Endocannabinoids Induce Ileitis in Rats via the Capsaicin Receptor (VR1)

DOUGLAS C. MCVEY, PATRICIA C. SCHMID, HARALD H. O. SCHMID, and STEVEN R. VIGNA

Departments of Cell Biology and Medicine, Duke University Medical Center, Veterans Affairs Medical Center, Durham, North Carolina (D.C.M., S.R.V.); and the Hormel Institute, University of Minnesota, Austin, Minnesota (P.C.S, H.H.O.S.)

Received August 4, 2002; accepted October 3, 2002

ABSTRACT

Intraluminal administration of the endocannabinoids N-arachidonoyl-ethanolamine (anandamide) and 2-arachidonoylglycerol (2-AG) causes inflammation similar to that caused by Clostridium difficile toxin A in the rat ileum. The effects of anandamide and 2-AG were significantly inhibited by pretreatment with the specific capsaicin receptor (vanilloid receptor subtype 1; VR1) antagonist capsazepine. Pretreatment with the CB1 and CB2 cannabinoid receptor antagonists N-piperidino-5-[(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide (SR141716) and N-[1S]-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]-5-[4-chloro-3-methylphenyl]-1-(4-methylbenzyl)-pyrazole-3-carboxamide (SR144528) did not affect the responses to anandamide. It has previously been shown that intraluminal toxin A stimulates substance P (SP) release from primary sensory neurons and that pretreatment with SP receptor [neurokinin (NK)-1 receptor] antagonists inhibits the inflammatory effects of toxin A. Anandamide stimulated SP release and this was blocked by capsazepine pretreatment. Also, pretreatment with the specific NK-1 receptor antagonist (2S,3S)-3-[(3,5-bis(trifluoromethyl)phenyl)methoxy]-2-phenylpiperidine (L-733,060) significantly inhibited the inflammatory effects of both toxin A and anandamide. Toxin A increased tissue concentrations of anandamide and 2-AG in the ileum, and these effects were enhanced after pretreatment with inhibitors of fatty acid amide hydrolase, a major endocannabinoid-degrading enzyme. The toxin A-stimulated release of anandamide but not 2-AG was selective over their congeners. These results demonstrate that the endocannabinoids anandamide and 2-AG stimulate intestinal primary sensory neurons via the capsaicin VR1 receptor to release SP, resulting in enteritis, and that endocannabinoids may mediate the inflammatory effects of toxin A.

Neurogenic mechanisms are known to play a role in intestinal inflammation caused by intraluminal inflammatory agents. For example, inhibition of the function of primary sensory nerves has been shown to inhibit intestinal inflammation in rats caused by toxin A from Clostridium difficile (Castagliuolo et al., 1994, 1998; Pothoulakis et al., 1994; Mantyh et al., 1996; McVey and Vigna, 2001) and by dextran sulfate sodium (Stucchi et al., 2000). In particular, inhibition of the release of the neuropeptide substance P (SP) from intestinal primary sensory nerves (Castagliuolo et al., 1994; Pothoulakis et al., 1994; Mantyh et al., 1996) or antagonism of the SP receptor (Pothoulakis et al., 1994; Stucchi et al., 2000) ameliorates intestinal inflammation in rats. However, the mechanisms by which the intraluminal inflammatory stimulus is transduced in the wall of the intestine, resulting in SP release and subsequent inflammation, are unknown.

