BASIC–ALIMENTARY TRACT

An Endogenous Cannabinoid Tone Attenuates Cholera Toxin–Induced Fluid Accumulation in Mice

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See editorial on page 973.

Background & Aims: Cholera toxin (CT) is the most recognizable enterotoxin causing secretory diarrhea, a major cause of infant morbidity and mortality throughout the world. In this study, we investigated the role of the endogenous cannabinoid system (i.e., the cannabinoid receptors and their endogenous ligands) in CT-induced fluid accumulation in the mouse small intestine. *Methods:* Fluid accumulation was evaluated by enteropooling; endocannabinoid levels were measured by isotope-dilution gas chromatography mass spectrometry; CB_1 receptors were localized by immunohistochemistry and their messenger RNA (mRNA) levels were quantified by reverse-transcription polymerase chain reaction (PCR). *Results:* Oral administration of CT to mice resulted in an increase in fluid accumulation in the small intestine and in increased levels of the endogenous cannabinoid, anandamide, and increased expression of the cannabinoid CB_1 receptor mRNA. The cannabinoid receptor agonist CP55,940 and the selective cannabinoid CB_1 receptor agonist arachidonoyl-chloro-ethanolamide inhibited CT-induced fluid accumulation, and this effect was counteracted by the CB_1 receptor antagonist SR141716A, but not by the CB_2 receptor antagonist SR144528. SR141716A, per se, but not the vanilloid VR1 receptor antagonist capsazepine, enhanced fluid accumulation induced by CT, whereas the selective inhibitor of anandamide cellular uptake, VDM11, prevented CT-induced fluid accumulation. *Conclusions:* These results indicate that CT, along with enhanced intestinal secretion, causes overstimulation of endocannabinoid signaling with an antisecretory role in the small intestine.

Diarrheal diseases continue to be a major cause of morbidity and mortality with a sadly impressive 3 million deaths per year seen in children worldwide.[1] These diseases are also a problem for people traveling to developing countries and have a major impact on military operations.[2,3] Cholera toxin (CT) is the most recognizable enterotoxin causing secretory diarrhea. Watery diarrhea in cholera reflects massive secretion of fluid and electrolytes by the small intestine, which is caused by the binding of *Vibrio cholerae* enterotoxin to GM_1-ganglioside receptors in the brush border membrane, resulting in adenylate cyclase activation and increased intracellular concentration of adenosine 3′,5′-cyclic monophosphate.[4,5] The profound dehydrating secretory diarrhea associated with CT may involve several intestinal secretory mechanisms, including activation of enteric neurones[6,7] and release and/or synthesis of endogenous secretagogues such as 5-hydroxytriptamine, prostaglandins, tackykinins, vasoactive intestinal polypeptide, and platelet activating factor.[8–15] When the level of fluid secretion increases beyond the ability of the colon to reabsorb water and electrolytes lost from the small intestine, diarrhea can lead to severe dehydration and eventually to death.[1]

Cannabis preparations have been used for millennia to treat a wide array of health problems, including many

Abbreviations in this paper: ACEA, arachidonoyl-chloroethanolamide; ChAT, choline acetyltransferase; CT, cholera toxin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; 2-AG, 2-arachidonylglycerol.

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gastrointestinal diseases.[16] Extracts of *Cannabis* were indicated for the treatment of diarrhea a century ago in the United States[17] and there are a number of anecdotal accounts of the effective use of *Cannabis* against dysentery and cholera.[18] Molecular targets of Δ^9-tetrahydrocannabinol (Δ^9-THC), the main active ingredient of *Cannabis*, are at least 2 types of receptors, that is, the cannabinoid CB_1 and CB_2 receptors, both of which are coupled to $G_{i/0}$ proteins.[19–21] CB_1 receptors are expressed mostly by central and peripheral neurons,[20,22] including human enteric nerves,[23,24] whereas CB_2 receptors are located mostly on immune cells.[22,25] Anandamide, 2-arachidonylglycerol (2-AG), and noladin ether are endogenous agonists for cannabinoid receptors (endocannabinoids),[21,26–28] the former 2 compounds having been detected also in animal intestinal tissues.[26,29–31] In vitro studies have shown that activation of CB_1 receptors in isolated rat intestinal tissues produces an antisecretory effect through a neural mechanism, which in all likelihood involves the inhibition of acetylcholine release from neurons of the submucosal plexus.[32]

In the present study we investigated the possible involvement of the endogenous cannabinoid system (i.e., the cannabinoid receptors and their endogenous ligands) in CT-induced intestinal secretion in mice. For this purpose we have used the nonselective cannabinoid agonist CP55,940, the selective CB_1 receptor agonist arachidonoyl-chloro-ethanolamide (ACEA), the selective CB_2 receptor agonist JWH-015, the selective CB_1 receptor and CB_2 receptor antagonists, SR141716A and SR144528, respectively, and the selective inhibitor of anandamide cellular re-uptake (and, hence, inactivation) VDM11.[22,33,34] In addition, we have measured the levels of endocannabinoids and the activity of anandamide amidohydrolase in both control and CT-treated mice, and have analyzed by immunohistochemistry the localization of CB_1 receptors in the mouse small intestine, and quantified CB_1 messenger RNA (mRNA) levels by reverse-transcription polymerase chain reaction (PCR).

Materials and Methods

Animals

Male ICR mice (Harlan Italy, Corezzana, MI) (18–22 g) generally were used. Mice were fed ad libitum with standard mouse food, except for the 16-hour period immediately preceding the experiments; water was available continuously during the 16-hour food deprivation period, but it was removed after CT administration.

