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# Inhibitory effect of palmitoylethanolamide on gastrointestinal motility in mice

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**1** We have studied the effect of palmitoylethanolamide (PEA, 2.5–30 mg kg$^{-1}$, i.p.) on upper gastrointestinal transit in control mice and in mice with chronic intestinal inflammation induced by croton oil.

**2** PEA significantly and dose-dependently decreased intestinal transit. The inhibitory effect of PEA (10 mg kg$^{-1}$) was not modified by the cannabinoid $CB_1$ receptor antagonist SR141716A (0.3 mg kg$^{-1}$, i.p.), the cannabinoid $CB_2$ receptor antagonist SR144528 (1 mg kg$^{-1}$, i.p.), $N^G$-nitro-L-arginine methyl ester (L-NAME, 25 mg kg$^{-1}$, i.p.), yohimbine (1 mg kg$^{-1}$, i.p.), naloxone (2 mg kg$^{-1}$, i.p.) or hexamethonium (1 mg kg$^{-1}$, i.p.).

**3** PEA levels were significantly decreased in the small intestine of croton oil-treated mice. In these animals, PEA also inhibited motility and this effect was not counteracted by SR141716A (0.3 mg kg$^{-1}$), or SR144528 (1 mg kg$^{-1}$).

**4** Pre-treatment of mice with the amidase inhibitor phenylmethyl sulphonil fluoride (PMSF, 30 mg kg$^{-1}$, i.p.) did not modify the inhibitory effect of PEA, either in control or in mice with inflammation.

**5** It is concluded that PEA inhibits intestinal motility with a peripheral mechanism independent from cannabinoid receptor activation. The decreased levels of PEA in croton oil-treated might contribute, at least in part, to the exaggerated transit observed during chronic intestinal inflammation.

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**Keywords:** Intestinal motility; cannabinoid receptors; anandamide; palmitoylethanolamide; intestine; inflammatory bowel disease; fatty acid amide hydrolase

**Abbreviations:** DMSO, dimethyl sulphoxide; L-NAME, $N^G$-nitro-L-arginine methyl ester; PEA, palmitoylethanolamide; PMSF, phenylmethyl sulphonil fluoride

## Introduction

Palmitoylethanolamide (PEA), a shorter and fully saturated analogue of the endocannabinoid anandamide, has been known for almost 40 years. It was first identified as the active principle of fractions with anti-inflammatory properties obtained from peanut oil, egg yolk and soybean lecithin (Kuehl *et al*., 1957). In 1965 Bachur and co-workers detected for the first time PEA in mammalian tissues, namely rat brain, liver and skeletal muscle (see for reference Lambert & Di Marzo, 1999). Since then, PEA has been detected in mouse peritoneal macrophages (Schmid *et al*., 1997), leukocytes (Bisogno *et al*., 1999), rat blood plasma (Giuffrida & Piomelli, 1998), canine heart (Epps *et al*., 1979), rat skin (Calignano *et al*., 1998) and rat testis (Kondo *et al*., 1998). PEA is produced in tissues through an enzymatic route similar to that of anandamide (Di Marzo *et al*., 1994) and can be co-released with anandamide from depolarized neurones (Di Marzo *et al*., 1994; Izzo *et al*., 2000).

Unlike anandamide, PEA does not bind efficiently to cannabinoid $CB_1$ and $CB_2$ receptors (Lambert & Di Marzo, 1999), but it does mimic anandamide in several assays; in fact, PEA, like anandamide, decreases spontaneous activity in mice (Adams *et al*., 1995), possesses analgesic (Calignano *et al*., 1998) and anti-inflammatory activity (Berdyshev *et al*., 1998) and relaxes the rat isolated mesenteric artery (White & Hiley, 1998). Anandamide reduces intestinal motility, both *in vitro* (Pertwee *et al*., 1995; Izzo *et al*., 1998) and *in vivo* (Fride, 1995; Calignano *et al*., 1997; Izzo *et al*., 2001a), while, to date, no data has been published on the effect of PEA on the digestive functions. In this study, we have evaluated the effect of PEA on upper gastrointestinal transit in mice; in addition, since PEA can be released after cellular injury (Lambert & Di Marzo, 1999), we have assayed endogenous PEA levels as well as investigated PEA effect on motility in a chronic model of intestinal inflammation. Preliminary accounts of some of these studies have been communicated

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## Methods

### *Animals*

Male ICR mice (Harlan Italy, Corezzana, MI) (24–26 g) were used after 1 week of acclimation (temperature $23\pm2^{\circ}C$; humidity 60%). Food was withheld 6 h before transit measurement and 18 h before the induction of chronic intestinal inflammation. All animal experiments complied with the Italian D.L. n 116 of 27 January 1992 and associated guidelines in the European Communities Council Directive of 24 November 1986 (86/609/ECC).