It is well known that primary sensory neurons are sensitive to the plant alkaloid capsaicin, the pungent ingredient in hot peppers. The basis for the capsaicin sensitivity of primary sensory neurons is the expression of the vanilloid receptor subtype 1 (VR1, also known as TRPV1), a nonspecific cation channel for which capsaicin is an agonist (Caterina et al., 1997). It has recently been shown that the selective capsaicin VR1 receptor antagonist capsazepine inhibits the inflammatory effects of Clostridium difficile toxin A in the rat ileum (McVey and Vigna, 2001), suggesting that toxin A causes capsaicin VR1 receptor activation as part of its inflammatory mechanism. In addition, intraluminal capsaicin, a direct activator of the capsaicin VR1 receptor, caused a pattern of intestinal damage, secretion, and inflammation virtually identical to that seen after toxin A, and capsazepine also blocked the effects of capsaicin (McVey and Vigna, 2001). These results suggested that the inflammatory effects of intraluminal toxin A are transduced by the capsaicin VR1
receptor expressed by primary sensory nerves in the intestine. However, the mechanism by which intraluminal toxin A activates the capsaicin VR1 receptor is unknown. One possibility is that toxin A stimulates the synthesis or release of a VR1 agonist molecule that diffuses from the mucosal epithelium to adjacent primary sensory nerve endings. Recent discoveries led us to hypothesize that anandamide (N-arachidonylethanolamine; 20:4n-6 NAE) or another endocannabinoid such as 2-arachidonoylglycerol (2-AG) may transduce the inflammatory signal generated by toxin A in the intestinal lumen. Anandamide was first isolated from brain (Devane et al., 1992), and 2-AG was first isolated from both brain (Sugiura et al., 1995) and small intestine (Mechoulam et al., 1995), and both were found to be endogenous cannabinoids receptor agonists. Both anandamide and 2-AG were also shown to induce vasodilation by activating capsaicin VR1 receptors expressed by perivascular sensory nerves, causing the release of calcitonin gene-related peptide in the rat and guinea pig (Zygmunt et al., 1999). Because anandamide was more potent than 2-AG as a VR1 receptor agonist (Zygmunt et al., 1999), it has received more attention in subsequent studies, including its role in the activation of the cloned human VR1 receptor (Smart et al., 2000). However, as derivatives of arachidonic acid, both anandamide and 2-AG share certain structural and functional characteristics, and we thus hypothesized that both are good candidates to serve as endogenous activators of intestinal capsaicin VR1 receptors in response to intraluminal toxin A.

To test this hypothesis, we examined the effects of anandamide, 2-AG, and their congeners, as well as synthetic cannabinoids on inflammation and SP release in the rat ileum. We show here that anandamide and 2-AG cause ileal inflammation in the rat that is very similar to that caused by toxin A. The endocannabinoid-induced inflammation is capsaicine-sensitive, indicating that it is mediated by the capsaicin VR1 receptor. This is confirmed by demonstration that anandamide also stimulates the capsaicine-sensitive release of SP in the ileum. Finally, intraluminal administration of toxin A causes increased concentrations of anandamide and 2-AG in the rat ileum and both toxin A-induced inflammation and anandamide and 2-AG release are potentiated by pretreatment with inhibitors of the endocannabinoid-degrading enzyme fatty acid amide hydrolase (FAAH).

Materials and Methods

Materials. Anandamide; 2-AG; WIN 55,212-2 mesylate, (R)-(+)-[2,3-dihydro-5-methyl-3-(morpholino)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthylethanolamine; HU 210, (6aR)-trans-3-(1,1-dimethylheptyl)-6a,7,10,10-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-methanol; palmitoylethanolamide; and methyl arachidonyl fluorophosphonate (MAFP) were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Capsazepine; L-733,060, (2S,3S)-3-[3,5-bis[trifluoromethyl]phenyl]methoxy)-2-phenylpipеридине; L-733,061, (2R,3R)-3-[3,5-bis[trifluoromethyl]phenyl]methoxy)-2-phenylpipеридине; and phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma-Aldrich (St. Louis, MO). SR141716, N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide, and SR144528, N-[15]-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl)-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrrolo-3-carboxamide, were gifts from Sanofi Recherche (Montpellier, France). C. difficile toxin A was a gift from TechLab, Inc. (Blacksburg, VA).

