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The endocannabinoid system and the molecular basis of paralytic ileus in mice

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ABSTRACT

The endocannabinoid system (i.e., the cannabinoid receptors and their endogenous ligands) plays an important role in the physiological control of intestinal motility. However, its participation in intestinal pathological states is still poorly understood. In the present study, we investigated the possible role of the endocannabinoid system in the pathogenesis of paralytic ileus, a pathological state consisting of decreased intestinal motility following peritonitis, surgery, or other noxious situations. Ileus was induced by i.p. administration of acetic acid, and gastrointestinal propulsion was assessed by the charcoal method. Endocannabinoid levels were measured by isotope-dilution gas chromatography-mass spectrometry, whereas cannabinoid CB_1 receptors were identified by immunohistochemistry. Acetic acid administration inhibited gastrointestinal transit (ileus), and this effect was accompanied by increased levels of the endocannabinoid anandamide compared with control mice and by overexpression of CB_1 receptors in myenteric nerves. Furthermore, acetic acid-induced ileus was alleviated by the CB_1 receptor antagonist SR141716A and worsened by VDM11, a selective inhibitor of anandamide cellular uptake (and hence inactivation). From these findings, it can be concluded that the intestinal hypomotility typical of paralytic ileus is due, at least in part, to the enhancement of anandamide levels and CB_1 expression during this condition, and that selective, nonpsychotropic CB_1 receptor antagonists could represent new drugs to treat this disorder.

Key words: anandamide transport • cannabinoid receptors • intestine • myenteric plexus

Paralytic ileus (also called adynamic, or digestive, ileus) is defined as a long-lasting inhibition of gastrointestinal motility observed in response to nociception initiated at the abdominal level. The many situations that can provoke paralytic ileus include peritonitis, trauma to the nerves supplying the gut wall during intra-abdominal surgery, decreased blood supply to the intestinal wall, spinal injury, pneumonia, pancreatitis, and myocardial infarction (1, 2). Patients with this disorder accumulate gas and secretions, leading to bloating, distension, emesis, and visceral pain (2). Two types of experimental models for paralytic ileus are commonly used. One model is based on abdominal surgery, and another model is based on peritoneal irritation. Both ileus models share numerous common pathophysiological features despite involving different stimuli (mechanical vs. chemical).

The etiology of ileus is poorly understood. It is believed that this clinical problem is due, at least in part, to activation of a neural reflex initiated by peritoneal incision or irritation (3). The afferent limb of this reflex might be due to stimulation of capsaicin-sensitive nerve fibers (with opioid κ receptors having an important role) (4, 5), whereas the efferent limb seems to involve sympathetic adrenergic neurones (with activation of α_2-adrenoceptors on the intrinsic cholinergic neurones) (3, 6, 7). Corticotropin releasing factor has been proposed as a link between the afferent and efferent limbs of the inhibitory nervous pathways, both in surgically (8) and chemically induced ileus (9). Enteric inhibitory nerves (7) and local factors such as inflammation (10), associated with induction of cycloxygenase-2 (11) and nitric oxide syntase (12), might also play a causative role.

Cannabis preparations have been used for millennia to reduce diarrhea and intestinal motility (13), due to the presence in this plant of the psychoactive natural product, Δ^9-tetrahydrocannabinol (THC) and to the existence in mammalian enteric neurones of selective receptors for THC, the cannabinoid CB_1 receptor (14, 15). Endogenous agonists for cannabinoid receptors (namely anandamide and 2-arachidonoylglycerol [2-AG]) have been identified in the rodent gut (16), and there is increasing evidence for the participation of at least one of these compounds, that is, anandamide, in the physiological regulation of intestinal motility (17). Like THC, anandamide, by acting at enteric CB_1 receptors, significantly inhibits intestinal motility in mice (18–20). Unlike THC, however, anandamide is subjected to rapid inactivation consisting of cellular reuptake, mediated by the anandamide membrane transporter (AMT), followed by intracellular hydrolysis, catalyzed by anandamide amidohydrolase (also known as fatty acid amide hydrolase; see ref 21 for review).

In the present study, we have investigated for the first time the possibility that alterations in the enteric endocannabinoid system (i.e., the cannabinoid receptors and their endogenous ligands) contribute to causing paralytic ileus. Apart from the inhibitory effects of endocannabinoids on intestinal motility (14, 15), this possibility also is supported by the finding of enhanced endocannabinoid levels during nociception and tissue damage (21). We employed a widely used mouse model of ileus, consisting of the nociceptive stimulation of the peritoneum by i.p. acetic acid (4, 5, 9, 22). We have measured, both in control and acetic acid-treated mice, the levels of the endocannabinoids and the activity of anandamide amidohydrolase, and have analyzed, by immunohistochemistry, the expression and localization of CB_1 receptors in the mouse small intestine in relation to cholinergic and/or substance P-releasing neurons. Furthermore, we have evaluated in both conditions the effect on intestinal motility of the selective CB_1 receptor

antagonist SR141716A (23) and of the selective inhibitor of the AMT (and, hence, of anandamide inactivation) VDM11 (24). We report data strongly suggesting that increased stimulation of enteric cannabinoid CB_1 receptors by anandamide is in part responsible for paralytic ileus in mice.

MATERIALS AND METHODS

Animals

Male ICR mice (Harlan Italy, Corezzana, MI) (20–25 g) were used after 1 wk of acclimation (temperature 23 ± 2°C; humidity 60%; 12-h light/dark cycles). All the experiments were performed after deprivation of food but not water for an 18-h period.

Ileus

Ileus was induced by i.p. administration of acetic acid (120 mM in saline, 0.1 ml/10 g mouse), and motility was studied 30 min later (4, 22). Control animals received saline.

Upper gastrointestinal transit

Gastrointestinal transit was measured 30 min after induction of peritoneal irritation as previously described (3, 20). At this time, 0.1 ml (10 g/mouse) of a black marker (10% charcoal suspension in 5% gum arabic) was administered orally, and after 20 min, the mice were killed by asphyxiation with CO_2 and the small intestine was 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.

Intracerebroventricular injections

Intracerebroventricular (i.c.v.) injections were performed as described by Haley and McCormich (25). Mice were anaesthetized with enflurane, and the drugs were delivered in a volume of 4 μl, using a Hamilton microliter syringe fitted with a 26-gauge needle. The site of injection was 2 mm caudal and 2 mm lateral to bregma and 3 mm in depth from the skull surface. Animals fully recovered within 10 min after the injection. In preliminary experiments, the correct location of the i.c.v. injection was verified by injecting 5 μl Evans blue dye (0.5%). The brains were then removed and examined to verify dye distribution.