Cholera Toxin–Induced Fluid Accumulation

Evaluation of the intraluminal accumulation of fluid into the small intestine was performed using the enteropooling technique.[35–37] Briefly, enteropooling is defined as the intraluminal accumulation of fluid into the small intestine and reflects, among other factors, the sum of the fluid excreted from the blood to the lumen and, to a lesser extent, the portion of fluid already in the lumen. CT (10 μg/mouse in 0.3 mL Tris buffer 0.05 mol/L, pH 7.4) was delivered by an orogastric feeding tube. After 6 hours of incubation, the entire mouse small intestine from the pylorus to the ileocecal junction was isolated, taking care to avoid tissue rupture and loss of fluid. The attached mesentery and connective tissue then was removed and the tissue was patted dry. To normalize the data, fluid accumulation was expressed as follows:[36]

$$(W_1 - W_2) \times 10^{-6}/W_2$$

where W_1 is the weight of the ileum after excision and W_2 is the weight of the ileum after expulsion of its content. The dose of CT administered and the time of incubation were selected on the basis of a previous report.[37]

Drug Administration

CP55,940 (0.03–3 mg/kg), ACEA (0.1–10 mg/kg), JWH-015 (1–10 mg/kg), SR141716A (0.1–3 mg/kg), SR144528 (0.1–3 mg/kg), VDM11 (10 mg/kg), chlorisondamine, capsazepine (3–30 mg/kg), or vehicle (either saline, dimethyl sulfoxide, or ethanol, 10 μL/mouse) were given intraperitoneally (IP) 30 minutes before CT (or vehicle) administration.

In other experiments, cannabinoid receptor antagonists (SR141716A or SR144528, at a dose of 0.3 and 3 mg/kg, respectively) or chlorisondamine (5 mg/kg) were given IP 10 minutes before the cannabinoid agonists. The dose of VDM11 and chlorisondamine were selected on the basis of a previous report.[30,38]

Identification and Quantification of Anandamide and 2-Arachidonylglycerol

The small intestines from either control or CT-treated mice were removed (6 hours after the oral administration of CT or vehicle) and tissue specimens were weighed immediately, immersed into liquid nitrogen, and stored at –70° until chromatographic separation of endocannabinoids. Tissues were extracted with chloroform/methanol (2:1, by volume) containing 1 nmol each of d_8-anandamide, d_4-palmitoylethanolamide, and d_8-2-AG, synthesized as described previously.[39] The lipid extracts were purified by silica column chromatography and normal-phase high-pressure liquid chromatography, performed as described previously,[39] and the fractions corresponding to either anandamide/palmitoylethanolamide (retention time 26–27 minutes) or 2-AG (retention time 18–22 minutes) were derivatized and analyzed by isotope dilution gas chromatography-mass spectrometry performed in the selected monitoring mode as described in detail elsewhere.[40] Results were expressed as pmol/g or nmol/g of wet tissue. As 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 minutes later), and little arachidonic acid is present on the *sn*-1(3) position of (phospho)glycerides, the amounts of 2-AG reported here represent the combined mono-arachidonyl-glycerol peaks.

Anandamide Amidohydrolase Activity

To measure anandamide amidohydrolase activity, [^{14}C]anandamide (5 mCi mmol^{-1}), synthesized as described previously from [^{14}C]ethanolamine and arachidonic acid, was used as the radioligand at a 10 μmol/L concentration. Membrane fractions prepared from small intestine of either control or CT-treated mice were assayed (6 hours after the oral administration of CT or vehicle).[39] The assay was performed in 50 mmol/L Tris-HCl, pH 9, at 37°C for 30 minutes. [^{14}C]ethanolamine produced from the reaction was quantified as described previously,[39] and the activity was expressed as pmol of [^{14}C]ethanolamine produced min/mg/protein.

Immunohistochemistry

Samples were taken from the middle jejunum of 5 controls and 10 CT-treated (6 hours after the oral administration of CT or vehicle) animals that had been anesthetized with isofluorane and euthanized by cervical dislocation. The material was fixed immediately for 2 hours in 2% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (PBS) (pH 7.4) on ice, washed several times in the same buffer, and transferred to PBS of increasing concentrations of sucrose (10% to 30%), at 4°C. Frozen sections of 10 μm thickness were cut in a cryostat and mounted onto slides pretreated with Vectabond (Vector Laboratory, Burlingame, CA). Single and double indirect immunofluorescent labelings were performed. Two rabbit polyclonal antisera raised against the extracellular N-terminal sequences of the human CB_1 (1-14 aa and 1-16 aa, respectively) were purchased from Cayman Chemicals (dilution: 1:600; Ann Arbor, MI) and from Biosource Int (dilution: 1:250; Camarillo, CA). They were used alone or together with a goat polyclonal anti-human choline acetyltransferase (ChAT) antibody, used at a dilution of 1:50 (Chemicon International, Temecula, CA). Affinity-purified IgG obtained from donkey (Jackson Immunoresearch, West Grove, PA) conjugated to fluorescein isothiocyanate and 7 amino-4-methylcoumarin-3-acetic acid were used as secondary antibodies. Briefly, tissue sections were rehydrated in PBS (pH 7.4) for 10 minutes, then incubated in 0.4% Triton X-100 and 3% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) in PBS for 30 minutes to block nonspecific binding.[30,31] Sections were incubated simultaneously with sets of 2 different primary antisera raised in rabbit and goat, respectively, overnight at room temperature. After 3 rinses in PBS, sections were incubated simultaneously with the appropriate secondary antibodies diluted in the same buffer, conjugated to the fluorescein isothiocyanate and 7 amino-4-methylcoumarin-3-acetic acid fluorochromes for 1 hour in the dark. After several rinses in PBS, coverslips were mounted with Vectashield (Vector Laboratory). All antisera used were diluted in a PBS solution containing 3% of mouse and donkey IgG. Controls included substitutions of the primary antisera with PBS. Preincubation for 24 hours of the anti-CB_1 antiserum with an excess of the blocking peptide (50 nmol/mL) (both from Biosource International) resulted in the absence of specific immunoreactivities in neuronal elements in both controls and treated animals (data not shown). A preabsorbing peptide for the anti-CB_1 antiserum from Cayman Chemicals was not available. Possible cross-reactivity between secondary reagents and primary reagents from inappropriate species were checked on sections. All controls gave negative results.