### *Chronic intestinal inflammation*

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

### *Upper gastrointestinal transit*

Gastrointestinal transit was measured in control and in croton oil-treated mice. At this time, 0.1 ml 10 g mouse$^{-1}$ of a black marker (10% charcoal suspension in 5% gum arabic) was administered orally to assess upper gastrointestinal transit as previously described (Puig & Pol, 1998). After 20 min the mice were killed by asphyxiation with $CO_2$ and the gastrointestinal tract removed. The distance travelled by the marker was measured and expressed as a percentage of the total length of the small intestine from pylorus to caecum.

### *Drug administration*

PEA (0.3–30 mg kg$^{-1}$) or vehicle (ethanol, 15 $\mu$l mouse$^{-1}$) were given intraperitoneally (i.p.) 20 min before charcoal administration, either to control mice or mice with chronic inflammation. In some experiments SR141716A (0.3 mg kg$^{-1}$), SR144528 (1 mg kg$^{-1}$), $N^G$-nitro-L-arginine methyl ester (25 mg kg$^{-1}$), yohimbine (1 mg kg$^{-1}$), naloxone (2 mg kg$^{-1}$), hexamethonium (1 mg kg$^{-1}$) or phenilmethylsulphonyl fluoride (PMSF, 30 mg kg$^{-1}$) were given (i.p.) before PEA administration (10 min before PEA for PMSF and 30 min before PEA for the other drugs). These doses were selected on the basis of previous published work (Santos & Rao, 1999; Izzo *et al*., 2001a; Wiley *et al*., 2000).

### *Identification and quantification of PEA*

The small intestines of control and croton oil-treated mice were removed and tissue specimens were immediately weighed, immersed into liquid nitrogen, then stored at $-70^{\circ}$ until analysis. Tissue was then extracted with chloroform/methanol (2 : 1, by volume) containing 5 nmol $d_4$-PEA, synthesized as described from $d_4$-palmitic acid (Cambridge, U.K.) and ethanolamine (Bisogno *et al*., 1997). The lipid extracts were purified by silica column chromatography and normal phase high pressure liquid chromatography (NP-HPLC), carried out as described previously (Bisogno *et al*., 1997). The fraction corresponding to PEA (retention time 26–27 min) was derivatized and analysed as the tris-methylsylyl-derivatives by isotope dilution gas chromatography-mass spectrometry (GC-MS) carried out in the selected monitoring mode as described in detail elsewhere (Bisogno *et al*., 1999). Selected ions were at m/z = 375 and 360, for $d_4$-PEA, and at m/z = 371 and 356, for endogenous PEA, and corresponded to the molecular ions or the loss of 15 mass units (loss of a methyl group). The areas of the peak at m/z = 360 and 356 were used for quantitative measurements. Results were expressed as pmol mg$^{-1}$ tissue.

### *Drugs*

Drugs used were: Palmitoylethanolamide (PEA) (Tocris Cookson, Bristol, U.K.), hexamethonium bromide, naloxone hydrochloride, $N^G$-nitro-methyl arginine methyl ester (L-NAME) hydrochloride, phenylmethylsulphonyl fluoride (PMSF), yohimbine hydrochloride (SIGMA, Milan, Italy). SR141716A [(N-piperidin-1-yl)-5-(4-chlorophenyl)-1-2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride and SR144528 (N-[-1S-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide-3-carboxamide) were a gift from Dr Madaleine Mossé and Dr Francis Barth (SANOFI-Recherche, Montpellier, France). PEA was dissolved in ethanol, SR141716A and SR144528 in dimethyl sulphoxide (DMSO), while the other drugs were dissolved in saline.

