Distribution and function of the cannabinoid-1 receptor in the modulation of ion transport in the guinea pig ileum: relationship to capsaicin-sensitive nerves

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1Mucosal Inflammation, 2Gastrointestinal, and 3Neuroscience Research Groups, Department of Physiology and Biophysics, University of Calgary, Calgary, Alberta, T2N 4N1, Canada; and 4Departments of Anesthesiology and Physiology and Biophysics, University of Washington, Seattle, Washington 98195
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Distribution and function of the cannabinoid-1 receptor in the modulation of ion transport in the guinea pig ileum: relationship to capsaicin-sensitive nerves. Am J Physiol Gastrointest Liver Physiol 286: G863–G871, 2004. First published December 30, 2003; 10.1152/ajpgi.00482.2003.—We investigated the distribution and function of cannabinoid (CB1) receptors in the submucosal plexus of the guinea pig ileum. CB1 receptors were found on both types of submucosal secretomotor neurons, colocalizing with VIP and neuropeptide Y (NPY), the noncholinergic and cholinergic secretomotor neurons, respectively. CB1 receptors colocalized with transient receptor potential vanilloid-1 receptors on paravascular nerves and fibers in the submucosal plexus. In the submucosal ganglia, these nerves were preferentially localized at the periphery of the ganglia. In denervated ileal segments, CB1 receptor immunoreactivity in submucosal neurons was not modified, but paravascular and intraganglionic fiber staining was absent. Short-circuit current (Isc) was measured as an indicator of net electrogenic ion transport in Ussing chambers. In the ion-transport studies, Isc responses to capsaicin, which activates extrinsic primary afferents, and to electrical field stimulation (EFS) were reduced by pretreatment with the muscarinic antagonist atropine, abolished by tetrodotoxin, but were unaffected by VIP receptor desensitization, hexamethonium, α-aminooxy-5-methyloxazole-4-propionic acid, or N-methyl-d-aspartate glutamate receptor antagonists. The responses to capsaicin and EFS were reduced by 47 ± 12 and 30 ± 14%, respectively, by the CB1 receptor agonist WIN 55,212–2. This inhibitory effect was blocked by the CB1 receptor antagonist, SR 141716A. Ios responses to forskolin or carbachol, which act directly on the epithelium, were not affected by WIN 55,212–2. The inhibitory effect of WIN 55,212–2 on EFS-evoked secretion was not observed in extrinsically denervated segments of ileum. Taken together, these data show cannabinoids act at CB1 receptors on extrinsic primary afferent nerves, inhibiting the release of transmitters that act on cholinergic secretomotor pathways.

submucosal plexus; vasoactive intestinal peptide; neuropeptide Y; transient receptor potential vanilloid-1 receptor

THE ENTERIC NERVOUS SYSTEM exerts tight control over electrolyte and water transport by the intestinal epithelium (5, 7). Specifically, submucosal secretomotor neurons release vasoactive intestinal polypeptide and acetylcholine to activate cAMP- or Ca2+-dependent pathways, respectively, which control the gating of chloride transport through apically situated chloride transporters in enterocytes. The apically directed transport of chloride occurs predominantly via the cystic fibrosis transmembrane conductance regulator or a member of the calcium-dependent chloride channel family (4). Whereas the intrinsic properties of these transporters have been, and continue to be, the subject of numerous studies, less well documented are the inputs that control the activity of the submucosal secretomotor neurons that release neurotransmitters to activate these channels.

The best characterized inhibitory inputs to the submucosal secretomotor neurons come from sympathetic nerves that release norepinephrine, which acts at α-adrenergic receptors (22, 28, 39) and opioid-containing enteric nerves that release enkephalin or dynorphin, which acts primarily at δ-opioid receptors (35–39). However, recent reports (1, 15, 40) suggest that cannabinoid (CB) receptors of the CB1 subtype may also suppress neurally evoked secretion. Tyler et al. (40) showed that CB1 receptors could reduce secretion evoked by electrical field stimulation (EFS) but not that evoked by acetylcholine in the rat ileum. The cellular location of the receptors, and what class of submucosal motoneuron they regulated to reduce secretory responses, was not examined. Similarly, CB1-receptor activation by the potent agonist HU-210 reduced neurogenic ion transport elicited by the proinflammatory peptide kallidin (15). Gastric secretion evoked by pentagastrin and 2-deoxy-D-glucose was also inhibited by CB1 receptor agonists, and the receptor was identified on myenteric cholinergic neurons and/or vagal pathways in the myenteric plexus (1). These studies have recently been extended to show that endogenous cannabinoids play a role in the regulation of fluid secretion. Experiments in mouse have shown that upregulated expression of CB1 receptors on enteric nerves is responsible for the protective actions of anandamide in cholera toxin-induced fluid secretion in vivo (20).