Slides were observed with a Nikon Eclipse 600 microscope (Nikon, Tokyo, Japan), equipped with 100-W high-pressure mercury lamps. Photography was performed with a Nikon photographic system. Provia 400 transparency film (Fuji, Tokyo, Japan) was used. Images were digitized using a FS 2710 scanner (Canon Inc, Tokyo, Japan) and the CanoCraft FS software interface provided by the same company and further processed with Adobe Photoshop software (version 6.0, Adobe Systems, San Jose, CA).

Semiquantitative Reverse-Transcription Polymerase Chain Reaction for CB_1 Messenger RNA

Total RNA from the middle jejunum of each animal (6 hours after the oral administration of CT or vehicle) was extracted using Trizol reagent according to the manufacturer's recommendations (Gibco BRL, San Giuliano Milanese, Italy). After extraction, RNA was precipitated using ice-cold isopropanol, resuspended in diethyl pyrocarbonate-treated water (Sigma). Integrity of RNA was verified after separation by electrophoresis into a 1% agarose gel containing ethidium bromide. RNA was treated further with RNAse-free DNAse I (Ambion DNA-free kit; Ambion CELBIO, Pero, Milan, Italy) according to the manufacturer's recommendations to digest contaminating genomic DNA and to subsequently remove the DNAse and divalent cations.

The expression of mRNAs for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and CB_1 receptors was examined by reverse transcription coupled to the PCR. Total RNA was reverse-transcribed using oligo dT primers. DNA amplifications were performed in PCR buffer (Q-Biogen, Rome, Italy) containing 2 μL of complementary DNA, 500 μmol/L deoxynucleoside triphosphate, 2 mmol/L $MgCl_2$, 0.8 μmol/L of each primer, and 0.5 U Taq polymerase (Q-Biogen). The thermal reaction profile consisted of a denaturation step at 94°C for 1 minute, annealing at 60°C for 1 minute, and an extension step at 72°C for 1 minute. A final extension step of 10 minutes was performed at 72°C. The PCR cycles were 30 and were observed to be optimal and in the linear portion of the amplification curve (data not shown). Reaction was performed in a PE Gene Amp PCR System 9600 (Perkin Elmer, Rome, Italy). After reaction, the PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide for ultraviolet visualization.

The specific oligonucleotides were synthesized on the basis of cloned complementary DNA sequences of GAPDH and CB_1 common to the rat and mouse. For GAPDH, the primer sequences were 5′-CCCTTCATTGACCTCAACTAC-ATGGT-3′ (nt 208–233; sense) and 5′-GAGGGCCATCCA-CAGTCTTCTG-3′ (nt 655–677; antisense). The CB_1 sense and antisense primers were 5′-GATGTCTTTGGGAAGAT-GAACAAGC-3′ (nt 365–373) and 5′-AGACGTGTCTGTG-GACACAGACATGG-3′ (nt 460–468), respectively. The expected sizes of the amplicons were 470 bp for GAPDH and 309 bp for CB_1. The GAPDH housekeeping gene expression was used to evaluate any variation in the RNA content and complementary DNA synthesis in the different preparations. No PCR products were detected when the reverse-transcriptase step was omitted (data not shown).

Drugs

CP55,940 ([-]-cis-3-[2-hydroxy-4-(-,–dimethylheptyl)phenyl]-trans-4-[3-hydroxypropyl]cyclohexanol), ACEA ([all *Z*]-N-[2-chloroethyl]-5,8,11,14-eicosatetraenamide), JWH-015 (2-methyl-1-propyl-1*H*-indol-3-yl)-1-naphthalenylmethanone, and chlorisondamine diiodide were purchased from Tocris Cookson (Bristol, UK); CT (from *Vibrio cholerae*) and capsazepine were purchased from Sigma Chemical Co. (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 gifts from Drs. Madaleine Mossè and Francis Barth (SANOFI-Recherche, Montpellier, France). VDM11 ([all *Z*] *N*-[2-methyl-3-hydroxy-phenyl]-5,8,11,14-eicosa-tetraenamide) was synthesized as described previously.[41]

CP55,940, JWH-015, capsazepine, SR141716A, and SR144528 were dissolved in dimethyl sulfoxide; ACEA and VDM11 were dissolved in ethanol; chlorisondamine was dissolved in saline. The drug vehicles dimethyl sulfoxide and ethanol (10 μL/mouse, IP) had no effect on intestinal fluid accumulation.

Statistics

Data are expressed as the mean ± SEM of experiments in n mice. To determine statistical significance, Student *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 less than 0.05 were considered significant.