### *Statistics*

Data are means $\pm$ s.e.mean. To determine statistical significance, Student's *t*-test for unpaired data or one-way analysis of variance followed by Tukey-Kramer multiple comparisons test was used. A *P*-value less than 0.05 was considered significant. $ED_{50}$ (the dose of PEA that produced 50% of maximal inhibition of motility) values were calculated using the computer program of Tallarida & Murray (1986).

## Results

### *Effect of PEA on motility in control mice*

PEA (2.5–30 mg kg$^{-1}$) inhibited the gastrointestinal propulsion of charcoal in control mice (Figure 1) (% inhibition of motility: 2.5 mg kg$^{-1}$ $4\pm3\%$, 5 mg kg$^{-1}$ $15\pm3\%$, 10 mg kg$^{-1}$ $46\pm3\%$, 20 mg kg$^{-1}$ $59\pm3\%$, 30 mg kg$^{-1}$ $59\pm2\%$). The inhibitory effect of PEA was significant starting from the dose of 10 mg kg$^{-1}$. The inhibitory effect of PEA 10 mg kg$^{-1}$ was not significantly modified by the $CB_1$ receptor antagonist SR141716A (0.3 mg kg$^{-1}$) or by the $CB_2$ receptor antagonist SR144528 (1 mg kg$^{-1}$) (Table 1). L-NAME (25 mg kg$^{-1}$), yohimbine (1 mg kg$^{-1}$), naloxone (2 mg kg$^{-1}$) or hexamethonium (1 mg kg$^{-1}$) also did not modify the inhibitory effect of PEA 10 mg kg$^{-1}$ (Table 1). PMSF (30 mg kg$^{-1}$) did not modify significantly the inhibitory effect of PEA (2.5–30 mg kg$^{-1}$) on intestinal

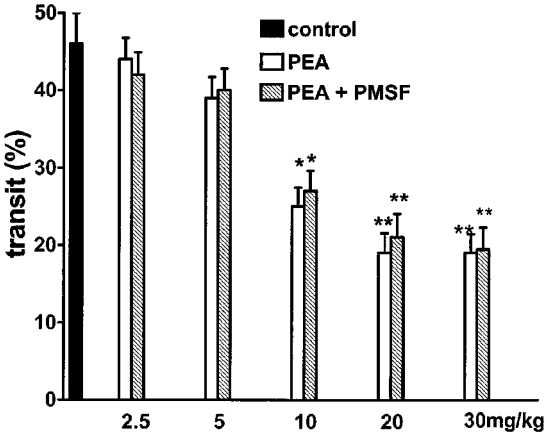


**Figure 1** Inhibitory effect of palmitoylethanolamide (PEA, 2.5–30 mg kg$^{-1}$, i.p.) on upper gastrointestinal transit: effect of a pretreatment with the amidase inhibitor phenylmethyl sulphonil fluoride (PMSF, 30 mg kg$^{-1}$ i.p.). Results are mean±s.e.mean of 10 animals for each experimental group. *$P<0.05$, **$P<0.01$ vs control.

**Table 1** Effect of PEA (10 mg kg$^{-1}$ i.p.) alone or in combination with various drugs on upper gastrointestinal transit in mice, either in control or in croton oil treated mice

| *Treatment* | *Dose* (mg kg$^{-1}$) | *Transit* (%) |
|---|---|---|
| Vehicle | | 48±3 |
| PEA | | 28±2* |
| PEA+SR141716A | 0.3 | 30±3* |
| PEA+SR1444528 | 1 | 25±3* |
| PEA+naloxone | 2 | 32±3* |
| PEA+yohimbine | 1 | 33±3* |
| PEA+L-NAME | 25 | 29±3* |
| PEA+hexamethonium | 1 | 35±2* |
| Croton oil | | 61±3* |
| +PEA | | 31±3# |
| +PEA+SR141716A | 0.3 | 31±3# |
| +PEA+SR144528 | 1 | 32±3# |

Results are mean±s.e.mean of 10 animals for each experimental group. *$P<0.05$ vs vehicle and #$P<0.05$ vs croton oil.

motility ($ED_{50}$ values (mg kg$^{-1}$) PEA 7.25±0.85; PMSF+PEA 7.55±0.91, $n=9-10$, $P>0.2$) (Figure 1).