Drug administration

SR141716A (1–20 mg/kg), SR144528 (1–10 mg/kg), ACEA (0.1–1 mg/kg), JWH-015 (10 mg/kg), VDM11 (10 mg/kg), carbachol (0.03 mg/kg), or vehicle (saline, dimethyl sulfoxide [DMSO], or ethanol, 10 μl/mouse) was given i.p. 20 min before acetic acid administration. In some experiments, ACEA (0.3 mg/kg), JWH-015 (10 mg/kg), or hexamethonium (10 mg/kg) was given i.p. 10 min before SR141716A (10 mg/kg, i.p.). In other experiments, SR141716A (1–20 mg/kg, i.p.) (4 μl/mouse) was given i.c.v. 20 min before acetic acid administration. SR141716A (1 mg/kg, i.p.) was also given 10 min before VDM11 (10 mg/kg, i.p.). SR141716A,

SR144528, and VDM11 were also evaluated in control mice (i.p., 20 min before saline). The doses of VDM11, SR144528, JWH-015, and hexamethonium were selected on the basis of previous work (16, 17, 26, 27). The dose of carbachol was selected in order to give approximately the same increase in motility as SR141716A.

Morphology

The small intestine was removed 30 min after induction of peritoneal irritation and was fixed with paraformaldehyde 4% for 48 h. Six cross sections were cut (each 6 μm thick) from the approximate midportion of the intestine and stained with hematoxylin and eosin to distinguish cell types. The sections were analyzed by using a standard light microscope (×20 objective), photographed under low power, videodigitized, and stored in the image analysis system (Qwin Lite 2.2, Leica).

Measurement of anandamide, 2-AG and palmitoylethanolamide (PEA)

The small intestines of the mice were removed, and the tissue specimens were immediately weighed, immersed in liquid nitrogen, and stored at –70° until lipid extraction. Tissues were extracted with chloroform/methanol (2:1, by volume) containing 1 nmol each of octa-deuterated (d_8) anandamide, tetra-deuterated (d_4) PEA, and d_8-2-AG, synthesized as described from the corresponding deuterated fatty acids and either ethanolamine or glycerol (28). The lipid extracts were purified by silica column chromatography and normal-phase high-pressure liquid chromatography (NP-HPLC), carried out as described previously (28), and the fractions corresponding to either anandamide/PEA (retention time 26–27 min) or 2-AG (retention time 18–22 min) were derivatized and analyzed by isotope dilution gas chromatography-mass spectrometry (GC-MS) carried out in the selected monitoring mode as described previously (29). Results were expressed as pmol or nmol per g wet tissue weight. Because during tissue extraction/purification, both d_8- and native 2-AG are partly transformed into the 1(3)-isomers (which are eluted from the GC column 0.5 min later), and only a little arachidonic acid is present on the *sn*-1(3) position of (phospho)glycerides, the amounts of 2-AG reported here were calculated from the combined mono-arachidonoyl-glycerol peaks.

Anandamide amidohydrolase activity

To measure anandamide amidohydrolase activity, [^{14}C]anandamide (5 mCi/mmol), synthesized as described previously from [^{14}C]ethanolamine and arachidonic acid (28), was used as the substrate at a 50 μM concentration. Membrane fractions prepared from small intestine of either control or acetic acid-treated mice were assayed (28).The assay was carried out in 50 mM Tris-HCl (pH 9), at 37°C for 30 min. The [^{14}C]ethanolamine produced from the reaction, quantified as described previously (28), was used as a measure of [^{14}C]anandamide hydrolyzed. The activity was expressed as pmol of [^{14}C]ethanolamine produced per mg protein × min.

Immunohistochemistry

Samples collected from the middle jejunum were immediately fixed for 2 h in 2% paraformaldheyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4) on ice, washed several

times in the same buffer, and stored in PBS containing increasing concentrations of sucrose (10–30%) at 4°C.

Frozen sections 10 μm thick were cut on a cryostat and mounted on slides pretreated with Vectabond (Vector, Burlingame, CA). The remaining part, stored in oxygenated Krebs on ice, was used for preparing laminar preparations. For whole-mount preparations, the jejunal segments were opened along the mesenteric border, stretched, and pinned flat (with the mucosal side down) onto strips of sylgard silicone rubber, fixed as described above for 1 h, and stored in 0.05% solution of sodium azide in PBS until further processing. Before staining, the lamina comprising the longitudinal and circular musculature with the myenteric plexus included was dissected out from the lamina submucosa/mucosa under a Nikon stereomicroscope.

The following antibodies were used: a rabbit polyclonal antiserum raised against the extracellular N-terminal sequence of human CB_1-receptors, at the dilution of 1:600 (Cayman Chemicals, Ann Arbor, MI); a goat polyclonal anti-human choline acetyltransferase (ChAT) antibody, at a dilution of 1:50 (Chemicon International, Temecula, CA); and a rat monoclonal anti-substance P antibody, at a dilution of 1:100. The secondary incubations comprised secondary antibodies (affinity-purified immunoglobulins G obtained from donkey: Jackson Immunoresearch, West Grove, PA) conjugated to fluorescein isotiocyanate (FITC), to Rhodamine Red-X, and 7 amino-4-methylcoumarin-3-acetic acid (AMCA). To chemically characterize CB_1-receptors-expressing neurons, we carried out double indirect immunofluorescent labelings as previously reported (17). In brief, tissue sections were rehydrated in PBS (pH 7.4) for 10 min, then incubated in a 0.4% Triton X-100 and 3% bovine serum albumin (BSA) (Sigma, St. Louis, MO) in PBS for 30 min to block nonspecific binding. Sections were simultaneously incubated with sets of two different primary antisera raised in rabbit and rat or goat, diluted together in 0.4% Triton X-100 and 3% BSA overnight at room temperature. After three rinses in PBS, sections were simultaneously incubated with appropriate secondary antibodies. Affinity-purified IgG preparations (from donkey) were used as secondary antibodies, conjugated to the fluorochromes FITC, rhodamine, and AMCA in PBS for 1 h in the dark. After several rinses in PBS, coverslips were mounted with Vectashield (Vector). The preparations had been preadsorbed on solid phase with serum proteins from multiple species (including donor species of the other antisera to be used at the same time). Secondary reagents were generally diluted together.

Controls included substitution of the primary antiserum with PBS. Possible cross-reactivities between secondary reagents and primary reagents from inappropriate species were checked on sections. All controls gave negative results. Whole-mount preparations were incubated in a 0.4% Triton X-100 and 3% BSA in PBS for 2 h as free-floating sections, then incubated with sets of two different primary antisera raised in rabbit and rat or goat, diluted in the same medium over three nights. Then, the material was rinsed in PBS several times and incubated overnight with appropriate secondary antibodies. All incubations were carried out at 4°C.