Results

Cholera Toxin–Induced Fluid Accumulation

Oral administration of CT to mice (10 μg/mouse) led to a significant accumulation of fluid in the small intestine (Figure 1). The cannabinoid agonists ACEA (0.1–10 mg/kg) and CP55,940 (0.03–3 mg/kg) produced a dose-dependent reduction of CT-stimulated intraluminal fluid accumulation (Figure 1). The inhibitory effect of both ACEA (10 mg/kg) and CP55,940 (0.3 mg/kg) was counteracted by the CB_1 receptor antagonist SR141716A (0.3 mg/kg), but not by the CB_2 receptor antagonist SR144528 (3 mg/kg) (Figure 2). The CB_2 receptor agonist JWH-015 (10 mg/kg) did not modify

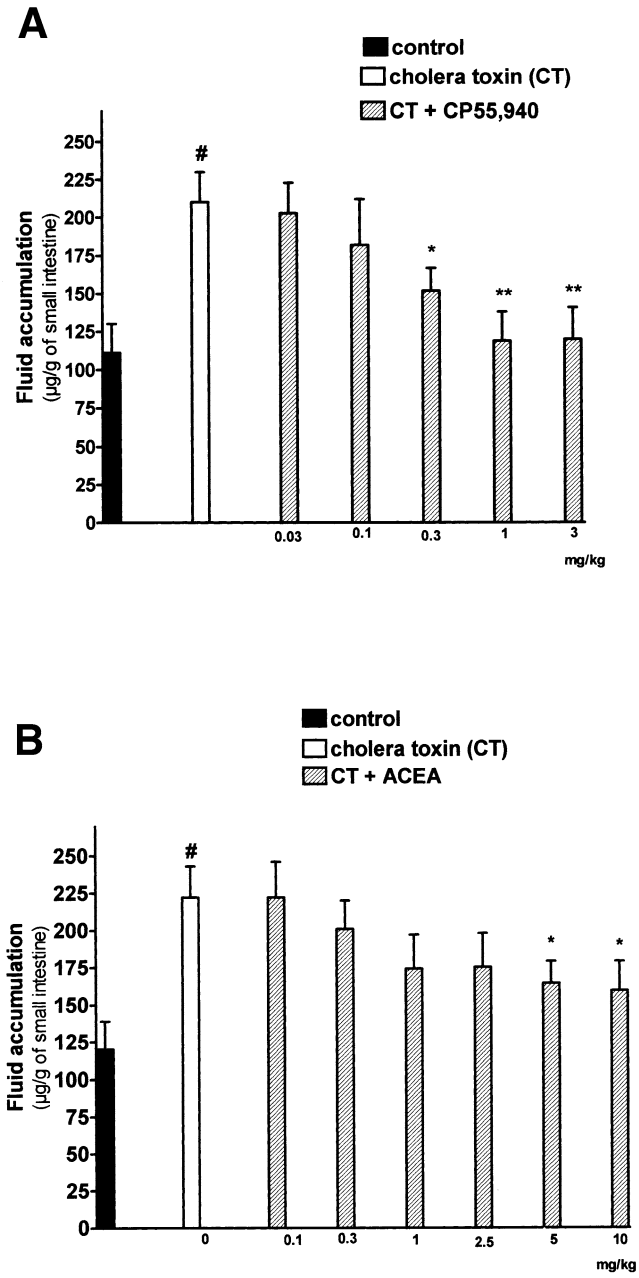

Figure 1. Effect of CP55,940 (*A*, 0.03–3 mg/kg IP) and ACEA (*B*, 0.1–10 mg/kg IP) on CT-induced fluid accumulation in mice. Fluid accumulation was measured 6 hours after CT (10 μg/mouse, per mouth) administration. Bars represent the mean ± SEM of 10–12 mice. #$P < 0.01$ vs. control; *$P < 0.05$ and **$P < 0.01$ vs. CT.

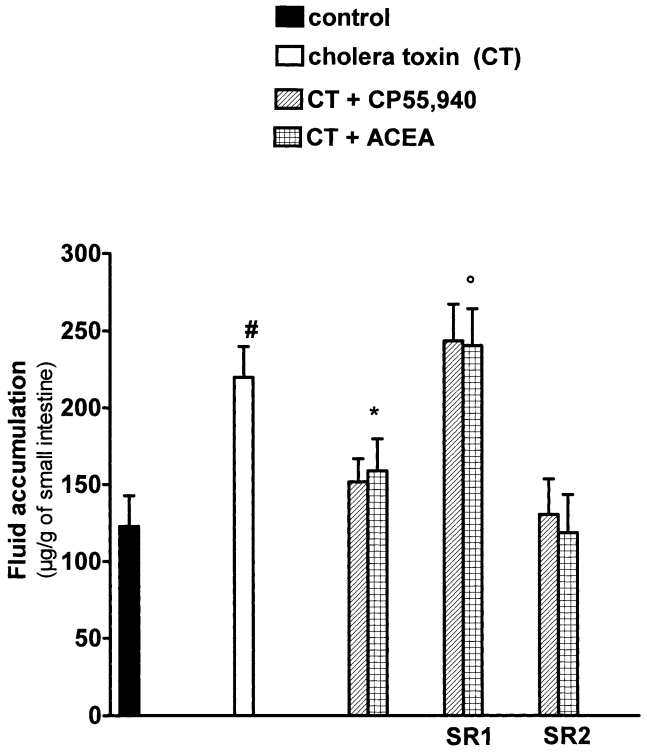

Figure 2. CT-induced fluid accumulation in mice. Antisecretory effect of the CP55,940 (0.3 mg/kg IP) and ACEA (10 mg/kg IP) alone or in mice pretreated with the CB_1 receptor antagonist SR141716A (SR1, 0.3 mg/kg IP) or the CB_2 receptor antagonist SR144528 (SR2, 3 mg/kg IP). Fluid accumulation was measured 6 hours after CT (10 μg/mouse per mouth) administration. Bars represent the mean ± SEM of 10–12 mice. #$P < 0.01$ vs. control; *$P < 0.05$ vs. CT; °$P < 0.05$ vs. CP55,940 (or ACEA).