In absence of any drugs (i.e. without PEA), SR141716A (0.3 mg kg$^{-1}$), SR144528 (1 mg kg$^{-1}$), L-NAME (25 mg kg$^{-1}$), yohimbine (1 mg kg$^{-1}$), naloxone (2 mg kg$^{-1}$), hexamethonium (1 mg kg$^{-1}$), PMSF (30 mg kg$^{-1}$), at the dosage used, did not modify significantly gastrointestinal transit (% transit: control: 48±4, SR141716A 59±4, SR144528 49±3, L-NAME 44±3, yohimbine 50±4, naloxone 51±3, hexamethonium 51±3, PMSF 50±5, $n=7-8$ for each experimental group). Ethanol (15 μl mouse$^{-1}$) or DMSO (10 μl mouse$^{-1}$) (alone or in combination) did not modify significantly intestinal motility, either in control or in croton-oil treated mice (data not shown).

### *Effect of PEA on motility in croton oil-treated mice*

As shown in Table 1, a charcoal meal administered intragastrically to control animals covered 48±3% of the total length of the small intestine in 20 min. Administration of croton oil (20 μl mouse$^{-1}$ on 2 consecutive days, 3 and 4 days before charcoal administration) induced a significant increase in gastrointestinal transit (Table 1). Pre-treatment of the animals with PEA (3–30 mg kg$^{-1}$) reversed the increase in gastrointestinal transit induced by croton oil in a dose-dependent fashion (Figure 2). The $ED_{50}$ value for PEA calculated in mice with inflammation (7.44±0.89 mg kg$^{-1}$) did not differ significantly ($P>0.2$, $n=10$) from the $ED_{50}$ value calculated in control mice (7.25±0.85 mg kg$^{-1}$). The inhibitory effect of PEA (10 mg kg$^{-1}$) was not significantly modified either by SR141716A (0.3 mg kg$^{-1}$) or by SR144528 (1 mg kg$^{-1}$) (Table 1). As in the case of control mice, PMSF (30 mg kg$^{-1}$) did not modify significantly the inhibitory effect of PEA (2.5–30 mg kg$^{-1}$) on intestinal motility in mice with intestinal inflammation (Figure 2) ($ED_{50}$ values (mg kg$^{-1}$): croton oil: 7.44±0.89, croton oil+PMSF 7.03±0.77, $n=10$, $P>0.2$).

SR141716A (0.3 mg kg$^{-1}$), SR144528 (1 mg kg$^{-1}$) or PMSF (30 mg kg$^{-1}$), administered alone, did not modify significantly gastrointestinal transit in croton oil-treated mice (data not shown).

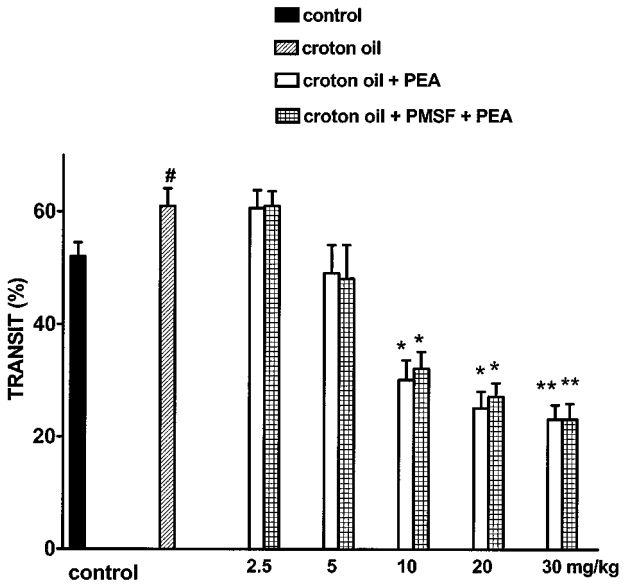


**Figure 2** Croton oil-treated mice: effect of the amidase inhibitor phenylmethyl sulphonil fluoride (PMSF, 30 mg kg$^{-1}$ i.p.) on palmitoylethanolamide (PEA, 2.5–30 mg kg$^{-1}$, i.p.)-induced delay in upper gastrointestinal transit. Results are mean±s.e.mean of 10 animals for each experimental group. #$P<0.05$ vs control, *$P<0.05$ and **$P<0.01$ vs croton oil.