Surgery. Isolated ileal segments were constructed in male Sprague-Dawley rats (150–175 g) as described previously (Pothoulakis et al., 1994; Mantyh et al., 1996). Isolated ileal segments 5 cm in length were constructed in anesthetized rats with silk sutures. Anandamide and 2-AG were dissolved in 100% ethanol to give stock solutions of 10 mg/ml and then further diluted (1:4) in saline. These compounds were injected at various doses from 1 to 100 μg in a volume of 400 μl into the lumen of the ileal segments using a 27-gauge syringe needle. Palmitoylethanolamide and the cannabinoid receptor agonists WIN 55,212-2 mesylate and HU 210 were dissolved in 100% ethanol, diluted 1:10 in saline to a concentration of 100 μM, and then injected into the lumen of the isolated segments in a volume of 400 μl (40 nmol/μl). Toxin A was administered at a dose of 5 μg in 400 μl of phosphate-buffered saline into the lumen of the isolated ileal segments. The CB1 receptor antagonist SR141716 (30 μmol/kg) was dissolved in DMSO/Tween 80 (1:1), diluted in saline, and then was injected i.p. 30 min prior to anandamide administration. The CB2 receptor antagonist SR144528 (1 mg/kg) was dissolved in DMSO and injected i.p. 1 h prior to anandamide administration. The CB1 and CB2 receptor antagonists were administered at doses shown to be effective at blocking CB1 and CB2 receptor mediated actions (Izzo et al., 1999; Massi et al., 2000). The NK-1R antagonist L-733,060 (3 mg/kg) and its inactive enantiomer L-733,061 (3 mg/kg) were dissolved in PBS and injected i.p. 10 min prior to anandamide or toxin A administration. The VR1 receptor antagonist, capsazepine, was dissolved in DMSO to give a stock solution of 0.1 M and then further diluted (1:10) in saline containing 10% Tween 50/10% ethanol. To determine the effects of VR1 receptor inhibition, capsazepine was injected s.c. (30 μmol/kg) 1 h before anandamide or 2-AG administration. This dose is within the range shown to be specific for VR1 antagonism of the effects of capsaicin on nociceptors (Dickenson and Dray, 1991; Perkins and Campbell, 1992). MAFP and PMSF were dissolved in 400 μl of 10% ethanol at a concentration of 100 μM and then injected into the lumen of the isolated ileal segments 30 min before toxin A. Control rats were prepared similarly and their isolated ileal segments were injected with the appropriate vehicles.

Luminal Fluid Accumulation. Luminal fluid accumulation was measured gravimetrically. After 3 h of treatment, the isolated ileal segments were removed, weighed, and their lengths were measured. Luminal fluid accumulation is expressed as milligrams of wet weight per centimeter of length.

Myeloperoxidase Activity. Myeloperoxidase (MPO) activity was determined as described previously (Bradley et al., 1982). Briefly, pieces of control and treated ileal segments were homogenized in 0.5% hexadechltrimethyleneammonium bromide in 50 mM KH2PO4 (pH 6), freeze/thawed three times, centrifuged at 4°C for 2 min, and then the absorbance of each supernatant was read at 460 nm at 0, 30, and 60 s after the addition of 2.9 ml of a solution containing 0.1 M phosphate-buffered saline. These compounds were injected at various doses from 1 to 100 μg in a volume of 400 μl into the lumen of the isolated ileal segments 30 min before toxin A. Control rats were prepared similarly and their isolated ileal segments were injected with the appropriate vehicles.

Substance P Release. Substance P release was assessed by analysis of NK-1R endocytosis as described previously (McVey and Vigna, 2001) with modifications. Briefly, pieces of ileal segments were collected from control, toxin A-treated, and capsaicine-pretreated toxin A-treated rats were fixed in freshly depolymerized 4% paraformaldehyde overnight at 4°C. The tissue was then washed and embedded in Tissue Tek O.C.T. compound (Sakura, Torrance, CA), frozen, sectioned at 20 μm, and mounted on Superfrost/Plus glass slides (Fisher Scientific Co., Pittsburgh, PA). After washing, the slides were stained using a rabbit antisem (#11886-5) specific for the C-terminal 15 amino acids of the rat NK-1R at a dilution of 1:3000 (Vigna et al., 1994). This was followed by incubation in a cyanine 3-conjugated donkey anti-rabbit IgG secondary antibody (Jackson Immunoresearch, West Grove, PA) at a dilution of 1:600.
Endocannabinoid Measurement. Anandamide and 2-AG were measured in ileal tissue extracts as described previously (Schmid et al., 2000). Briefly, pieces of toxin A- and vehicle-treated rat ileal segments and the segmental luminal contents were frozen on dry ice and stored at −80°C until lipid extraction. Total N-acylethanolamines (NAEs) and 2-monoacylglycerols (2-MAGs) were isolated from the lipid extracts by solid phase extraction and converted into tert-butyldimethylsilyl (t-BDMS) derivatives in the presence of deuterated analogs. The tBDMS derivatives were analyzed by gas chromatography/mass spectrometry using selected ion monitoring programs specific for NAE and 2-MAG. Because toxin A causes severe ileitis resulting in widespread sloughing off of the ileal mucosa, including villi, we added the endocannabinoid content of the ileal tissue extracts to the endocannabinoid content of the corresponding luminal contents (containing sloughed mucosal tissue plus secreted/extravasated fluid) and expressed the results as micromoles of endocannabinoid per micromole of tissue lipid phosphorus (Bartlett, 1959). In some cases, luminal contents and tissue were combined before lipid extraction.