CT-stimulated fluid accumulation (μg/g small intestine: CT, 221 ± 25; CT + JWH-015, 239 ± 21; n = 8; $P > 0.2$ vs. CT). In addition, CT-induced fluid accumulation was not significantly modified by the vanilloid VR1 receptor antagonist capsazepine (μg/g small intestine: CT, 231 ± 21; CT + capsazepine 3 mg/kg, 228 ± 19; capsazepine 10 mg/kg, 235 ± 22; capsazepine 30 mg/kg, 219 ± 20; n = 10–12; $P > 0.2$ vs. CT).

The ganglionic blocking compound chlorisondamine (5 mg/kg) reduced the CT-induced fluid accumulation by about 50%. When tested after chlorisondamine, CP55,940 (1 mg/kg) significantly ($P < 0.05$) inhibited CT-induced fluid accumulation (μg/g small intestine: control, 128 ± 21; CT, 235 ± 26; CT + chlorisondamine, 178 ± 15; CT + chlorisondamine + CP55,940, 131 ± 21; n = 8–10).

In the absence of cannabinoid receptor agonists, SR141716A (0.1–3 mg/kg) produced a dose-dependent increase of intraluminal fluid accumulation, an effect that attained statistical significance at 1 mg/kg (Figure 3). In contrast, SR144528 (0.1–3 mg/kg) did not modify CT-induced fluid accumulation.

The selective anandamide re-uptake inhibitor VDM11 (10 mg/kg) significantly prevented CT-induced intraluminal fluid accumulation, and this effect was counteracted by a per se noneffective dose (0.3 mg/kg) of SR141716A (Figure 4).

At the highest doses tested, CP55,940, ACEA, SR141716A, VDM11, capsazepine, and JWH-015 did not modify intraluminal fluid content in control (i.e., animals receiving the vehicle used to dissolve CT) mice (data not shown).

Endocannabinoid, Palmitoylethenolamide, and Anandamide Amidohydrolase Levels

Isotope gas chromatography-mass spectrometry analysis of lipid extracts from the small intestine of control and CT-treated mice showed that the levels of anandamide, but not 2-AG or palmitoylethanolamide, increased after CT administration (Table 1). The effect on anandamide levels was not caused by a changed enzymatic hydrolysis of anandamide because no significant effect by CT was observed on the capability of small intestine homogenates to hydrolyze [^{14}C]anandamide (Table 1).

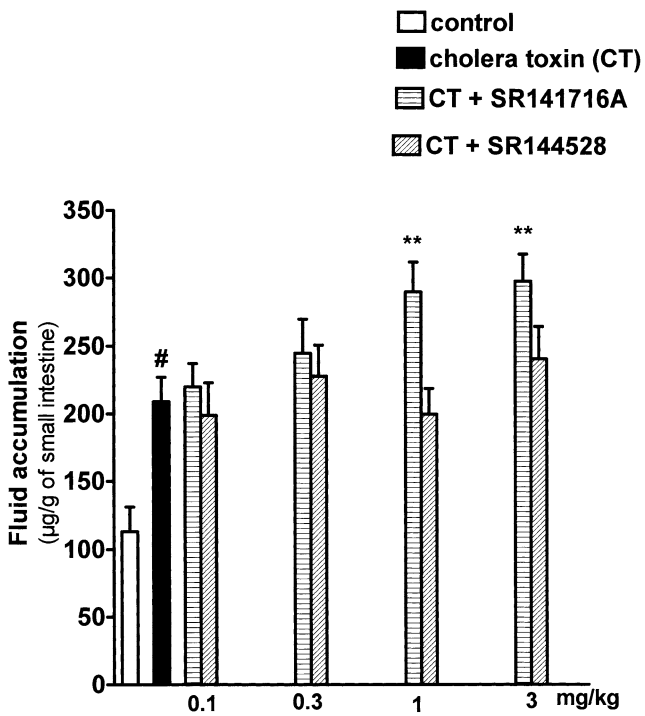

Figure 3. Effect of the CB_1 receptor antagonist SR141716A (0.1–3 mg/kg IP) and the CB_2 receptor antagonist SR144528 (0.1–3 mg/kg IP) on CT-induced fluid accumulation in mice. Fluid accumulation was measured 6 hours after CT (10 μg/mouse per mouth) administration. *Bars* represent the mean ± SEM of 10–12 mice. #$P < 0.01$ vs. control; **$P < 0.01$ vs. CT.