Slides were observed with a Nikon Eclipse 600 microscope, equipped with 100-W high-pressure mercury lamps. Photography was carried out with a Nikon photographic system. Provia 400 transparency film (Fuji, Tokyo, Japan) and Kodachrome 400 were used. Images were digitized using a FS 2710 scanner (Canon, Japan) and the CanoCraft FS software interface provided by the

same factory and were further processed with Adobe Photoshop software (version 6.0, Adobe Systems, San Jose, CA).

Drugs

Drugs used: ACEA (all Z)-N-(2-chloroehyl)-5,8,11,14-eicosatetraenamide) and JWH-015 (2-methyl-1-propyl-1*H*-indol-3-yl)-1-naphthalenylmethanone were purchased from Tocris Cookson (Bristol, UK), and hexamethonium bromide and carbachol were purchased from 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 Mossè and Dr. Francis Barth (SANOFI-Recherche, Montpellier, France). VDM11 was synthesized as described previously (24). SR141716A and SR144528 were dissolved in DMSO; ACEA and VDM11 were dissolved in ethanol; and carbachol and hexamethonium were dissolved in saline.

Statistics

Data are mean ±SE. To determine statistical significance, the unpaired Student's *t* test or one-way ANOVA followed by Tukey-Kramer multiple comparisons test were used. A P value <0.05 was considered significant. ED_{50} (the dose of SR141716A that produced 50% of maximal increase of motility) values were calculated using the computer program of Tallarida and Murray (30).

RESULTS

Acetic acid administration

Administration, i.p., of acetic acid induced a mild inflammatory response (Fig. 1) in the small intestine, which was associated with a strong reduction of gastrointestinal transit. Representative cross sections of the jejunum are shown in Figure 1. A mild infiltration of leukocytes can be observed in sections of intestine obtained from mice subjected to peritonitis (n=6) compared with the sections of intestine of control mice (n=6) (Fig. 1).

Endocannabinoids and amidohydrolase activity

Table 1 shows that anandamide, 2-AG, and PEA were detected in control tissues and that the levels of anandamide (but not 2-AG or PEA) were significantly increased in the small intestine of acetic acid-treated mice. By contrast, no significant difference in amidohydrolase activity was observed between control and acetic acid-treated mice.

Immunohistochemistry

In control animals, CB_1-receptor immunoreactivity (CB_1-IR) was observed in neural perikarya and fibers of the myenteric plexus, in nerve fibers running through the circular muscle layer (Fig. 2), and, rarely, in nerve fibers through the submucosa and mucosa.

Double labeling showed CB_1-IR in ChAT-immunoreactive (ChAT-IR) myenteric perikarya, a marker of cholinergic neurons (Fig. 2b and 2b'; Fig. 2c and 2c') and in fibers to the circular muscle (Fig. 2c and 2c'). A few CB_1-IR fibers, which were not cholinergic, as well as cholinergic nerve fibers that did not exhibit CB_1-IR, were also observed (Fig. 2c and 2c'). CB_1-IR was also exhibited by substance P-IR and non-IR perikarya of the myenteric plexus (Fig. 2d and 2d').

In acetic acid-treated animals, a slightly increased neural density of CB_1-IR was observed within the interganglionic strands and the ganglia of the myenteric plexus (Fig. 2e, 2f, and 2h), in nerve fibers to the circular muscle (Fig. 2e and 2h), in submucosal neural perikarya and fibers (Fig. 2g), and in nerve fibers that extended up the mucosa, among the glands (Fig. 2i). CB_1-IR was observed in myenteric (Fig. 2f and 2f') and submucosal ChAT-reactive perikarya (Fig. 2g and 2g'), in substance P-IR perikarya and nerve fibers contained within the myenteric plexus (Fig. 2h and 2h'; Fig. 2i and 2i'), as well as in a part of substance P-containing nerve bundles and fibers to the circular muscle layer (Fig. 2h and 2h'). The CB_1-containing periglandular nerve fibers did not exhibit substance P-IR (Fig. 2i and 2i').

Effect of the CB_1 receptor antagonist SR141716A on gastrointestinal transit

The cannabinoid CB_1 receptor antagonist SR141716A (1–20 mg/kg, i.p.), but not the selective CB_2 antagonist SR144528 (10 mg/kg, i.p.), significantly and dose-dependently increased intestinal motility in mice with peritoneal irritation (Fig. 3). Lower doses of SR144528 (1 and 3 mg/kg) were without effect (% transit: ileus 25±4; ileus +SR144528 1 mg/kg 26±5; ileus + SR144528 3 mg/kg 23±5; n=6, P>0.2). SR141716A was significantly more potent in control mice (ED_{50} 0.406 ± 0.07 mg/kg) than in acetic acid-treated mice (ED_{50} 2.59 ± 0.29 mg/kg). The selective CB_2 receptor antagonist SR144528 (10 mg/kg, i.p.) was without effect also in control mice (data not shown).

The effect of SR141716A (10 mg/kg, i.p.) was reduced by a per se ineffective dose of the selective CB_1 receptor agonist ACEA (0.3 mg/kg, i.p.), but not by the selective CB_2 receptor agonist JWH-015 (10 mg/kg, i.p.) (Fig. 4). Higher doses of ACEA administered alone (1 mg/kg, i.p.) further delayed the impaired motility in acetic acid-treated mice (% transit: ileus 26 ± 3; ileus + ACEA 16 ± 2; n=12, P<0.05), whereas JWH-015 (10 mg/kg, i.p.), given alone, was without effect (% transit: ileus 26 ± 3; ileus + JWH-015 27 ± 4; n=12, P>0.2).

ACEA (0.3 mg/kg, i.p.) was unable to modify the prokinetic effect of carbachol (0.03 mg/kg, IP) in acid acetic-treated mice (% transit: ileus 24 ± 4; ileus + carbachol 46 ± 4 ; ileus + carbachol + ACEA 42 ± 4; n=12).

Hexamethonium (10 mg/kg, i.p.) did not modify the gastrointestinal transit in acetic acid-treated mice (% transit: ileus 26 ± 3; ileus + hexamethonium 28 ± 2; n=12), nor it significantly modify the prokinetic effect of SR141716A (10 mg/kg, i.p.) (% transit: ileus 25 ± 2; ileus + SR141716A 49 ± 7; ileus + SR141716A + hexamethonium 46 ± 6; n=12).