Statistical Analysis. Results are expressed as mean ± S.E.M. Mean differences among two groups were examined by the Student’s t test and mean differences among several groups by one-way analysis of variance with the Dunnett’s or Tukey-Kramer post tests, using GraphPad InStat version 3.00 for Windows 95 (GraphPad Software Inc., San Diego, CA). P values <0.05 were considered significant.

Results

Intraluminal injection of anandamide caused intense and dose-dependent (1–100 µg) inflammation in the rat ileum as measured by histological damage and by stimulation of luminal fluid accumulation and MPO activity (Fig. 1). These effects of intraluminal anandamide were similar to the effects of toxin A from C. difficile (Pothoulakis et al., 1994;
Mantyh et al., 1996). Pretreatment of the rats with a subcutaneous injection of the capsaicin VR1 receptor antagonist capsazepine (30 μmol/kg) had no effect when given alone but significantly inhibited anandamide-induced luminal fluid accumulation (Fig. 1a), MPO activity (Fig. 1b), and histological damage (Fig. 1c). Intraluminal administration of 2-AG duplicated the effects of anandamide on luminal fluid accumulation (Fig. 2a), MPO activity (Fig. 2b), and histological damage (Fig. 2c), and capsazepine pretreatment significantly inhibited these effects. However, two synthetic cannabinoid agonists, WIN 55,212-2 mesylate and HU 210, and the cannabinoid receptor-inactive congener of anandamide, palmitoylethanolamide (16:0 NAE), did not affect the ileum at similar doses (data not shown). In these and subsequent experiments reported here, the vehicles in which the test compounds were dissolved had no effects (data not shown). These results showed that anandamide and 2-AG can cause enteritis in the rat similar to that caused by toxin A and that pretreatment of the rats with a specific capsaicin VR1 receptor antagonist significantly inhibits the effects of the endocannabinoids just as it inhibits the effects of toxin A.

To determine the specificity of the effects of anandamide for the capsaicin VR1 receptor versus endogenous CB1 and CB2 cannabinoid receptors, we tested the effects of pretreating the rats with subcutaneous injections of the specific CB1 receptor antagonist SR 141716, and the specific CB2 receptor antagonist SR144528. Neither cannabinoid receptor antagonist had an effect on the ileum when given alone and neither compound inhibited the effects of intraluminal anandamide on luminal fluid accumulation or MPO activity (Fig. 3), demonstrating that the inflammatory effects of anandamide were not mediated by cannabinoid receptors.

Intraluminal administration of toxin A stimulates the release of SP (Mantyh et al., 1996) and increases SP responses in the intestine (Castagliuolo et al., 1997), and pretreatment of rats with SP receptor antagonists (Pothoulakis et al., 1994; Mantyh et al., 1996) or with capsazepine (McVey and Vigna, 2001) inhibits the inflammatory effects of intraluminal toxin A. These results suggest that intraluminal toxin A causes the release of endogenous SP in the intestine, via the capsaicin VR1 receptor, and that SP then proceeds to stimulate the inflammatory cascade. Therefore, if an endocannabinoid mediates the effects of intraluminal toxin A, then administration of exogenous anandamide should also stimulate SP release and this effect should be blocked by capsazepine pretreatment. We therefore tested the effects of pretreatment with either capsazepine or a SP receptor antagonist on anandamide-induced SP release and intestinal inflammation. We used immunocytochemical assessment of NK-1R endocytosis in myenteric plexus neuronal cell bodies as an index of endogenous SP release as described previously (Mantyh et al., 1995, 1996; McVey and Vigna, 2001). Intraluminal administration of anandamide caused NK-1R endocytosis, an index of endogenous SP release, and this effect was blocked by pretreatment with the specific capsaicin VR1 antagonist capsazepine (Fig. 4). This is consistent with the hypothesis that toxin A causes endocannabinoid release to activate VR1, resulting in SP release and subsequent neurogenic inflammation.