### *PEA levels in the small intestine*

Figure 3 shows that PEA was detected in the small intestine of control mice (77.5±6.3 pmol g$^{-1}$ tissue) and that this production was significantly ($P<0.05$) decreased in the small intestine of croton oil-treated mice.

## Discussion

PEA is an endogenous fatty acid ethanolamide that shares some pharmacological actions with $\Delta^9$-tetrahydrocannabinol,

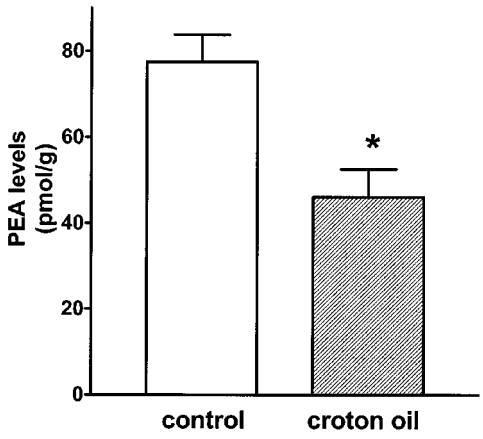


**Figure 3** Levels of palmitoylethanolamide (PEA) in the mouse small intestine during control and croton oil-induced gut inflammation. Results are mean ± s.e.mean of 4–5 animals for each experimental group. $*P<0.05$ vs control.

the main ingredient of marijuana, and with the endocannabinoids anandamide and 2-arachidonylglycerol (Lambert & Di Marzo, 1999). However, PEA does not bind to cannabinoid $CB_1$ and $CB_2$ receptors and therefore it cannot be defined as an 'endocannabinoid' according to the definition given by Di Marzo (1998). Previous studies have shown that PEA possesses analgesic (Calignano *et al*., 1998), anti-inflammatory (Berdyshev *et al*., 1998) and central depressant activity (Adams *et al*., 1995). In the present study, we have revealed its presence in the small intestine and its ability to reduce intestinal motility, both during physiological conditions and in a pathological state.

### *Control mice*

Previous studies have shown that anandamide as well as other natural and synthetic cannabinoid agonists, by activating enteric $CB_1$ receptors, can reduce intestinal motility either *in vitro* (Pertwee *et al*., 1995; Izzo *et al*., 1998) or *in vivo* (Fride, 1995; Calignano *et al*., 1997; Izzo *et al*., 2001a). In the present study we have shown that the selective cannabinoid $CB_1$ receptor antagonist SR141716A, at doses able to counteract the inhibitory effect of anandamide (Izzo *et al*., 2001a), was not able to counteract the inhibitory effect of PEA on intestinal motility. There is also some evidence in literature that some effect of PEA can be mediated by as-yet uncharacterized '$CB_2$-like' receptors, because some pharmacological effects of PEA can be counteracted by the selective $CB_2$ receptor antagonist SR144528 (Facci *et al*., 1995; Calignano *et al*., 1998). In the present study, however, the effect of PEA on intestinal motility was not modified by SR144528. The dose of SR144528 used in the present study was 10 fold higher than the dose of SR144528 able to counteract the analgesic effect of PEA (Calignano *et al*., 1998). Collectively, these results indicate that the effect of PEA on intestinal motility is not mediated by activation of cannabinoid receptors. Presynaptic/prejunctional systems, such as $\alpha_2$-adrenoceptors or opioid receptors, which, if activated, are known to inhibit intestinal motility, are not involved in the inhibitory effect of PEA. In fact, naloxone or yohimbine, antagonists of opioid or $\alpha_2$-adrenoceptors, respectively, failed to modify PEA-induced changes in motility. In addition, the effect of PEA was not modified by the ganglion blocker hexamethonium, thus suggesting a peripheral site of action. Moreover, it is unlikely that the inhibitory effect of PEA could derive from modulation of NO production, as pre-treatment of mice with the NO synthase inhibitor L-NAME did not modify PEA-induced changes in motility. Others have shown that PEA inhibits NO production in murine macrophages and that this effect does not appear to be mediated by cannabinoid receptors (Ross *et al*., 2000).