SR141716A was approximately equipotent in increasing gastrointestinal transit after both i.p. and i.c.v. routes of administration in acetic acid-treated mice. The ED_{50} value of i.c.v.-administered SR141716A was 2.01 ± 0.25 mg/kg, which was not statistically different from the

ED_{50} value of SR141716A observed after i.p. administration (2.59 ± 0.29 mg/kg). Saline, DMSO, and ethanol (4 μl/mouse, i.c.v., or 10 μl/mouse, i.p.) had no effect on the response under study either in control mice or in acetic acid-treated mice (data not shown).

Effect of the anandamide transporter inhibitor VDM11 on gastrointestinal transit

The selective anandamide transporter inhibitor, VDM11 (10 mg/kg, i.p.), did not modify the gastrointestinal transit in control mice (% transit: control 54 ± 4; VDM11 44 ± 4; n=10, P>0.2), but it further delayed gastrointestinal transit in acetic acid-treated mice in a way sensitive to SR141716A (10 mg/kg, i.p.) (Fig. 5).

DISCUSSION

Paralytic ileus is a common complication whose pathogenesis is still under debate. Several mechanisms for this disorder have been proposed, including the involvement of facilitated extrinsic and intrinsic neural activity, the release of inhibitory humoral agents, and other factors such as local inflammation (see introduction). In the present work, we provide, for the first time, functional, biochemical and immunohistochemical evidence that alterations in the enteric endocannabinoid system contribute to causing paralytic ileus.

We found that acetic acid administration (i.p.), which induces peritonitis, strongly inhibited intestinal motility, thus resulting in paralytic ileus. This pathological state was accompanied by significantly increased intestinal levels of anandamide, but not of 2-AG, compared with the small intestine of saline-treated mice. We did not observe significant differences between control and acetic acid-treated mice in small intestine anandamide amidohydrolase activity, which, at any rate, was sufficient to ensure the degradation of anandamide under both physiological and pathological conditions. These findings suggest that the increase of anandamide levels found in the intestine of acetic acid-treated mice was not due to a reduction of the rate of its inactivation by FAAH. By contrast, in a previous study (16), croton oil-induced small intestine inflammation in mice, which is accompanied by increased intestinal motility, was found to cause an enhancement of anandamide amidohydrolase activity and no change in anandamide levels. It is very unlikely that the anandamide congener, PEA (which is inactive at cannabinoid receptors and reduces intestinal motility via an unknown mechanism [31]), contributes to motility changes in ileus, because we did not observe any difference in its levels between control and acetic acid-treated mice. However, we cannot rule out that the other endocannabinoid 2-AG, whose levels in the small intestine were not increased following acetic acid treatment but were nevertheless already very high in control mice, might also participate in generating paralytic ileus.

Anandamide is known to inhibit intestinal motility through activation of enteric CB_1 receptors (14, 15, 19, 20). In a variety of species, acetylcholine and substance P have been shown to be the major excitatory neurotransmitters of motor neurons in the gastrointestinal tract (32–35). Acetylcholine is also the primary motor neurotransmitter for the external muscle of the mouse intestine (36–39) and partially coexists with substance P in a large subpopulation of neurons that project for long distances (40). Previous immunohistochemical studies have found CB_1 receptors on enteric nerves (17, 41–44), with the majority of CB_1 receptor-expressing neurons being cholinergic (17, 42). In the present study, we have observed an increased number and density of

CB_1 receptors in both myenteric plexus and in nerve bundles and fibers of the external muscle of the jejunum in acid acetic-treated mice compared with controls, thus strongly suggesting an overexpression of these receptors. Such overexpression of CB_1 receptors pairs with the increased levels of anandamide observed in acetic acid-treated mice, thus potentially resulting in the overstimulation of the endocannabinoid system in myenteric neurons, which coexpress CB_1 receptors with acetylcholine and substance P. Because CB_1 receptor stimulation by various agonists, including anandamide, reduces acetylcholine release from enteric nerves and inhibits nonadrenergic noncholinergic (NANC) excitatory trasmission (which is mediated by the release of endogenous tackykinins such as substance P) (45–48), we hypothesize that, following peritonitis-induced ileus, overactivity of CB_1 receptors on the enteric cholinergic/substance P neurons leads to a reduced release of both neurotransmitters, with subsequent delayed motility. However, we cannot rule out the possibility that yet other mechanism(s), such as non-CB_1 and non-CB_2 receptors for anandamide (49), might contribute to anandamide-induced delay in small intestine transit. Furthermore, in our study, CB_1 receptors were also found in nonchemically identified myenteric neurons, both in control and treated animals. It is unlikely that such neurons are nitrergic because, in the mouse colon, CB_1 receptor and NO synthase immunoreactivities did not overlap with myenteric or submucosal neurones and fibers (17). Finally, we did not address the possibility that cannabinoid CB_2 receptors, for which 2-AG acts as a more efficacious agonist than anandamide, might also be regulated during ileus. Indeed, with the exception of one study (50), CB_2 receptors have not been implicated so far in the control of intestinal motility.

In order to demonstrate the existence of a cause-and-effect relationship between the enhancement of anandamide/CB_1 receptor levels in acetic acid-treated vs. saline-treated mice and decreased intestinal motility, we carried out a series of pharmacological experiments. First, we studied the effect of the selective CB_1 receptor antagonist SR141716A. If the enhanced endocannabinoid tone observed in the small intestine of acetic acid-treated mice was causative of the decreased intestinal motility, we should have observed an alleviation of this typical sign of paralytic ileus, as was indeed the case. As previously reported, ileus was quantified in terms of gastrointestinal transit assessed using a charcoal “meal” (3). This method is not quantitative in the same sense that radioisotopic methods are (51) because it relies merely on the leading edge of the visible marker. Nevertheless, our data clearly show that the leading front of the charcoal progressed significantly less in acetic acid-treated animals and that SR141716A restored a normal intestinal motility. This effect was very likely mediated uniquely by the blockade of cannabinoid CB_1 (and not CB_2) receptors because it was counteracted by a per se ineffective dose of the selective cannabinoid CB_1 receptor agonist, ACEA, but not by the selective cannabinoid CB_2 receptor JWH-015. Moreover, although activation of cannabinoid CB_2 receptors was shown to inhibit intestinal motility (50), the selective CB_2 receptor antagonist SR144528 did not modify acetic acid-induced ileus.

