl)-1-naphthalenymethanone; KOR, κ-opioid receptor; MAG, monoacylglycerol; SR144528, *N*-[-1*S*-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; WIN 55,212-2, (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-gmorpholinylmethyl)pyrrolo [1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone

Introduction

Salvinorin A is the main pharmacologically active ingredient of *Salvia divinorum*, a hallucinogenic plant that has been used for centuries for divination and shamanism by *curanderos* in Mexico and other areas (Roth *et al*., 2004). *S. divinorum* leaf preparations (occasionally fortified with extracted salvinorin A) are widely available in western Europe and the USA, notably on internet sites. The lipid-like salvinorin A molecule is chemically and structurally unique in that it represents the only known psychoactive diterpenoid, and

Correspondence: Dr R Capasso or Professor AA Izzo, Department of Experimental Pharmacology, University of Naples Federico II, Via D Montesano 49, 80131 Naples, Italy.
E-mail: rafcapas@unina.it or aaizzo@unina.it
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was the first non-nitrogenous hallucinogen to be identified (Vortherms and Roth, 2006). Additionally, salvinorin A was reported to be the most potent naturally occurring hallucinogen, with an effective dose, when smoked, of 200–1000 μg in humans (Siebert, 1994; Sheffler and Roth, 2003).

Salvinorin A was found to be a strong and selective agonist of the κ-opioid receptor (KOR) (Roth *et al*., 2002; O'Connor and Roth, 2005). KOR-mediated salvinorin A effects *in vivo* include antinociceptive (McCurdy *et al*., 2006), antipruritic (Wang *et al*., 2005) and motor effects (Fantegrossi *et al*., 2005), inhibition of striatal dopamine levels in mice (Zhang *et al*., 2005) and induction of discriminative stimuli in rhesus monkeys (Butelman *et al*., 2004). We have recently shown that salvinorin A inhibited enteric cholinergic transmission in the isolated guinea-pig ileum (Capasso *et al*., 2006) and reduced motility in the croton oil model of intestinal inflammation in mice through activation of KORs (Capasso *et al*., 2008b). Moreover, croton oil-induced gut inflammation increased the potency of salvinorin A, but not of the reference KOR agonist U-50488, suggesting that salvinorin A may have target(s) other than KORs in the inflamed gut (Capasso *et al*., 2008b).

Because upregulation of cannabinoid CB_1 receptors and increased expression of fatty acid amide hydrolase (FAAH, one of the enzymes responsible for endocannabinoid inactivation) were observed in the intestine of mice treated with croton oil (Izzo *et al*., 2001), and as salvinorin A exhibits rewarding effects that are attenuated by a cannabinoid CB_1 receptor antagonist in zebrafish and rats (Braida *et al*., 2007, 2008), here we investigated the possible involvement of the endocannabinoid system, specifically, cannabinoid receptors, FAAH, monoacylglycerol (MAG) lipase (which is involved in the inactivation of the endocannabinoid 2-arachidonoylglycerol) and endocannabinoid membrane transporter (EMT), in salvinorin A-induced delay in motility during intestinal inflammation.

Methods

Animals

All animal procedures were in conformity with the principles of laboratory animal care (NIH publication no. 86-23, revised 1985) and with 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). Male ICR mice (Harlan Italy, S Pietro al Natisone UD, Italy) (24 ± 26 g) were used after 1 week of acclimation. Food was withheld 6 h before transit measurement and 18 h before the induction of intestinal inflammation.

Intestinal inflammation

Inflammation was induced as previously described (Puig and Pol, 1998; Borrelli *et al*., 2006). Mice received orally two doses of croton oil (20 μL per mouse) in 2 consecutive days. Motility was measured 4 days after the first administration of croton oil. This time point was selected on the basis of a previous work (Puig and Pol, 1998; Izzo *et al*., 2001), in which maximal inflammatory response occurred 4 days after the first treatment.

In vivo *studies on transit*

Transit was measured *in vivo* by evaluating the intestinal location of rhodamine-B-labelled dextran (Capasso *et al*., 2005). Animals were given fluorescently labelled dextran (100 μL of 25 mg mL^{-1} stock solution) via a gastric tube into the stomach. Twenty minutes after administration, the animals were killed by asphyxiation with CO_2 and the entire small intestine with its content 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 rhodamine. The supernatant was centrifuged at 35 *g* to precipitate the intestinal chyme. The fluorescence in duplicate aliquots of the cleared supernatant was read in a multiwell 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:

$GC = \Sigma$(fraction of fluorescence per segment × segment number)

GC ranged from 1 (minimal motility) to 10 (maximal motility). This procedure yielded an accurate, non-radioactive measurement of intestinal transit (Capasso *et al*., 2005).

Isolation and identification of salvinorin A from S. divinorum

Salvinorin A was isolated from the leaves of *S. divinorum* (purchased from The Sage Wisdom Salvia Shop, Malibu, CA, USA) using the method described elsewhere (Munro and Rizzacasa, 2003) The identity and purity of the colourless, crystalline material was determined by spectroscopic and chromatographic techniques. The sample used for biological experiments was recrystallized twice from ethanol and had a melting point of 242–244 °C (Valdes *et al*., 1984; melting point 242–244 °C) and specific optical rotation $[\alpha]_D^{25}$ −41.8° ($c = 1$, $CHCl_3$) (O'Neil *et al*., 2006; $[\alpha]_D^{25}$ −41°) ($c = 1$, $CHCl_3$). Purity of the sample was determined as 99% by HPLC (Delta Prep 4000, Waters Corporation, Milford, MA, USA; 5 μm Waters XTerra C18 column 7.8 × 100 mm, isocratic mobile-phase MeCN:H_2O (40:60), UV detector ($\lambda = 210$ nm)).