Pretreatment of the rats with a subcutaneous injection of the specific NK-1R antagonist L-733,060 had no effect on luminal fluid accumulation or MPO activity when tested
alone but significantly inhibited anandamide-stimulated intestinal fluid accumulation and MPO activity (Fig. 5). The low-affinity enantiomer of L-733,060, L-733,061, had no effect on anandamide-induced fluid accumulation or capsazepine inhibition of anandamide-induced SP release. The percentage of NK-1R-ir neuronal cell bodies with >10 NK-1R-ir endosomes was determined in 20 cells per animal for each treatment as an index of endogenous SP release. Anandamide stimulated SP release and this effect was significantly inhibited by capsazepine. * P < 0.01 versus control. Scale bar, 5 μm.

**Fig. 4.** Effect of capsazepine treatment before intraluminal anandamide administration on substance P release in the ileum revealed by endogenous SP-mediated NK-1R endocytosis in myenteric plexus ganglionic cell bodies. a, laser scanning confocal fluorescent micrographs of neurons immunostained for the NK-1R from ileal segments treated for 3 h with vehicle (A), 100 μg of anandamide (B), or 100 μg of anandamide after 1-h pretreatment with 30 μmol/kg capsazepine s.c. before anandamide administration (C). b, quantitation of anandamide-induced SP release and capsazepine inhibition of anandamide-induced SP release. The percentage of NK-1R-ir neuronal cell bodies with >10 NK-1R-ir endosomes was determined in 20 cells per animal for each treatment as an index of endogenous SP release. Anandamide stimulated SP release and this effect was significantly inhibited by capsazepine. * P < 0.01 versus control. Scale bar, 5 μm.

**Fig. 5.** Effects of the specific NK-1R antagonist L-733,060 (3 mg/kg i.p.) and its inactive enantiomer L-733,061 (3 mg/kg i.p.) administered 10 min before anandamide (100 μg) or toxin A (5 μg) on stimulated luminal fluid accumulation (a) and MPO activity (b). L-733,060 significantly inhibited both anandamide- and toxin A-stimulated luminal fluid accumulation and MPO activity, whereas the inactive enantiomer L-733,061 had no effect. *, P < 0.001 versus anandamide alone; #, P < 0.001 versus toxin A alone.

VR1 agonist, they were tested for their effects on two indices of intestinal inflammation, luminal fluid accumulation and MPO activity, in response to intraluminal toxin A with and without capsazepine pretreatment. MAFP and PMSF both significantly increased luminal fluid and MPO activity in the rat ileum in response to toxin A, and these effects were strongly inhibited by capsazepine pretreatment (Fig. 7), suggesting that MAFP and PMSF do indeed inhibit a VR1 receptor agonist-degrading enzyme, such as FAAH.

To determine whether the toxin A-induced increases in ileal anandamide and 2-AG were specific or perhaps simply a result of global tissue lipolysis, the tissue endocannabinoid concentrations were also related to total NAE and total MAG concentrations. The increased anandamide concentrations in response to toxin A alone and toxin A plus MAFF or PMSF further increased the toxin A-induced generation of the two endocannabinoids (Fig. 6, b and c). In addition, pretreatment with MAFF and PMSF also increased toxin A-induced luminal fluid accumulation (Fig. 6a). To rule out the possibility that MAFF and PMSF were acting by mechanisms not involving a

**Discussion**

The present results demonstrate that intraluminal administration of exogenous anandamide or 2-AG in the rat ileum
causes neuronal SP release via stimulation of the capsaicin VR1 receptor, resulting in intestinal inflammation, and that these endocannabinoids may mediate the intestinal inflammatory effects of C. difficile toxin A. This ability to cause tissue inflammation via the VR1 receptor is not common to all cannabinoids because the synthetic cannabinoids WIN 55,212-2 mesylate and HU 210 did not have the same effects at similar concentrations. This is consistent with the demonstration that WIN 55,212-2 mesylate and HU 210 do not interact with VR1 receptors in vitro (Zygmun et al., 1999).

Because anandamide and 2-AG act at G protein-coupled CB1 and CB2 cannabinoid receptors as well as at the capsaicin VR1 receptor, it was important to determine whether the inflammatory effects of the endocannabinoids could be accounted for by their actions on CB1 or CB2 receptors. Pretreatment of the rats with specific cannabinoid receptor antagonists had no effect on the inflammatory activities of the two endocannabinoids, thus supporting the conclusion that cannabinoid receptors are not involved in the intestinal inflammatory effects of anandamide and 2-AG. This is also consistent with the localization of cannabinoid receptors in the rat intestine only in Peyer’s patches (Herkenham, 1995) and enteric ganglia (Pinto et al., 2002) and not in the mucosa where the inflammation was observed.