In agreement with previous observations (16), we found that SR141716A enhances small intestine transit also in vehicle-treated mice. Indeed, the antagonist was significantly more potent in control than in acetic acid-treated mice. Although in most, but not all, previous studies, lower doses of SR141716A were found to be needed to observe significant results, this different potency is possibly due to the increased levels of anandamide and CB_1 receptors that we observed in acetic acid-treated mice. In fact, a greater amount of antagonist is expected to be needed to counteract the effect of higher amounts of anandamide on overexpressed enteric CB_1

receptors. Therefore, the low potency of SR141716A in acetic acid-treated mice lends further, albeit indirect, support to our hypothesis that an enhanced anandamide tone is partly responsible for reduced intestinal motility during paralytic ileus.

With the aim of further challenging this hypothesis, we wanted to assess whether the pharmacological manipulation not only of anandamide CB_1-mediated actions with SR141716A, but also of anandamide levels with an inhibitor of its inactivation, would result in an effect on intestinal motility during ileus. We reasoned that any agent that prolongs the effects of endogenous anandamide by selectively retarding its degradation would worsen the intestinal hypomotility in acetic acid-treated mice. Anandamide is removed from its extracellular sites of action by cellular uptake, which is facilitated by the AMT and driven by intracellular anandamide hydrolysis catalyzed by FAAH (21). Thus, anandamide reuptake by cells represents the first and key step in the termination of anandamide actions at CB_1 receptors. Therefore, we tested the selective inhibitor of the AMT, VDM11 (24), on intestinal motility. We found that this compound delayed gastrointestinal transit in acetic acid-treated, but not in control, mice, thus indicating not only a functional role of anandamide transport in terminating the biological action of anandamide during paralytic ileus, but also the participation of endogenous anandamide in the reduction of motility during this pathological state. The effect of VDM11 was fully antagonized by a submaximal dose of SR141716A, indicating that it was due to the enhancement of endocannabinoid levels, and therefore to indirect activation of CB_1 receptors. Importantly, this compound has been found so far to exert anandamide-like effects only in those pathological conditions in which an enhanced endocannabinoid tone was demonstrated (26, 27).

Previous investigators have given significant attention to the central mechanisms that might cause ileus (9); thus, we were interested to determine to what extent the effect of SR141716A is due to blockade of a central or a peripheral site of action. This is because the CB_1 receptor is located both in the central and in the enteric nervous system (14, 15). In previous work, carried out in control mice, we showed that SR141716A was significantly more active in increasing motility in mice when administered i.c.v. than when administered i.p. The higher central-to-peripheral potency of SR141716A suggested a central site of action (52). However, central CB_1 receptors probably contribute little to the effects of peripherally administered SR141716A in control mice, as the effect of i.p.-injected SR141716A was not modified by the ganglionic blocker hexamethonium (52). Likewise, in the present study, the prokinetic effect of SR141716A very probably involves, at least in part, peripheral (enteric) CB_1 receptors because SR141716A, when injected i.c.v., increased transit, but only at doses that were also active when injected i.p. (i.e., SR141716A was equally active after i.p. or i.c.v. administration; it is very likely that SR141716A diffuses out of the brain and undergoes distribution equal to that induced by i.p. injection) and because hexamethonium, at doses previously shown to antagonize the effect of i.c.v.-injected cannabinoid drugs (17), did not alter the effect of i.p.-injected SR141716A. The lack of effect of hexamethonium on acetic acid-induced decreased motility, when administered alone, is somewhat surprising in the light of the accepted hypothesis of an important role of nicotinic receptors is the control of intestinal motility, during both physiological and pathological states (e.g., ileus). The reasons of this discrepancy remain to be examined. However, it has been reported that hexamethonium does not modify small intestinal transit either in rats (53) or mice (20) under physiological conditions.

In conclusion, we have shown here that paralytic ileus-induced decreased intestinal motility in response to peritoneal irritation is due, at least in part, to increased levels in the small intestine of anandamide, which contributes to reducing motility by acting on overexpressed enteric CB_1 receptors. The causative role of anandamide in paralytic ileus is suggested not only by its increased levels during peritonitis, but also by functional studies on motility with the blocker of anandamide uptake VDM11 (which worsens transit), and with the selective CB_1 receptor antagonist SR141716A (which restores the impaired motility). Our data not only provide new insights in the possible etiology of paralytic ileus, but they also open the possibility for the use of selective, nonpsychotropic CB_1 receptor antagonists, or yet to be developed inhibitors of anandamide biosynthesis, as new pharmacological tools for the clinical management of paralytic ileus.

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Table 1

Levels of anandamide, 2-arachidonylglycerol, palmitoylethanolamide, and anandamide amidohydrolase activity in the mouse small intestine during control and ileus conditions[a]

	Control	Ileus
Anandamide (pmol/g/tissue)	25±2.4	56.3±5.4*
2-Arachidonylglycerol (nmol/g/tissue)	31.1±7.7	37.8±8.7
Palmitoylethanolamide (pmol/g/tissue)	598.8±82.9	696.9±84.3
Anandamide amidohydrolase (pmol/mg protein × min)	12.7±2.8	11.9±2.9

[a]Results are expressed as the mean ±SE of six animals. *P<0.05 vs. control. On average, 1 g of tissue yields 50–100 mg of protein. In a previous study (16), lower anandamide amidohydrolase activity was detected in the mouse small intestine, probably due to the use of a lower concentration of substrate.

Fig. 1

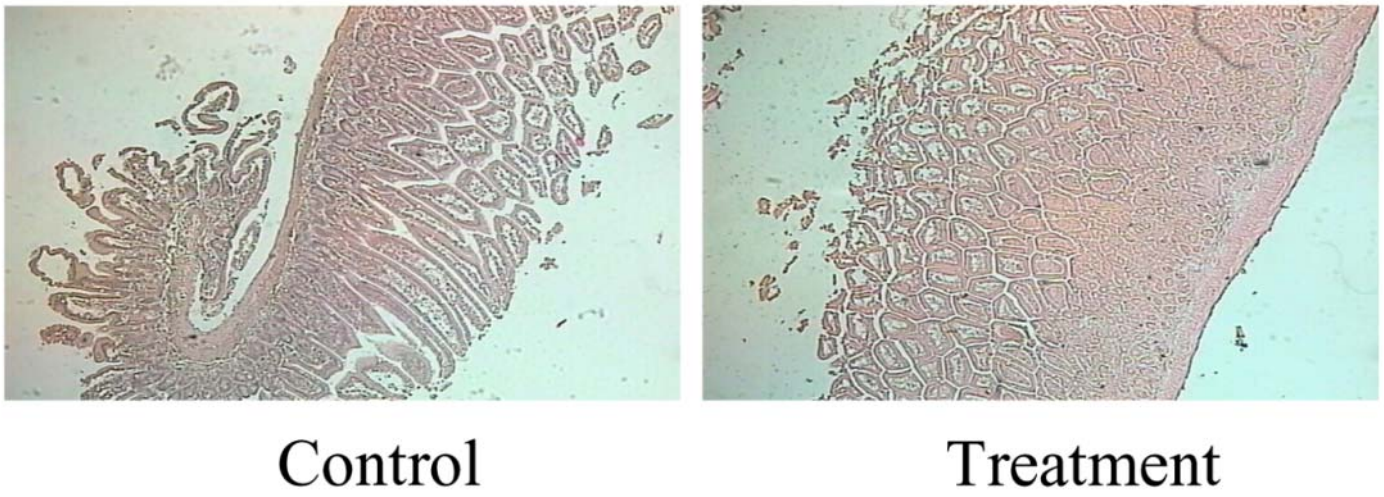

Figure 1. Photomicrographs showing representative cross sections (20x magnification) of jejunum from control and acetic acid-treated mice. The photographs are representative of six experiments.