All spectroscopic data (IR, UV, NMR and MS) were in full agreement with the published values (Ortega *et al*., 1982; Valdes *et al*., 1984; Barnes *et al*., 2006; Giner *et al*., 2007). Salvinorin A HPLC retention time was 18 min.

Drug administration in vivo

All drugs were given intraperitoneally (i.p.). Salvinorin A (3 mg kg^{-1}), U-50488 (30 mg kg^{-1}), JWH 015 ((2-methyl-1-propyl-1*H*-indol-3-yl)-1-naphthalenymethanone; 10 mg kg^{-1}) and arachidonyl-2-chloroethylamide (ACEA) (0.2 mg kg^{-1}) were administered 30 min before rhodamine administration

to mice with inflammation. In some experiments, nor-binaltorphimine (20 mg kg^{-1}, to block KOR), rimonabant (0.1 mg kg^{-1}, to block cannabinoid CB_1 receptors) or SR144528 (*N*-[-1*S*-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; 1 mg kg^{-1}, to block cannabinoid CB_2 receptors) was given 30 min before the KOR or the cannabinoid receptor agonists. In some experiments, salvinorin A (3 mg kg^{-1}), U-50488 (30 mg kg^{-1}) or ACEA (0.1 mg kg^{-1}) (alone or in mice pretreated with rimonabant 0.1 mg kg^{-1}) was given 30 min before rhodamine administration to control mice (that is, mice not treated with croton oil). The doses of antagonists used in this study (that is, rimonabant, SR144528 and nor-binaltorphimine) were selected on the basis of previous work (Capasso *et al*., 2001, 2008b); the doses of salvinorin A (3 mg kg^{-1}) and U-50488 (30 mg kg^{-1}) were derived from dose–response curves previously published (Capasso *et al*., 2008b). The dose of ACEA was selected on the basis of preliminary experiments, which showed that this cannabinoid CB_1 receptor agonist had an inhibitory effect on motility, which was similar to that produced by 3 mg kg^{-1} salvinorin.

Cannabinoid CB_1 and CB_2 receptor binding assays

For receptor binding assays, the compound was tested using membranes from HEK cells transfected with either the hCB_1 or hCB_2 receptor and [^{3}H]-CP-55,940 ([^{3}H]-(−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)-phenyl]-*trans*-4-(3-hydroxypropyl)-cyclohexanol; $K_d = 0.18$ nM for CB_1 receptor and $K_d = 0.31$ nM for CB_2 receptor) as the high-affinity ligand as described by the manufacturer (Perkin Elmer, Monza, Italy) (Brizzi *et al*., 2005). Some experiments were also carried out using membranes prepared from the rat brain and the same radioligand. Displacement curves were generated by incubating drugs with [^{3}H]-CP-55,940 (0.084 nM for CB_2 and 0.14 nM for CB_1 binding assay). In all cases, K_i values were calculated by applying the Cheng-Prusoff equation to the IC_{50} values (obtained by GraphPad) for the displacement of the bound radioligand by increasing concentrations of the test compound.

FAAH assay

The effect of increasing concentrations of the test compound on the enzymic hydrolysis of [^{14}C]anandamide was studied as described previously (Maurelli *et al*., 1995), by using membranes prepared from rat brain. In brief, the whole rat brain was homogenized at 4 °C in 50 mM Tris-HCl buffer, pH 7.0, by using an ultraturrax and a dounce homogenizer. Homogenates were first centrifuged at 800 *g* for 15 min to get rid of the debris and the supernatant was centrifuged at 10 000 *g* for 30 min. The pellet from this latter centrifugation was used for the assay. Membranes (70–100 μg) were incubated with increasing concentrations (up to 50 μM) of the test compounds and [^{14}C]anandamide (10 000 c.p.m., 1.8 μM, synthesized in our laboratory from arachidonoyl chloride and [^{14}C]ethanolamine) in 50 mM Tris-HCl, pH 9, for 30 min at 37 °C. Water-soluble [^{14}C]ethanolamine produced from [^{14}C]anandamide hydrolysis was used to calculate FAAH activity and was measured by scintillation counting of the aqueous phase after extraction of the incubation mixture with 2 volumes of $CHCl_3/CH_3OH$ (1:1 by volume). Data are expressed as IC_{50} values.

Endocannabinoid cellular uptake (EMT) assay

The effect of compound on the uptake of anandamide by RBL-2H3 cells was studied as described previously (Di Marzo *et al*., 2002). Cells were incubated with [^{14}C]anandamide (4 μM) for 5 min at 37 °C in the presence or absence of varying concentrations of the test compound. Residual [^{14}C]anandamide in the incubation media after extraction with $CHCl_3/CH_3OH$ (2:1 by volume) was used as a measure of the anandamide that was taken up by cells. Data are expressed as the concentration exerting 50% inhibition of anandamide uptake (IC_{50}) calculated with GraphPad.