PMSF is a non-specific irreversible amidase inhibitor that inhibits the action of fatty acid amide hydrolase. Previous investigators have shown that PMSF enhanced the pharmacological activity of anandamide (Wiley *et al*., 2000; Lambert & Di Marzo, 1999), including its ability to reduce intestinal motility (Pertwee *et al*., 1995). In the present study, PMSF, at doses previously shown to be effective (Wiley *et al*., 2000), did not modify the inhibitory effect of PEA on intestinal motility. The lack of effect of PMSF is not surprising in the light of the observation the PEA is not hydrolyzed by fatty acid amide hydrolase as efficiently as anandamide (Lambert & Di Marzo, 1999), and that another amidase insensitive to PMSF has been identified for PEA (Ueda *et al*., 1999).

### *Mice with intestinal inflammation*

Croton oil is an irritant that produces experimental chronic inflammation in the mouse small intestine. Inflammation is characterized by a clear disruption of the mucosa and an infiltration of lymphocyte in the submucosa (Puig & Pol, 1998). Macroscopic observation and increased wet weight, which is considered a reliable and sensitive indicator of the severity and extent of the inflammatory response, confirmed that inflammation occurred in our experimental conditions. Previous investigators have shown that the chronic intestinal inflammation induced by two consecutive doses of croton oil given 24 h apart (as in this study), produces maximal inflammatory response and maximal increase in gastrointestinal motility 4 days after the first dose of croton oil (Puig & Pol, 1998). Therefore the influence of PEA on intestinal motility, as well as the levels of PEA in the small intestine, were studied at this time point.

We have recently shown that chronic inflammation enhances the potency of cannabinoid receptor agonists on intestinal motility by up-regulating $CB_1$ receptor expression in the small intestine (Izzo *et al*., 2001b). In the present study we have observed that PEA decreased intestinal motility in mice with inflammation, although with the same potency observed in control mice. In addition, consistent with the results obtained in control mice, the inhibitory effect of PEA was not mediated by activation of cannabinoid receptors and remained unchanged after pretreatment with the amidase inhibitor PMSF.

PEA has been detected in the rat brain, liver, skin, testis and skeletal muscle, in the canine heart and in mouse peritoneal macrophages, and it can be released following cellular injury (for review see Lambert & Di Marzo, 1999). Previous studies have also shown that PEA can be released with the endocannabinoid anandamide from different mammalian tissues (Di Marzo *et al*., 1994). We have recently detected anandamide in the small intestine of both control

mice and in mice treated with croton oil (as in the present study) (Izzo *et al*., 2001b). However, no significant differences were observed in anandamide levels between control and inflamed mice (Izzo *et al*., 2001b). In the present study we have detected PEA in the small intestine of both control and inflamed mice and, in addition, we have observed a significant reduction of PEA level in the small intestine of croton oil-treated mice. The amounts of PEA that we found in the small intestine from control animals was higher than the levels previously described for anandamide in the same tissue (Izzo *et al*., 2001b) and in the high pmol $g^{-1}$ tissue range of concentrations. If we assume an equal distribution of PEA throughout the small intestine, these amounts would correspond to an average concentration of about 70 nM, which would be not enough to influence the activity of $CB_1$ or $CB_2$ receptors, in agreement with the non-$CB_1$, non-$CB_2$ mechanism of action suggested here for exogenous PEA effect. In fact, some effect of PEA on cannabinoid receptors was only observed at concentrations $>10$ $\mu$M (Griffin *et al*., 2000). Nevertheless, this concentration of PEA might be sufficient to activate yet to be discovered sites of action for this compound, possibly involved in the control of intestinal motility. If this was the case, the decrease of PEA observed here in inflamed small intestine might contribute in part to the increased motility observed in croton oil-treated mice.

## *Conclusions*

In the present study we have shown that PEA is able to inhibit intestinal motility in mice with a mechanism independent from cannabinoid receptor activation, under both physiological and pathological conditions. The decreased levels of PEA in croton oil-treated mice might contribute, at least in part, to the exaggerated transit observed during chronic inflammation. The previously undetected effect of PEA on intestinal motility broadens its pharmacological spectrum of actions and opens the possibility that this compound, which, unlike anandamide, has weak psychotropic effects, is used as a possible therapeutic drug for the treatment of intestinal hypermotility during inflammatory bowel diseases.

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