Fig. 2

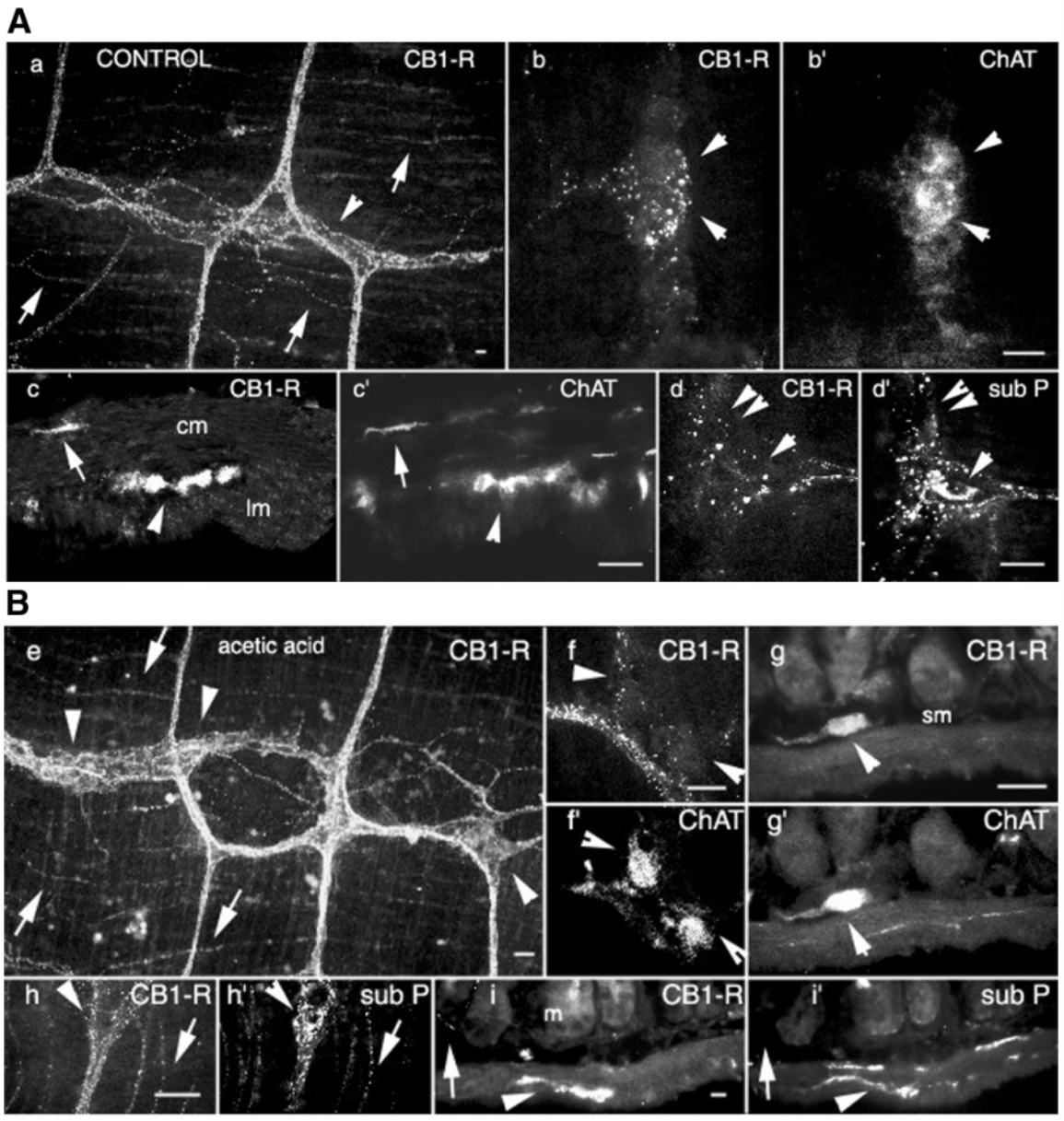

Figure 2. A) General distribution of CB_1 receptor (CB1-R) immunoreactivity (CB_1-IR) in laminar preparation of the myenteric plexus in the small intestine of control animals. CB_1-IR are found in nerve fibers of the strands, in weakly reactive and nonreactive ganglionic perikarya (arrowheads), and in nerve bundles and fibers to the circular muscle layer (arrows). CB_1 receptors are present in ChAT- (b-b', c-c') myenteric neural perikarya and in nerve fibers to the circular muscle layer (c-c': arrows) as well as in substance P myenteric containing perikarya (d-d'). CB_1 receptors are also present in nonreactive myenteric neurons (d-d': arrows). a,b-b', d-d': laminar preparations; c-c': cryosections. **B)** General distribution of CB_1-IR in laminar preparation of the myenteric plexus in the small intestine of acetic acid-treated animals (e): A higher density and immunoreactive intensity is found in myenteric neural perikarya (arrowheads) and in nerve bundles and fibers to the circular muscle layer (e: arrows). Immunoreactive CB_1 receptors are observed in ChAT-ir perikarya in the myenteric (f-f: arrowheads) and submucous (g-g': arrowheads) plexuses. CB_1-IR is also observed in substance P-myenteric neurons (h-h', i-i': arrowheads) and in some nerve bundles and fibers to the circular muscle layer (h-h': arrows). CB_1-IR nerve fibers to the mucosal glands do not contain substance P (i-i': arrows). e, f-f', h-h': laminar preparations; g-g', i-i': cryosections. Calibration bar: 100 μm. ChAT, choline acetyl transferase; lm, longitudinal muscle; cm, circular muscle; sm, submucosa; m, mucosa.