MAG lipase enzymatic assay

Cytosolic fractions obtained by differential centrifugation from COS-7 cells were incubated in the presence of test compounds with 2-[^{3}H]-arachidonoylglycerol in 50 mM Tris-HCl, pH 7, for 20 min at 37 °C. After the incubation, lipids were extracted with 2 volumes of $CHCl_3$/MeOH 2:1 (by vol). The organic phases containing lipids were fractionated by TLC on silica on polypropylene plates using $CHCl_3$/MeOH/NH_4OH (85:15:1 by vol) as the eluting system. The bands corresponding to free fatty acids were cut and radioactivity was measured with a beta-counter.

Electrical field stimulation in the isolated mouse ileum

Segments (1–1.5 cm) of the terminal ileum from control mice (killed by asphyxiation with CO_2) were removed, flushed free of luminal contents and placed in Krebs' solution (composition in mM: NaCl 119, KCl 4.75, KH_2PO_4 1.2, $NaHCO_3$ 25, $MgSO_4$ 1.5, $CaCl_2$ 2.5 and glucose 11). The isolated organ was set up to record contractions from the longitudinal axis in an organ bath filled with warm (37 °C), aerated (95% O_2/5% CO_2) Krebs' solution in the presence of the acetylcholinesterase inhibitor neostigmine (1 μM), to potentiate cholinergic neurotransmission (Mulè *et al*., 2007). The tissues were connected to an isotonic transducer (load 0.5 g) connected to a 'Gemini' recording apparatus (Ugo Basile, Comerio, Italy). Stable and reproducible contractions for a time period of 4 h were obtained with electrical field stimulation (EFS, 8 Hz for 10 s, 400 mA, 1 ms pulse duration) every 2 min. Contractions were expressed as % of contractions produced by 10^{-3} M acetylcholine; this concentration of acetylcholine produced a maximal contractile response (100% contraction). After stable control contractions evoked by EFS had been recorded, the contractile responses were observed in the presence of increasing cumulative concentrations of salvinorin A (10^{-11}–10^{-6} M). The contact time for each concentration was 15 min. Preliminary experiments showed that this contact time was sufficient for salvinorin A to achieve maximal effect. The effect of salvinorin A was also evaluated after the administration in the bath (contact time 30 min) of the KOR antagonist nor-binaltorphimine

(3×10^{-8} M) or of the CB_1 receptor antagonist rimonabant (3×10^{-8} M). The concentration of nor-binaltorphimine was selected on the basis of a previous study (Capasso *et al*., 2006), in which we showed that this KOR antagonist counteracted the inhibitory effect of salvinorin A on EFS-induced contractions in the isolated guinea-pig ileum. The concentration of rimonabant was selected on the basis of preliminary experiments, in which we showed that this antagonist, at the concentration used, *per se*, did not modify EFS-induced contractions (see also the Results section).

In preliminary experiments, the effects of the neuronal blocker tetrodotoxin (3×10^{-7} M), the muscarinic receptor antagonist atropine (10^{-6} M), the cannabinoid receptor agonist WIN 55,212-2 ((*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone; 10^{-6} M) or the α_2-adrenergic agonist clonidine (10^{-6} M) were evaluated on EFS-induced contractions. The effect of salvinorin A (10^{-6} M) was also evaluated (contact time 15 min) on the contractions produced by exogenous acetylcholine (10^{-6} M). This concentration of acetylcholine gave a contractile response that was similar in amplitude to that of electrical stimulation. Acetylcholine was left in contact with the tissue for 60 s and then washed out. The interval between each contraction was 15 min.

Statistics

Data are expressed as the mean ± s.e.mean 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 analysis of variance followed by a Tukey–Kramer multiple comparisons test was used for analysis of multiple treatment means. *P*-values <0.05 were considered significant. IC_{50} (the concentration of salvinorin A that produced 50% of maximal inhibition of EFS-induced contractions) and E_{max} (maximal inhibitory effect on EFS-induced contractions) were used to characterize the potency and the efficacy of salvinorin A, respectively. The IC_{50} and E_{max} values (geometric mean ± 95% CL) were calculated by nonlinear regression analysis using the equation for a sigmoid concentration–response curve (GraphPad Prism, Instat Program Version 4.01).

Drugs

The drug and molecular target nomenclature used in this paper are in agreement with BJP's Guide to Receptors and Channels (Alexander *et al*., 2007). Acetylcholine chloride, clonidine hydrochloride, neostigmine bromide, tetrodotoxin, U-50488 hydrochloride and croton oil were purchased from Sigma (Milan, Italy); ACEA, nor-binaltorphimine, JWH 015 and WIN 55,212-2 ((*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone) mesylate were purchased from Tocris Cookson (Northpoint, UK); salvinorin A (purity: 99% by HPLC) was isolated from *S. divinorum* leaves as described above. Rimonabant and SR144528 were a kind gift from Drs Madaleine Mossè and Francis Barth (Sanofi-Aventis, Montpellier, France). Salvinorin A, ACEA, JWH 015, rimonabant, WIN 55,212-2 and SR144528 were dissolved in dimethyl sulphoxide, and U-50488 and nor-binaltorphimine were dissolved in saline. The drug vehicles (dimethyl sulphoxide, 4 μL per mouse; dimethyl sulphoxide/Tween 80, 20 mL per mouse; saline, 0.1 mL per mouse; dimethyl sulphoxide, 0.01% *in vitro*) had no significant effect on the responses under study, both *in vitro* and *in vivo*.