Analysis of endocytosis of the NK-1 receptor is an excellent method of assessing release of endogenous SP in various tissues, including the central nervous system and the intestine (Mantyh et al., 1995; Southwell et al., 1996). Unlike measurements of tissue concentrations of SP or immunohistochemical staining of SP in which it is impossible to distinguish between stored versus released SP or to distinguish between changes in SP biosynthesis versus SP degradation, NK-1 receptor endocytosis is an easily measured index of the concentration of physiologically released SP at the surface of its target cells. In the rat and guinea pig ileum, NK-1 receptor endocytosis has been shown to be SP concentration-dependent, inhibited by specific NK-1 receptor antagonists, reversible, and sensitive to release of endogenous SP (Southwell et al., 1998a,b; Mann et al., 1999; Jenkinson et al., 2000). Using this assay, toxin A was found to cause SP release from primary sensory nerves in the rat ileum (Mantyh et al., 1996; McVey and Vigna, 2001). In addition, depletion of endogenous SP by destruction of primary sensory nerves or pharmacological antagonism of the NK-1 receptor protects the rat ileum against toxin A-induced inflammation (Pothoulakis et al., 1994; Mantyh et al., 1996). Therefore, it was important to determine the effects of endocannabinoids on SP release and the effects of an NK-1 receptor antagonist on endocannabinoid-induced inflammation. The finding that anandamide stimulated intestinal SP release and that this effect was abolished by pretreatment with the specific VR1 receptor antagonist capsazepine supports the hypothesis that anandamide causes intestinal inflammation via capsaicin VR1 receptor-mediated SP release from primary sensory nerves in the intestinal mucosa. This conclusion was further supported by the demonstration that pretreating the rats with the specific NK-1 receptor antagonist L-733,060 strongly inhibited both toxin A- and anandamide-induced ileitis in this model. These observations are also consistent with the previous demonstration that exogenous anandamide increases basal acetylcholine release and muscle tension in the guinea pig ileum by stimulating the release of endogenous tachykinins via the VR1 receptor (Mang et al., 2001). It is also possible that intrinsic afferent neurons in the intestine may mediate these effects of endocannabinoids in view of the demonstration that these nerves also express immunoreactive VR1 receptors in the rat ileum (Anovi-Goffer et al., 2002).

These results raised the question whether intraluminal toxin A causes intestinal inflammation by releasing endogenous anandamide or 2-AG to activate VR1 receptors, result-
ing in SP release. It is likely that the inflammatory effects of intraluminal toxin A are mediated by an endogenous mechanism, because toxin A binds to brush-border receptors on the epithelial enterocytes lining the intestinal lumen and thus does not enter the body (Torres et al., 1990). Therefore, we measured the levels of anandamide and 2-AG in the ileum after toxin A treatment. Toxin A caused about a 4-fold increase in ileal anandamide concentration and about a 3-fold increase in ileal 2-AG concentration over vehicle control levels after 3 h; both responses were statistically significant. In addition, we tested the effects of pretreatment with FAAH inhibitors before toxin A administration on ileal anandamide and 2-AG concentrations. FAAH has been shown to be a major determinant of endocannabinoid signaling in vivo because it is the major endogenous degradative enzyme of anandamide and can also degrade 2-AG (Mechoulam et al., 1998; Cravatt et al., 2001). Even though 2-AG is most likely degraded primarily by monoglyceride lipase activity (Karlishson et al., 1997), it is also a substrate for FAAH and FAAH inhibitors may not be highly specific (for review, see Deutsch et al., 2002). Thus, we reasoned that if a FAAH-sensitive endocannabinoid mediates the inflammatory effects of toxin A on the ileum, pretreatment of the ileum with FAAH inhibitors such as MAFP and PMSF (Ross et al., 2001) before toxin A administration should result in increased anandamide and 2-AG levels and more ileitis than observed after administra-

![Graph](image)