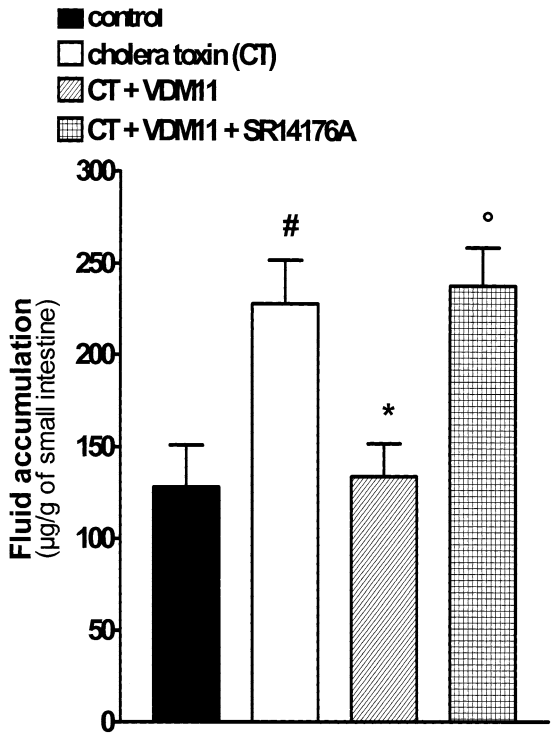

Figure 4. Effect of the anandamide re-uptake inhibitor VDM11 (10 mg/kg IP), alone or in mice pretreated with the CB_1 receptor antagonist SR141716A (0.3 mg/kg IP) on intestinal fluid accumulation stimulated by CT. Fluid accumulation was measured 6 hours after CT (10 μg/mouse per mouth) administration. Bars represent the mean ± SEM of 8–10 mice. #$P < 0.01$ vs. control; **$P < 0.01$ vs. CT; #$P < 0.01$ vs. VDM11.

Immunohistochemistry

Both anti–CB1-R antisera used in this study displayed similar patterns of receptor immunoreactivity. In control animals, CB_1-receptor immunoreactivity was displayed mainly by myenteric neurons (Figure 5*A*), and occasionally by submucous neurons. Immunoreactive nerve fibers to the circular muscle layer also were scarce. Double labeling showed that CB_1-receptor immunoreactive neurons co-contain ChAT, a marker of cholinergic neurons (Figure 5*A* and *B*).

In CT-treated animals, a slightly higher CB_1 and ChAT immunoreactivity was observed in the myenteric and, frequently, in the submucosal neurons, as well as in nerve fibers to the circular muscle layer (Figure 5*C* and *D*).

Study of CB_1 Messenger RNA Expression by Semiquantitative Reverse-Transcription Polymerase Chain Reaction

Agarose gel analysis of reverse-transcription PCR reactions from total RNA from mouse small intestine showed intense bands of the sizes expected for a CB_1 mRNA transcript (309 bp, see earlier in the Materials and Methods section) and a GAPDH mRNA transcript (470 bp), when using primers selective for CB1 and GAPDH, respectively. The absence of CB1 amplicons when the reverse-transcription reaction was omitted confirmed the absence of genomic DNA contamination in the RNA sample. When analyzed by densitometry scanning, and normalized to the respective GAPDH transcript bands, CB_1 transcript bands from the small intestine RNA of CT-treated mice were consistently and significantly more intense than those from untreated mice (Figure 6). This finding suggests that the enhanced CB_1 immunoreactivity found in the small intestine of CT-treated mice (see earlier) was owing to a higher expression of CB_1 receptor at the transcriptional level.

Discussion

The list of antisecretory drugs is impressively and disappointingly short.[1] Oral rehydration therapy has a significant impact on the morbidity and mortality of patients with acute diarrhea; however, oral rehydration therapy does not interfere with the secretory process nor does it diminish diarrhea.[42] New, safe, and effective antisecretory agents are therefore being sought. Any substance that decreases net fluid secretion, by either inhibiting the secretory process or by enhancing fluid absorption, is a potential antidiarrheal drug.[1,4,5] In the present work, we provide functional, biochemical, and immunohistochemical evidence that the enteric endocannabinoid system could represent a molecular target for new antidiarrheal agents.

Previous studies have shown that activation of enteric CB_1 inhibits esophageal[43] and gastrointestinal motility,[44–46] including in isolated human tissues,[23,24] and in an experimental model of diarrhea in the mouse.[47] In this study, we were able to show that the nonselective can-

Table 1. Effect of CT Treatment on Anandamide, 2-Arachidonoyl-Glycerol, and Palmitoylethanolamide Levels, and on Anandamide Amidohydrolase Activity, in the Mouse Small Intestine

	Control	CT treated
Anandamide (pmol/g tissue)	33.2 ± 7.2	92.8 ± 4.6[a]
2-Arachidonoyl-glycerol (nmol/g tissue)	46.8 ± 8.2	93.8 ± 32.3 (NS)
Palmitoylethanolamide (pmol/g tissue)	526.0 ± 93.2	458.0 ± 40.0 (NS)
Anandamide amidohydrolase (pmol/min × mg protein)	14.6 ± 1.6	16.2 ± 2.0 (NS)

NOTE. Data are means ± SEM of n = 4 different determinations, each performed in duplicate.
[a]$P < 0.01$ vs. control, as determined by analysis of variance followed by the Bonferroni test.
NS, not significantly different from control, $P > 0.05$.