Results

In vivo *studies on transit*

Figure 1 shows the effect of i.p. injected salvinorin A (3 mg kg^{-1}), U-50488 (30 mg kg^{-1}) and ACEA (0.2 mg kg^{-1}) in animals treated with croton oil. Oral administration of croton oil produced significantly increased intestinal transit (shown as higher values of the GC), which was reversed by salvinorin A (3 mg kg^{-1}), U-50488 (30 mg kg^{-1}) and ACEA (0.2 mg kg^{-1}). The effect of these three agonists was counteracted by the cannabinoid CB_1 receptor antagonist rimonabant (0.1 mg kg^{-1}), whereas the KOR antagonist nor-binaltorphimine (20 mg kg^{-1}) counteracted the inhibitory effect of U-50488 (30 mg kg^{-1}) and salvinorin A (3 mg kg^{-1}), but not the effect of ACEA (0.2 mg kg^{-1}; Figure 1). The cannabinoid CB_2 receptor antagonist SR144528 (1 mg kg^{-1}) counteracted the inhibitory effect of JWH 015 (10 mg kg^{-1}), but not the inhibitory effect of salvinorin A (3 mg kg^{-1})

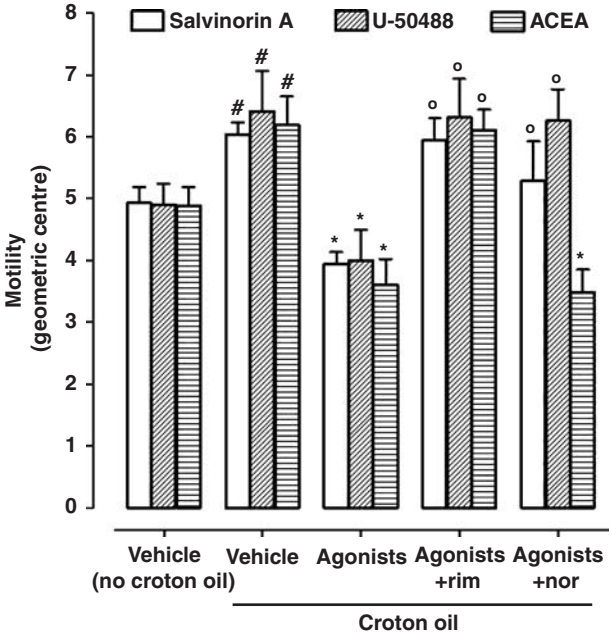

Figure 1 Croton oil-treated mice: effect of salvinorin A (3 mg kg^{-1}; i.p.), U-50488 (30 mg kg^{-1}; i.p.) and ACEA (0.2 mg kg^{-1}; i.p.) (alone or in the presence of the cannabinoid CB_1 receptor antagonist rimonabant (rim, 0.1 mg kg^{-1}, i.p.) or in the presence of the KOR antagonist nor-binaltorphimine (nor, 20 mg kg^{-1}, i.p.)) on intestinal transit. Transit was expressed as the GC of the distribution of a fluorescent marker along the small intestine (see the Methods section). Bars represent the mean ± s.e.mean of 8–10 animals. $^{\#}P<0.05$ vs control, $^{*}P<0.05$ vs vehicle (no croton oil) and $^{\circ}P<0.05$ vs the corresponding agonists (i.e. vs salvinorin A, U-50488 or ACEA).

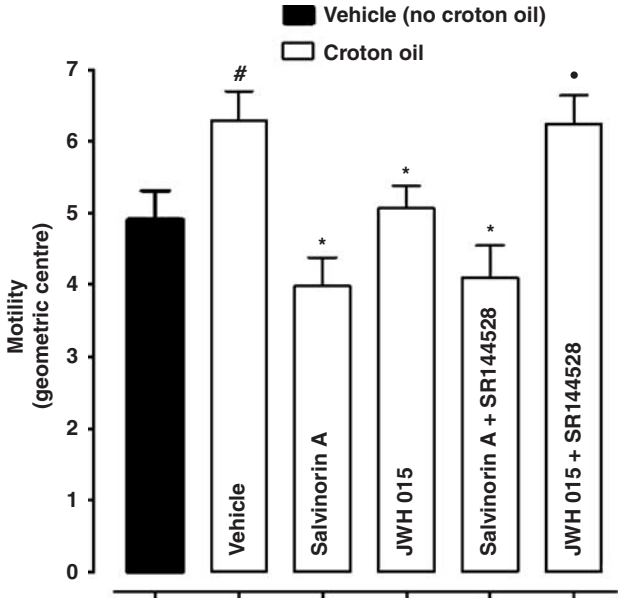

Figure 2 Croton oil-treated mice: effect of salvinorin A (3 mg kg^{-1}) and JWH 015 (10 mg kg^{-1}) (alone or in the presence of the cannabinoid CB_2 receptor antagonist SR144528 (10 mg kg^{-1}, i.p.)) on intestinal transit. Transit was expressed as the GC of the distribution of a fluorescent marker along the small intestine (see the Methods section). Bars represent the mean ± s.e.mean of 9–10 animals. $^{\#}P<0.05$ vs control, $^{*}P<0.05$ vs croton oil (vehicle) and $^{\circ}P<0.05$ vs JWH 015.