Fig. 3

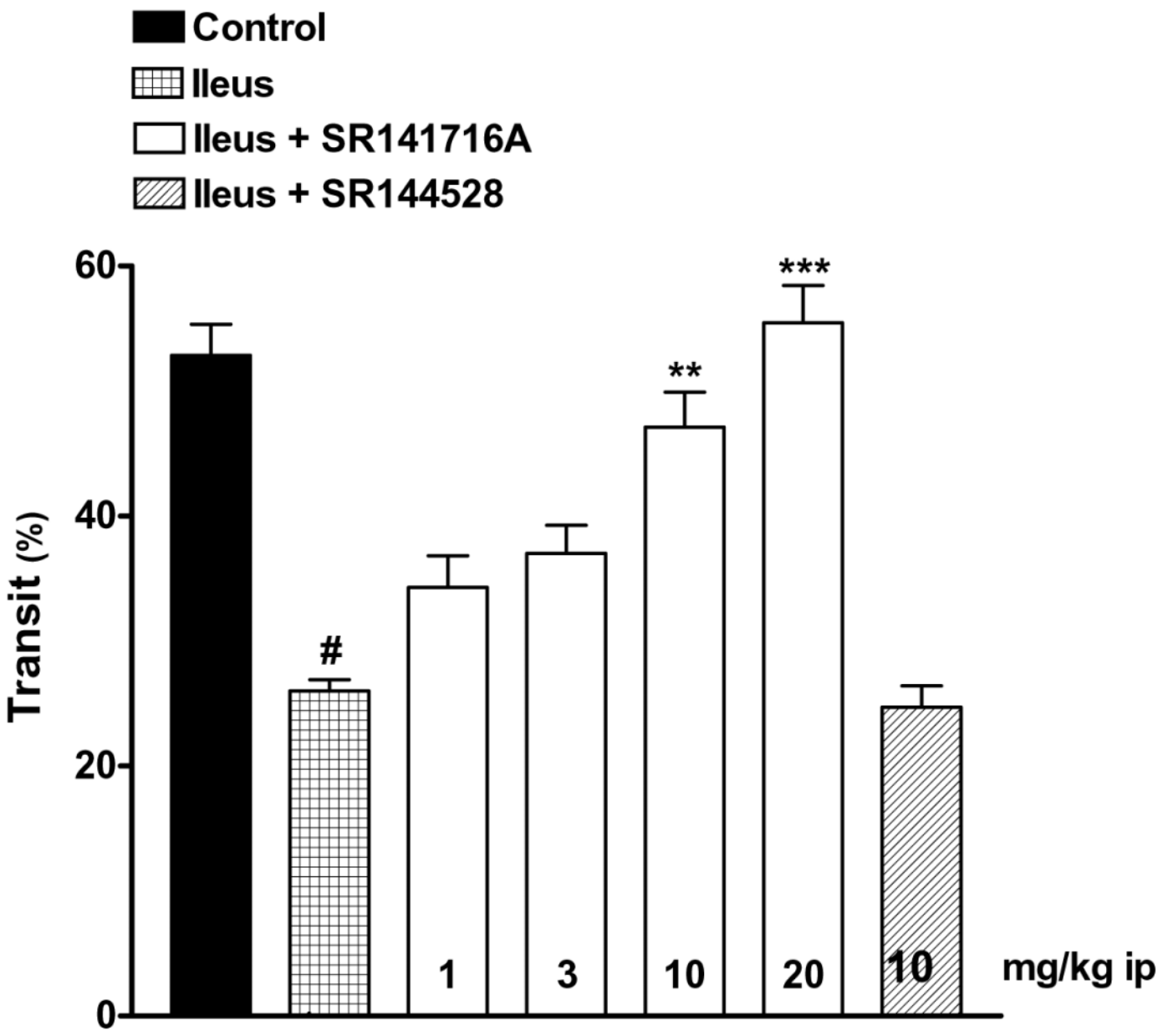

Figure 3. Effect of the CB_1 receptor antagonist SR141716A (1–20 mg/kg, i.p.) or SR144528 (10 mg/kg, i.p.) on gastrointestinal transit in experimental ileus induced by acetic acid in mice. SR141716A (or SR144528) was given 20 min before acetic acid administration. Each point represents the mean ±SE of the data obtained from 10–12 mice. #P<0.01 vs. control **P<0.01 and ***P<0.001 vs. ileus.