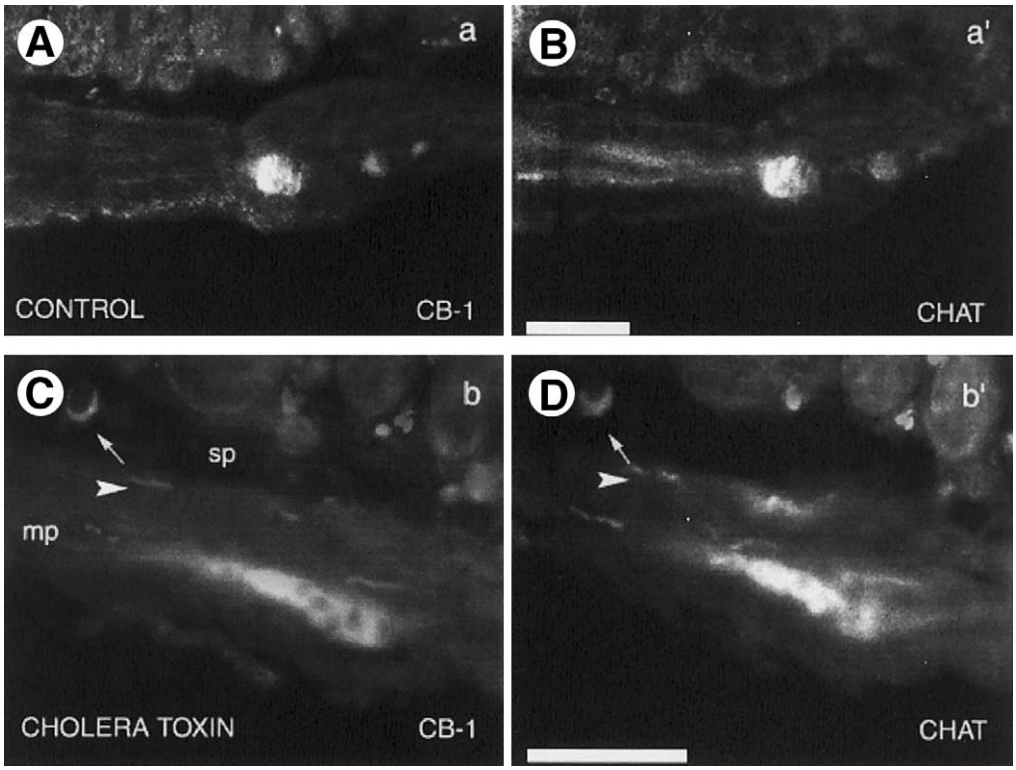

Figure 5. (*A* and *B*) CB_1 and ChAT immunoreactive neural perikarya in the myenteric plexus of control mice. (*C* and *D*) A higher density and immunoreactivity is observed in the CB_1/ChAT displaying neurons of the myenteric plexus, in a submucous neuron (*arrow*), as well as in nerve fibers to the circular muscle (*arrowhead*) of CT-treated mice. Calibration bar 200 μm. Mp, myenteric plexus; sp, submucous plexus.

nabinoid receptor agonist CP55,940 and the selective CB_1 receptor agonist ACEA decreased CT-stimulated fluid accumulation in the mouse small intestine. The antidiarrheal effect of the cannabinoid agonists examined here is very likely mediated uniquely by CB_1 receptors because: (1) the effect of both CP55,940 and ACEA was counteracted by the selective CB_1 receptor antagonist SR141716A; (2) the CB_2 receptor antagonist SR144528 did not modify the antisecretory effect of CP55,940; (3) the CB_1 selective agonist ACEA reduced CT-stimulated

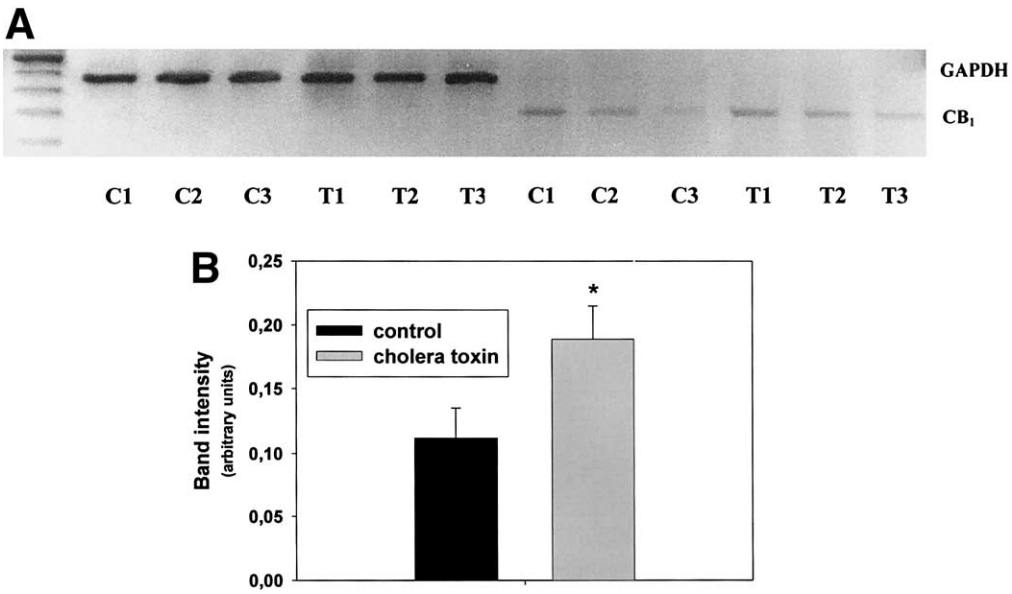

Figure 6. (*A*) Agarose gel analysis of reverse-transcription PCR reactions from total RNA from the small intestine of mice treated with vehicle (C1, C2, C3) or CT (T1, T2, T3). Bands of the sizes expected from CB_1 (309 bp) and GAPDH (470 bp) mRNA transcripts when using primers selective for CB_1 and GAPDH, respectively, are shown. A 100-bp ladder is shown on the left lane. (*B*) When analyzed by densitometry scanning, background substracted, and normalized to the respective GAPDH transcript bands, CB_1 transcript bands from the small intestine RNA of CT-treated mice were consistently and significantly more intense than those from untreated mice (means ± SEM, N = 3, $P < 0.05$ by Student *t* test).

fluid accumulation; and (4) the CB_2 receptor agonist JWH-015 was without effect.

The antisecretory effect of cannabinoid agonists likely involves peripheral mechanisms because CP55,940 and ACEA, when tested after the ganglionic blocking compound chlorisondamine, still inhibited CT-induced fluid accumulation. In agreement with our in vivo results, Tyler et al.[32] showed that the cannabinoid receptor agonist WIN55,212-2 effectively inhibited (via activation of CB_1 receptors) neuronal-mediated ileal secretion in vitro, measured electrically as an increase in I_{sc} (short circuit current).