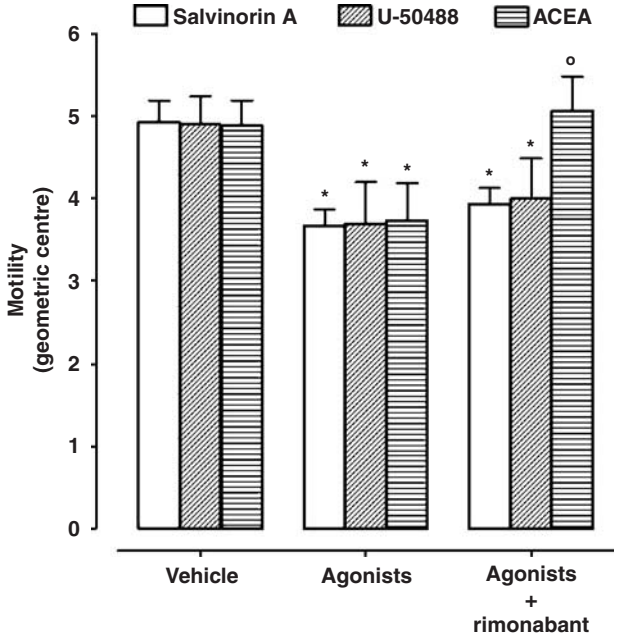

Figure 3 Effect of salvinorin A (3 mg kg^{-1}, KOR agonist), U-50488 (30 mg kg^{-1}, KOR agonist) and ACEA (0.2 mg kg^{-1}, cannabinoid CB_1 receptor agonist) (alone or in the presence of the cannabinoid CB_1 receptor antagonist rimonabant (0.1 mg kg^{-1}, i.p.)) on intestinal transit in control mice. Transit was expressed as the GC of the distribution of a fluorescent marker along the small intestine (see the Methods section). Bars represent the mean ± s.e.mean of 9–11 animals. $^{*}P<0.05$ vs vehicle and $^{\circ}P<0.05$ ACEA alone (i.e. without rimonabant).

(Figure 2). When given (i.p.) to control animals (that is, mice not treated with croton oil) salvinorin A (3 mg kg^{-1}), U-50488 (30 mg kg^{-1}) and ACEA (0.2 mg kg^{-1}) reduced motility (Figure 3). Rimonabant (0.1 mg kg^{-1}) counteracted the inhibitory effect of ACEA (0.2 mg kg^{-1}), but not the effect of U-50488 (30 mg kg^{-1}) or salvinorin A (3 mg kg^{-1}) (Figure 3).

In the absence of any agonist, the cannabinoid CB_1 receptor antagonist rimonabant (0.1 mg kg^{-1}) or the KOR antagonist nor-binaltorphimine (20 mg kg^{-1}) did not modify intestinal transit, both in control mice (GC: control 5.10 ± 0.39, nor-binaltorphimine 5.01 ± 3.8, rimonabant 5.60 ± 0.46; $n=9$–11) and in croton oil-treated animals (GC: croton oil 6.76 ± 0.51, nor-binaltorphimine 6.76 ± 0.53, rimonabant 6.99 ± 0.62; $n=9$–10). Also, SR144528 (1 mg kg^{-1}) did not modify motility in croton oil-treated mice (data not shown).

Binding, cellular uptake and enzymatic assays

In radioligand displacement binding assays carried on membranes containing the human recombinant CB_1 or the rat native CB_1 receptor, salvinorin A did not significantly displace the binding of [^{3}H]-CP-55,940 at 10 μM, and exerted <20% displacement at 25 μM (Table 1). By contrast, salvinorin A did exhibit some affinity for the human recombinant CB_2 receptor ($K_i=7.9$ μM). Under the same conditions, the CB_1/CB_2 agonist WIN 55,212-2 exhibited $K_i=21\pm1.1$ and 2.1 ± 0.1 nM at human recombinant CB_1 and CB_2, respectively.

Salvinorin A exhibited less than 20% inhibition of [^{14}C]anandamide hydrolysis by rat brain membranes or cellular uptake by RBL-2H3 cells at concentrations of 50 and 25 μM, respectively (Table 1). Under the same conditions, the FAAH inhibitor arachidonoyl-serotonin (synthesized in our laboratory) exhibited 85% of inhibition at 25 μM and the EMT inhibitor OMDM-2 (a generous gift from Professor G Ortar, University of Rome 'La Sapienza') exhibited 70% of inhibition at 10 μM (Table 1). Finally, salvinorin A (10 and 50 μM) exhibited less than 20% inhibition of 2-[^{3}H]-arachidonoylglycerol hydrolysis by COS-7 cell fractions, whereas the MAG lipase inhibitor methylarachidonoylfluorophosphonate (Cayman, Ann Arbor, MI, USA) inhibited 2-[^{3}H]-arachidonoylglycerol hydrolysis by 92% at a concentration of 200 nM (Table 1).

EFS in the isolated mouse ileum

EFS (8 Hz for 10 s, 400 mA, 1 ms pulse duration) of the mouse ileum evoked a contractile response that was 58 ± 4% of the contraction produced by 10^{-3} M acetylcholine. EFS-induced contractions were abolished by tetrodotoxin (3×10^{-7} M), atropine (10^{-6} M), by the cannabinoid receptor agonist WIN 55,212-2 or by the α_2-adrenoceptor agonist clonidine (10^{-6} M). These results suggest that these contractions were due to the release of acetylcholine from enteric nerves and that they can be modulated by drugs that act on prejunctional receptors.