**Fig. 7.** Effects of capsazepine pretreatment on toxin A-stimulated (5 μg) or toxin A (5 μg) plus MAFP- or PMSF-stimulated (100 μM) ileal luminal fluid accumulation (a) and ileal MPO activity (b). *, P < 0.001 versus toxin A + MAFP; #, P < 0.001 versus toxin A + PMSF.
of toxin A alone. Indeed, we observed significantly greater inflammation and significantly higher ileal levels of both anandamide and 2-AG when the ileum was pretreated with the FAAH inhibitors MAFP or PMSF before toxin A administration. These results are consistent with the finding that the rat small intestine contains high levels of FAAH (Katayama et al., 1997). In addition, the effects of MAFP + toxin A and PMSF + toxin A on both luminal fluid accumulation and tissue MPO levels were strongly inhibited by pretreatment with capsazepine, a specific VR1 antagonist. These data strongly suggest that toxin A causes ileitis in this model by causing the release of anandamide and/or 2-AG followed by activation of VR1 receptors and SP release.

We also examined the specificity of the toxin A-stimulated anandamide and 2-AG responses by comparing the tissue levels of these two endocannabinoids to the total amounts of lipids in the same family of compounds. Thus, anandamide levels were compared with all NAEs and 2-AG levels were compared with all 2-MAGs. We found that toxin A and toxin A + MAFP or PMSF caused a selective and statistically significant increase of anandamide over the summed total of all NAEs in the ileum, but the levels of 2-AG simply mirrored the total 2-MAG concentrations. This suggests that toxin A has a specific effect on anandamide release in the ileum, whereas the 2-AG responses may just reflect the effect of toxin A on overall tissue lipolysis. Thus, even though the total amount of 2-AG released by toxin A in the ileum is greater than the total amount of anandamide released, the nonspecificity of the 2-AG release may simply represent an effect of toxin A-induced tissue inflammation. In contrast, the specific release of anandamide in response to toxin A may account for mediation of the effects of toxin A on VR1 receptor activation and subsequent ileitis. This hypothesis is supported by the similar pattern of effects of toxin A and the FAAH inhibitors on luminal fluid accumulation and anand-

Fig. 8. Effects of toxin A (Tx A; 5 μg), toxin A (5 μg) + MAFP (100 μM), and toxin A (5 μg) + PMSF (100 μM) on ileal concentrations of anandamide and total NAEs (a) and on ileal concentrations of 2-AG and total 2-MAGs (b). The NAEs included in total NAEs include 16:0, 18:1(n-9), 18:1(n-7), 18:2(n-6), and 20:4(n-6). The 2-MAGs included in total 2-MAGs include 18:1(n-9), 18:1(n-7), 18:2(n-6), and 20:4(n-6). * P < 0.05 versus corresponding total NAE.
amide release versus luminal fluid accumulation and 2-AG release (compare Fig. 6, a and b, versus a and c).

It has recently been reported that the levels of anandamide and 2-AG in the small intestine of mice are not changed after oral administration of the intestinal inflammatory agent croton oil (Izzo et al., 2001). However, croton oil caused an approximate doubling of intestinal levels of FAAH in this model, suggesting that croton oil-induced inflammation caused a more rapid turnover (biosynthesis plus degradation) of endocannabinoids in this model. Whether the differences in results between the present rat study and this previous mouse study are due to species differences or to different responses to toxin A versus croton oil remain to be resolved by further research.

The observations that anandamide has anti-inflammatory effects in rat skin (Richardson et al., 1998) and that endocannabinoid levels are unchanged in a model of skin inflammation (Beaulieu et al., 2000) suggest that there may be differences among organs in terms of the effects of endocannabinoids on inflammation. Indeed, anandamide can exert either proinflammatory or anti-inflammatory actions, depending on whether it activates VR1 or cannabinoid receptors (Szallas and DiMarzo, 2000). Our results clearly demonstrate that anandamide and 2-AG primarily activate VR1 receptors in the rat ileum resulting in endogenous substance P release and subsequent inflammation. Although there is no information available on the possible role of these mechanisms in human intestinal inflammation, it is interesting to note that increased VR1 immunoreactivity has been observed in colonic tissue from human inflammatory bowel disease patients (Yiangou et al., 2001).

Acknowledgments
We thank TechLab, Inc. for the gift of toxin A and Sanofi Recherche for the gifts of SR 141716 and SR 144528.

References
Terjesen J, Wennng E, Lange S, and Lonnroth I (1990) Enterotoxins from Clostrid-
**Cite this paper:**


**Address correspondence to:** Steven R. Vigna, Department of Cell Biology, Box 3709, Duke University Medical Center, Durham, NC 27710. E-mail: srv@duke.edu