Another important finding of the present study was that CT-induced fluid accumulation was associated with a significant increase in anandamide (but not 2-AG) levels in the small intestine, as compared with control mice. The intestinal content of palmitoylethanolamide (a saturated fatty acid ethanolamide that is co-released with anandamide by mammalian tissues) was not modified after CT challenge. We did not observe significant differences between control and CT-treated mice in the activity in the small intestine of anandamide amidohydrolase (also known as fatty acid amide hydrolase), the enzyme responsible for anandamide degradation, which, at any rate, was sufficient to ensure the degradation of anandamide under both physiologic and pathologic conditions. These findings suggest that the increase of anandamide levels found in the intestine of CT-treated mice were not caused by a reduction of the rate of its metabolic degradation. The presence of anandamide amidohydrolase in the mouse[29–31] and rat[48] intestine has been documented previously.

CT is known to evoke secretory nervous reflexes in the enteric nervous system and cholinergic neurones are implicated in the activation of such reflexes.[6] Immunohistochemical studies showed the presence of CB_1 receptors on enteric nerves of various species, including mice, rats, guinea pigs, pigs, and ferrets.[30–31,49–53] CB_1 immunoreactivity is highly colocalized with immunoreactivity for ChAT in enteric neurons and fibers, which is consistent with the ability of cannabinoid receptor agonists to reduce (via CB_1 receptors) acetylcholine release from myenteric nerves.[54] In the present study we have shown, for the first time, an overexpression of CB_1 receptor mRNA during an intestinal hypersecretory state. Intestinal overexpression of CB_1 receptors has been documented previously in a model of intestinal inflammation[29] and during experimental ileus.[31] This finding, together with the enhanced amounts of anandamide in the small intestine found here after CT administration, suggests that endocannabinoid signaling via cannabinoid CB_1 receptors is enhanced during CT-induced intestinal hypersecretion.

In view of the present observation that stimulation of CB_1 receptors abolishes the effects of CT on intestinal secretion, it seemed reasonable to hypothesize that the levels of both anandamide and CB_1 receptors are enhanced in response to CT to counteract CT-induced intestinal secretion. To verify this hypothesis we performed a series of experiments using cannabinoid receptor antagonists. If an endogenous cannabinoid tone exists to control the secretory response to CT, CB_1 receptor antagonism should be expected to have a worsening effect on CT-stimulated secretion. This was indeed the case, as shown by the increase in fluid accumulation after SR141716A in CT-treated, but not in control, mice. In a different species (i.e., the rat) and with a different time-course of drug administration, it also has been shown that SR141716A affects basal intraluminal fluid accumulation.[55]

Previous investigations suggest that anandamide activates vanilloid VR1 receptors[56] and that vanilloid VR1 immunoreactivity occurs in the rodent intestine.[57] However, it is very unlikely that endogenous anandamide, or any other endovanilloid, exerts its antisecretory effect via such receptors, as, in the present study, we have shown that the vanilloid VR1 receptor antagonist capsazepine (up to 30 mg/kg dose) did not modify CT-induced fluid accumulation. Others have reported recently that intraluminal administration of anandamide causes inflammation similar to that caused by *Clostridium difficile* toxin A in the rat ileum, and that this effect was inhibited significantly by capsazepine at a dose of 11.3 mg/kg.[58]

Finally, we wanted to assess whether the pharmacologic manipulation not only of anandamide CB_1-mediated actions by SR141716A, but also of anandamide levels with an inhibitor of its inactivation, would result in an effect on intestinal secretion in CT-treated mice. After receptor activation, anandamide can be removed from its site of action by carrier-mediated transport into the cell (anandamide transport or re-uptake), where it is hydrolyzed by an anandamide amidohydrolase. Therefore, we investigated the effect on diarrhea of the selective re-uptake inhibitor, VDM11.[41] We found that VDM11 prevented CT-induced fluid accumulation without affecting control fluid secretion, thus indicating not only a functional role of anandamide transport in terminating the biologic action of anandamide during CT-stimulated fluid accumulation, but also the protective (antisecretive) role of endogenous anandamide during this specific disease. The effect of VDM11 was antagonized by a per se noneffective dose of SR141716A,

indicating that it was caused by the enhancement of endocannabinoid levels, and hence to indirect activation of CB_1 receptors. Importantly, this compound has been found so far to exert anandamide-like effects only in those pathophysiologic states in which an enhanced endocannabinoid tone was shown,[59,60] including an intestinal motility disorder (i.e., paralytic ileus).[31] When tested in control mice in vivo at the dose used here, VDM11, unlike CB_1 receptor agonists, has been shown to reduce motility in the colon,[30] but not in the small intestine.[31]

In conclusion, our data indicate that endogenous anandamide exerts a protective role on CT-induced fluid accumulation via activation of overexpressed CB_1 receptors on enteric cholinergic nerves. From the clinical point of view, our findings indicate that 2 possible strategies might be pursued to obtain new drugs potentially able to block diarrhea without provoking unacceptable systemic (i.e., psychotropic, cardiovascular) side effects: first, to develop selective CB_1 agonists that do not cross the blood-brain barrier, in a manner similar to the antidiarrheal opiate loperamide; and second, to use inhibitors of endocannabinoid inactivation (in a manner similar to the recently developed enkephalinase inhibitor acetorphan)[1] which, by increasing local intestinal (pathologic) levels of anandamamide (where ongoing production is occurring), can have greater pharmacologic selectivity than drugs directly acting on CB_1 cannabinoid receptors.

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