Table 1 Effect of salvinorin A on CB_1 receptors, FAAH, the putative EMT and MAG lipase

Drug	*[^{3}H]-CP-55,940 displacement (from HEK-293 cell membranes overexpressing the human CB_1)*	*[^{14}C]anandamide hydrolysis (by rat brain membranes)*	*[^{14}C]anandamide uptake (by RBL-2H3 cells)*	*2-[^{3}H]-AG hydrolysis (by COS-7 cell cytosol)*
Salvinorin A, 10 μM	2.5 ± 1.1%	NT	9.1 ± 2.1%	5.1 ± 1.2%
Salvinorin A, 25 μM	16.8 ± 1.7%	8.5 ± 1.3%	19.1 ± 1.8%	NT
Salvinorin A, 50 μM	NT	17.9 ± 2.1%	NT	15.9 ± 2.5%
AA-5-HT, 25 μM	NT	85.0 ± 3.8%	NT	NT
OMDM-2, 10 μM	NT	NT	70.0 ± 5.1%	NT
MAFP, 200 nM	NT	NT	NT	92.0 ± 6.9%

Abbreviations: AA-5-HT, arachidonoylserotonin; EMT, endocannabinoid membrane transporter; FAAH, fatty acid amide hydrolase; MAFP, methylarachidonyl-fluorophosphonate; MAG, monoacylglycerol; NT, not tested; OMDM-2, (*S*)-*N*-oleoyl-(1′-hydroxybenzyl)-2′-ethanolamine.
The affinity for CB_1 receptors was assessed by measuring the displacement of [^{3}H]-CP-55,940 from membranes of HEK-293 cells overexpressing human recombinant CB_1 receptors. The effect on FAAH was measured by assessing the inhibition of [^{14}C]anandamide hydrolysis by rat brain membranes. The effect on the putative EMT was evaluated by measuring the inhibition of [^{14}C]anandamide uptake by intact RBL-2H3 cells. Finally, the effect on MAG lipase was assessed by measuring the inhibition of 2-[^{3}H]-arachydonoyl (2-AG) hydrolysis by COS-7 cell cytosol. Salvinorin A never attained 50% inhibition at the doses tested in each assay. However, salvinorin A exhibited weak affinity for the CB_2 receptor ($K_i = 7.9$ μM) using the displacement of [^{3}H]-CP-55,940 from membranes of HEK-293 cells overexpressing the human recombinant CB_2 receptors, and under conditions in which the CB_1/CB_2 agonist WIN 55,212-2 ($K_i = 21 \pm 1.1$ nM for CB_1 receptors) exhibited a K_i of 2.1 ± 0.1 nM.
Data are expressed as per cent of inhibition and are means ± s.e.mean of $n = 3$ and are expressed as % of inhibition. AA-5-HT, MAFP and OMDM-2 served as positive control.

Salvinorin A significantly and in a concentration-dependent manner inhibited EFS-induced contractions (Figure 4) (IC_{50} (95% CL): 3.1×10^{-9} (7×10^{-10}–1.5×10^{-8}) M; E_{max} (95% CL): 38.0 (27.4–48.7)%). The effect of salvinorin A was reduced by the KOR antagonist nor-binaltorphimine (3×10^{-8} M) and by the CB_1 receptor antagonist rimonabant (3×10^{-8} M). In the absence of any drugs, nor-binaltorphimine and rimonabant, at the concentration used, did not modify EFS-induced contractions (% inhibition: 3×10^{-8} M rimonabant, 4 ± 7%; 3×10^{-8} M nor-binaltorphimine, 2 ± 1%; $n = 8$–10). In preliminary experiments, we found that higher concentrations of rimonabant reduced EFS-induced contractions (% inhibition: 10^{-7} M rimonabant, 25 ± 3%; 10^{-6} M rimonabant, 86 ± 10%; $P < 0.01$, $n = 4$–6).

Salvinorin A (10^{-6} M) did not modify significantly the contractions induced by exogenous acetylcholine (% inhibition: 10 ± 2%; $n = 8$).

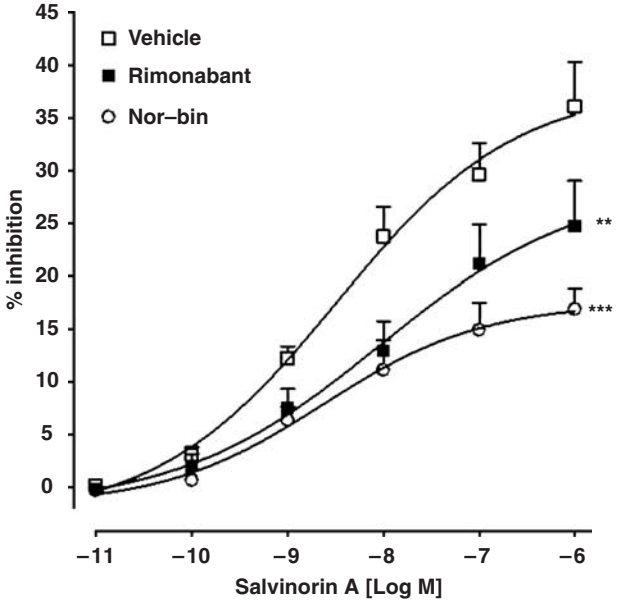

Figure 4 Electrical field stimulation (EFS)-induced contractions of the isolated ileum of control mice: effect of salvinorin A (10^{-11}–10^{-6} M) alone (vehicle) or in the presence of the cannabinoid CB_1 receptor antagonist rimonabant (3×10^{-8} M) or the KOR antagonist nor-binaltorphimine (nor-bin, 3×10^{-8} M). Each point represents mean ± s.e.mean of 8–10 experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs vehicle (i.e. the rimonabant and nor-binaltorphimine concentration–response curves were significantly different from the vehicle curve).