Fig. 4

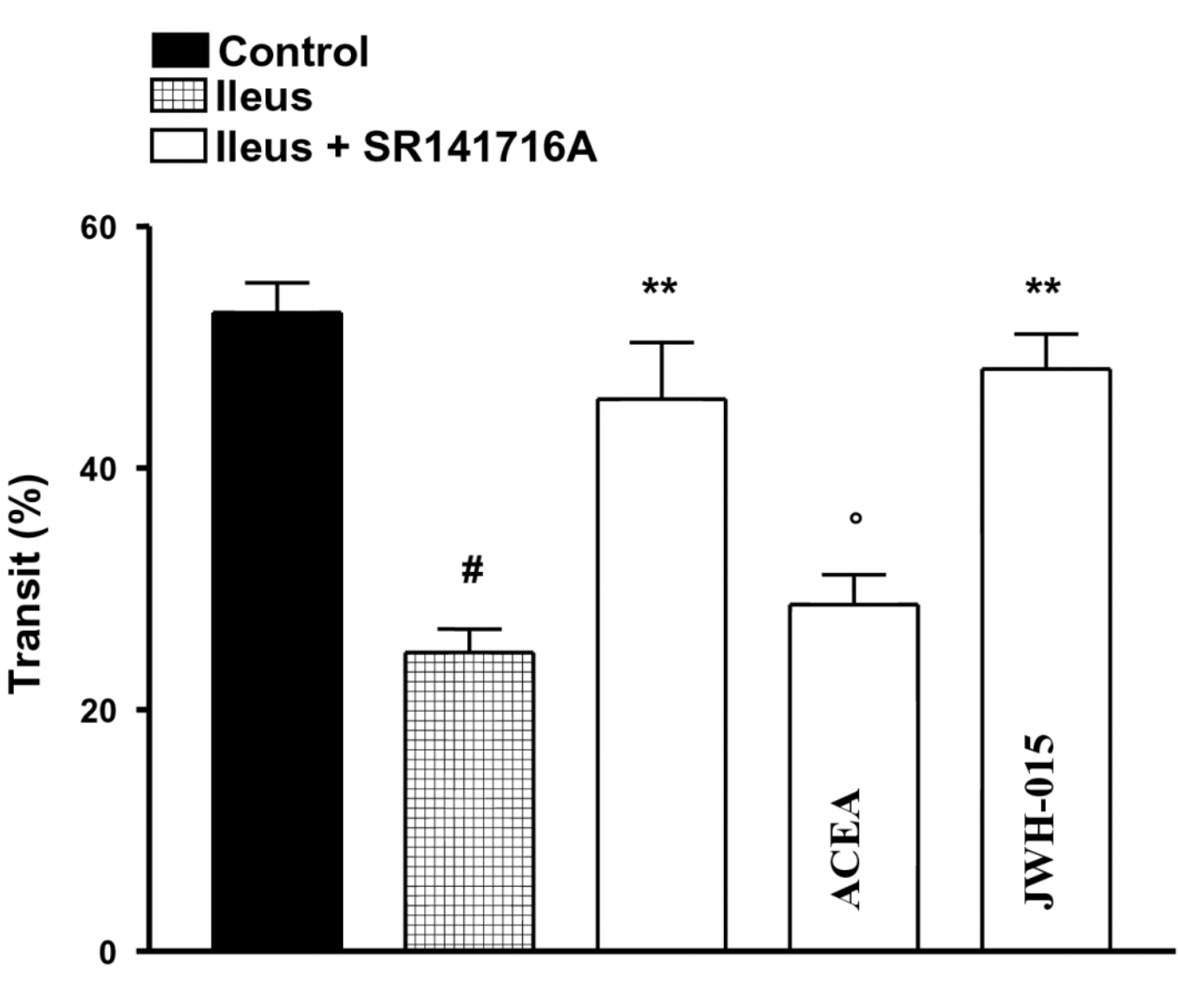

Figure 4. Prokinetic effect of SR141716A (10 mg/kg, i.p.) alone or in mice pretreated with the CB_1 receptor agonist ACEA (0.3 mg/kg, i.p.) or the CB_2 receptor agonist JWH-015 (10 mg/kg, i.p.). ACEA (or JWH-015) was given 10 min before SR141716A. SR141716A was given 20 min before acetic acid administration. Each point represents the mean ±SE of the data obtained from 10–12 mice. #P<0.001 vs. control; **P<0.01 vs. ileus; °P<0.01 vs. ileus + SR141716A.

Fig. 5

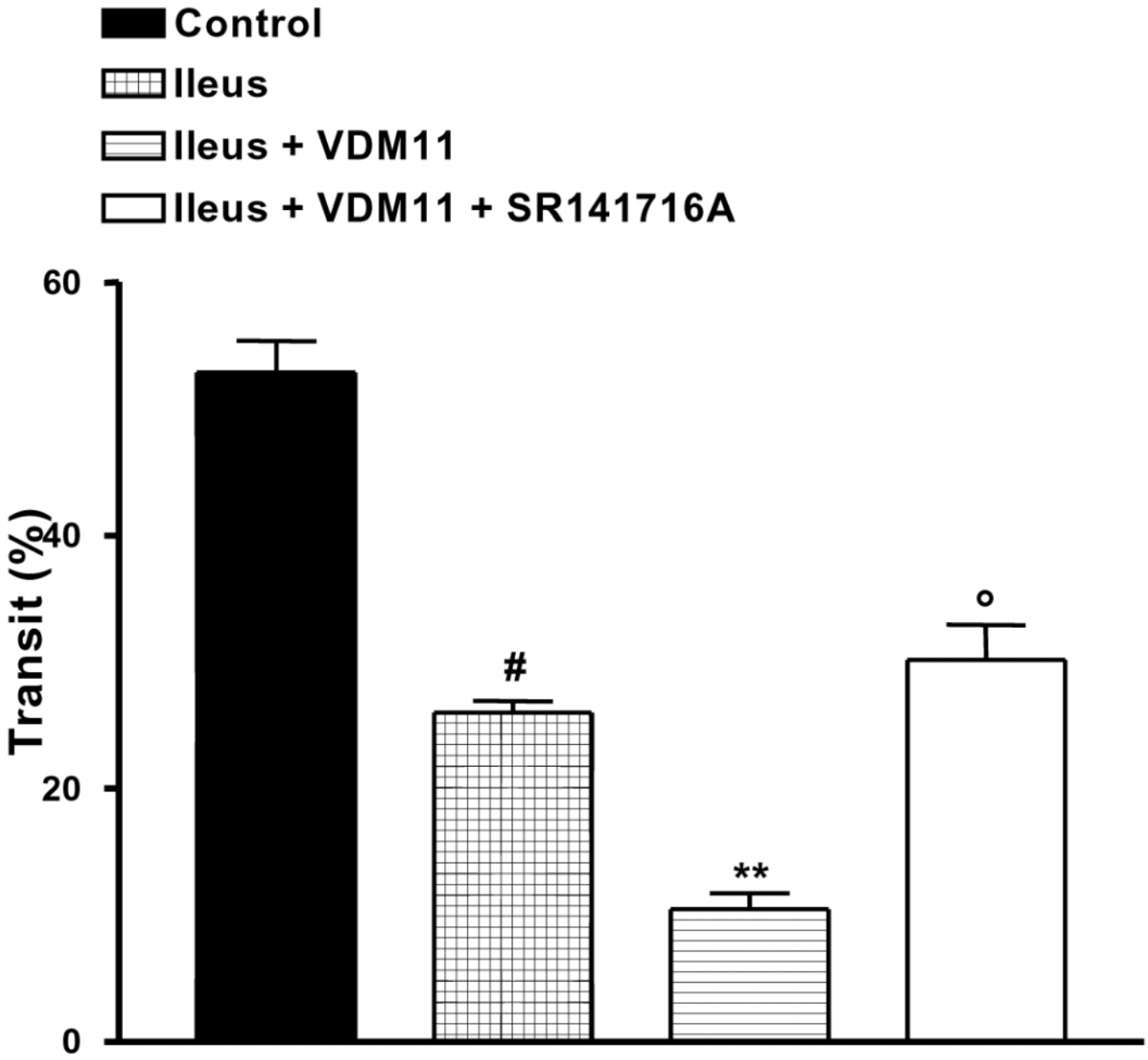

Figure 5. Effect of the anandamide membrane transporter inhibitor VDM11 (10 mg/kg, i.p.), alone or in mice pretreated with the CB_1 receptor agonist SR141716A (1 mg/kg, i.p.) on gastrointestinal transit in experimental ileus induced by acetic acid in mice. VDM 11 was given 20 min before acetic acid administration. SR141716A was given 10 min before VDM11 (i.e., 30 min before acetic acid administration). Each point represents the mean ±SE of the data obtained from 10–12 mice. #P<0.001 vs. control; **P<0.01 vs. ileus; °P<0.01 vs. ileus + VDM11.