Discussion

Salvinorin A, a highly potent and selective KOR agonist, is the most potent naturally occurring hallucinogen known (Sheffler and Roth, 2003). Previous findings have shown that this diterpenoid may have target(s) other than KOR in the inflamed gut (Capasso *et al*., 2008b). Here, we have shown that salvinorin A reduced motility in a model of small bowel disease by a mechanism indirectly involving cannabinoid CB_1 receptors.

Cannabinoid and opioid systems share neuroanatomical, neurochemical and pharmacological features (Corchero *et al*., 2004): both cannabinoids and opioids induce analgesia, catalepsy, hypothermia, motor depression, hypotension, immunosuppression, sedation, rewarding effects (Vigano *et al*., 2005) and, importantly, inhibition of gastrointestinal motility (Duncan *et al*., 2005). Cannabinoid CB_1 and opioid receptors (μ-opioid receptor, δ-opioid receptor and KOR) are expressed presynaptically/prejunctionally and their activation elicits inhibition of excitatory transmission, which is associated with a decrease in acetylcholine release and a reduction in peristalsis (Coutts and Izzo, 2004; Wood and Galligan, 2004; Duncan *et al*., 2005; Izzo and Camilleri, 2008). Moreover, cannabinoid CB_1 receptor colocalization has been demonstrated with KOR in porcine cultured myenteric neurons (Kulkarni-Narla and Brown, 2001). Evidence has been provided demonstrating a functional cross-talk between the cannabinoid and opioid systems in the central nervous system (Corchero *et al*., 2004; Fattore

et al., 2004), although current evidence does not suggest a cross-talk between opioid and cannabinoid receptors in the digestive tract (Massa and Monory, 2006; Sanger, 2007). For example, Carai *et al*. (2006) showed that the cannabinoid CB_1 receptor antagonist rimonabant failed to block the inhibitory effect of morphine and loperamide on transit in small intestine. However, it should be emphasized that morphine and loperamide inhibit intestinal motility through activation of μ-opioid receptor and δ-opioid receptor, but not KOR (Hurwitz *et al*., 1994). Thus, there have been no functional studies that have examined specifically KOR and cannabinoid CB_1 interactions in the gastrointestinal tract to date, although an interaction between both KOR and the cannabinoid system on self-administration effects has been previously reported (Mendizábal *et al*., 2006).

In this study, we have shown that salvinorin A inhibited motility in the croton oil model of ileitis. The effect of salvinorin A was counteracted by both the selective KOR antagonist nor-binaltorphimine and the selective CB_1 receptor antagonist rimonabant. U-50488, a synthetic KOR agonist, also inhibited motility in a KOR- and a CB_1-antagonist sensitive manner. Collectively, such findings suggest a cross-talk between KORs and cannabinoid CB_1 receptors in the regulation of intestinal motility under inflammatory conditions. Because rimonabant is known to exert pro-kinetic effects both in control and in croton oil-treated mice (Izzo *et al*., 2001), here, in the agonist/antagonist studies, we used a dose of rimonabant ($0.1\,mg\,kg^{-1}$) that, *per se*, did not affect intestinal motility. In addition, we have recently shown that this dose of rimonabant did not modify the inhibitory effect of loperamide (μ-opioid receptor and δ-opioid receptor agonist) on intestinal motility in croton oil-treated animals (Capasso *et al*., 2008a). Collectively, these results suggest that the interaction between cannabinoid CB_1 receptors and KORs is specific and does not involve other opioid receptor subtypes. Interestingly, we have shown that the inhibitory effect on motility of the selective CB_1 receptor agonist ACEA in croton oil-treated mice was not modified by the selective KOR antagonist nor-binaltorphimine, suggesting an 'unidirectional' cross-talk. This is possibly because KORs are not overexpressed in myenteric nerves of mice treated with croton oil (Pol and Puig, 2004), whereas CB_1 receptors are overexpressed (Izzo *et al*., 2001). Indeed, the cannabinoid CB_1/KOR cross-talk is restricted to pathophysiological states only, as the inhibitory effect of salvinorin A (as well as the inhibitory effect of U-50488) on motility was not modified by rimonabant under physiological conditions, that is, in non-croton oil-treated mice. Again, the increased expression of CB_1 receptors in the small intestine of mice treated with oral croton oil (Izzo *et al*., 2001) could explain why the cannabinoid CB_1/KOR interaction is observed in intestinal pathophysiological states only. The study of the mode of action of salvinorin A in reducing motility in control mice represents a separate aim of investigation, which falls outside the scope of this work. Salvinorin A has been shown to reduce motility through a non-CB_1 non-KOR-mediated mechanism in control mice (Capasso *et al*., 2008b). Also, an involvement of CB_2 receptors seems very unlikely, as these receptors are involved in the control of intestinal motility in pathophysiological states only (Izzo and Camilleri, 2008; Wright *et al*., 2008).

We have recently shown that gut inflammation increases the potency of salvinorin A; the increased potency was mediated by KOR, but it was not shared by the prototypical KOR agonist U-50488, suggesting that salvinorin A may have target(s) other than KORs in the inflamed gut (Capasso *et al*., 2008b). Because the endocannabinoid system undergoes adaptive changes in the inflamed gut (that is, increased expression of CB_1 and CB_2 receptors, increased levels of endocannabinoids) (Di Marzo and Izzo, 2006; Izzo, 2007; Wright *et al*., 2008) and because the rewarding effects of salvinorin A in zebrafish and rats involve cannabinoid CB_1 receptors (Braida *et al*., 2007, 2008), we hypothesized one such target might be the endocannabinoid system. Thus, and in view of the results shown above, next we investigated the possibility that salvinorin A activates CB_1 or CB_2 receptors (which are known to reduce intestinal motility under inflamed conditions) (Di Marzo and Izzo, 2006; Izzo and Camilleri, 2008) either directly or by increasing the levels of endocannabinoids through inhibition of the mechanisms involved in their inactivation (that is, the putative EMT, FAAH and MAG lipase). Binding and enzymatic experiments revealed that salvinorin A had no effect on anandamide inactivation, nor any strong affinity for CB_1 receptors, although it did exhibit a very weak affinity for CB_2 receptors, which have been shown to be involved in the control of intestinal motility in the inflamed gut (Mathison *et al*., 2004; Duncan *et al*., 2008), including the croton oil model of intestinal inflammation (Capasso *et al*., 2008a). However, it is very unlikely that the weak affinity for CB_2 receptors could contribute to the inhibitory effect of salvinorin A on motility because its effect on transit was not modified by the selective CB_2 antagonist SR144528. The dose of SR144528 used in this study has been previously shown to counteract the inhibitory effect of the CB_2 receptor agonist JWH 015 on intestinal transit in croton oil-treated mice (Capasso *et al*., 2008a). These findings strongly suggest that (1) the observation that the effect of salvinorin A in croton oil-treated mice is also antagonized by rimonabant is not due to its direct or indirect activation of CB_1 receptors, as indicated also by the fact that rimonabant also antagonized the effect of U-50488, and (2) the higher potency of salvinorin A with respect to U-50488 under these inflammatory conditions is also not due to its additional activation of CB_1 receptors.

Although the present experiments allow us to exclude the possibility that salvinorin A binds to cannabinoid receptors or influences endocannabinoid transport and enzymatic degradation, the explanation of why intestinal inflammation increased the potency of salvinorin A but not that of the synthetic KOR agonist U-50488 previously reported (Capasso *et al*., 2008b) still remains to be investigated. However, it should be considered that salvinorin A was significantly more efficacious than the KOR agonist U-50488 (Chavkin *et al*., 2004) and binds to and activates KOR through hydrophobic—and not ionic—interactions (Grundmann *et al*., 2007). Moreover, salvinorin A is unusual as a KOR agonist as it is 40-fold less potent in promoting

internalization of the human KOR and causes less down-regulation of surface receptors than U-50488 (Wang *et al*., 2005).

Finally, to test whether the interaction between cannabinoid CB_1 receptors and KOR occurs at the level of enteric nerves, we performed experiments on the isolated mouse ileum. We found that salvinorin A inhibited EFS-induced contractions in this preparation. The IC_{50} value found in our study (3.2×10^{-9} M) was similar to that previously calculated in the isolated guinea-pig ileum (Capasso *et al*., 2006). However, compared with the guinea-pig ileum (Capasso *et al*., 2006), here we found a lower efficacy of salvinorin A at reducing EFS-induced contractions, as indicated by the E_{max} values (that is, 38% in the mouse and 56% in the guinea-pig). Nevertheless, these results are in line with the ability of KOR opioid agonists to produce a partial inhibition of electrically evoked acetylcholine release from guinea-pig myenteric neurons (Kojima *et al*., 1994). The inhibitory effect of salvinorin A on EFS-induced contractions was reduced by both the KOR antagonist nor-binaltorphimine and the cannabinoid CB_1 receptor antagonist rimonabant (Figure 4). These results suggest that the CB_1/KOR interaction occurs, at least in part, on enteric nerves. In contrast to the *in vivo* results, here we have found that the interaction between cannabinoid CB_1 receptors and KORs *in vitro* occurs also under physiological conditions. In addition, we found that concentrations of rimonabant higher than 3×10^{-8} M reduced EFS-induced contractions *in vitro*. This unexpected result is not in agreement with similar studies in the isolated guinea-pig ileum (Pertwee *et al*., 1996; Izzo *et al*., 1998; Begg *et al*., 2002) and, notably, with *in vivo* studies on motility in mice, which consistently showed a pro-kinetic effect of rimonabant (Calignano *et al*., 1997; Izzo *et al*., 2000; Carai *et al*., 2006). Discrepancies between *in vitro* and *in vivo* actions of cannabinoids, which have been previously documented in the digestive tract (Coruzzi *et al*., 2006; Capasso *et al*., 2008a), need further investigation.

In conclusion, from the study of the inhibition of intestinal motility by the hallucinogenic compound salvinorin A, we have shown, for the first time, a selective functional interaction between cannabinoid CB_1 receptors and KORs in the inflamed gut *in vivo*. The identification of the biochemical nature of this interaction will require additional *ad hoc* studies.

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Conflict of interest

The authors state no conflict of interest.

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