Capsaicin-sensitive extrinsic primary afferents are also involved in the control of epithelial secretory function, through activation of transient receptor potential vanilloid-1 (TRPV1) receptors (25, 42, 44). In in vitro preparations of guinea pig ileum mounted in Ussing chambers, exposure to capsaicin resulted in an increase in short-circuit current (Isc), mediated by apically directed chloride secretion (25, 42, 44). Extrinsic denervation revealed that this was due to the activation of substance P-containing extrinsic primary afferent nerves that release neurotransmitter locally from terminals of axon collaterals to stimulate submucosal secretomotorneurons. The population of submucosal secretomotorneurons mediating the secre-
tory response to capsaicin has not been elucidated. Furthermore, it is not known whether CB1 receptors are localized on primary afferent nerves of the submucosal plexus and whether they can modulate the responses to capsaicin.

In this study, we sought to determine the role of CB1 receptors in gastrointestinal secretory function through a detailed assessment of the expression of CB1 receptors in the submucosal plexus of the guinea pig ileum. With this information in hand, we then determined the role of CB1 receptors in mediating electrolyte transport evoked by capsaicin and EFS using ileal preparations mounted in Ussing chambers.

MATERIALS AND METHODS

Animals. Male Hartley guinea pigs (300–500 g) were obtained from Charles River and were housed at constant temperature (22°C) and photoperiod (12:12-h light-dark cycle) in group cages in the University of Calgary Animal Resources Center. The guinea pigs had free access to standard guinea pig chow and tap water and were allowed to acclimatize to their housing conditions for at least 7 days before inclusion in an experiment. Animals were not fasted before surgery or euthanasia. All procedures were approved by the University of Calgary Animal Care Committee and followed the guidelines established by the Canadian Council on Animal Care.

Surgical denervation. In some experiments, segments of the ileum were subjected to surgical extrinsic denervation. Animals (n = 11) were anesthetized with 4% halothane in oxygen, and the distal portion of the ileum was exteriorized through a midline incision in the abdomen. An ileal segment 5–8 cm in length was denervated by crushing both the mesenteric vascular pedicles and accompanying nerve bundles 1–2 cm from the wall of the ileum in three to four consecutive mesenteric arcades with forceps for 30 s. The denervated region was demarcated by placing sutures at the proximal and distal borders. Seven to ten days later, the animals were anesthetized with an intraperitoneal injection of pentobarbital sodium, and the affected region of ileum and a control segment 20 cm proximal to it were removed. One-half of the experimental and control tissue obtained was immediately placed into cold (4°C) Krebs buffer for secretion studies (see Electrolyte transport studies), whereas the other half was placed in ice-cold PBS containing 1 μM nifedipine for 10 min for immunohistochemistry. Animals were subsequently euthanized by exsanguination.

Immunohistochemistry. Segments of distal ileum (n = 4–6) were opened along the mesenteric border, stretched, and pinned mucosal-side-up on a Sylgard-coated petri dish. Tissues were fixed overnight with 4% paraformaldehyde (4°C), and then rinsed for 3 × 10 min in PBS (pH 7.4). The submucosal plexus was separated from the mucosa and muscle layers by dissection with fine forceps using a dissecting microscope. Tissues were washed for 3 × 10 min in PBS containing 0.1% Triton X-100, incubated with primary antibodies as outlined in Table 1 for 48 h (4°C), washed for 3 × 10 min in PBS, and incubated with secondary antibodies for 90 min (room temperature), followed by washing (3 × 10 min in PBS) and mounting in bicarbonate-buffered glycerol (pH 8.6). In some cases, tissues were subsequently incubated with a second or third primary antibody of a different species followed by the appropriate secondary antibody to determine colocalization. Secondary antibodies used were donkey anti-rabbit, rat, or goat conjugated to CY5 or FITC; donkey anti-rabbit conjugated to CY5; or goat anti-rabbit or mouse conjugated to FITC (1:50–1:100; Biocan Scientific, Mississauga, ON). In all cases, preabsorption of the primary antibody with the original hapten (1–10 nmol/ml in the diluted antibody, 24 h at 4°C) abolished all immunoreactivity.

Confocal microscopy. Samples were viewed on an Olympus Fluoview FV300 microscope system using krypton-argon and helium-neon lasers. Differential visualization of the fluorophores FITC (excitation 490 nm and emission 520 nm), CY3 (excitation 552 nm and emission 565 nm), and CY5 (excitation 650 nm and emission 677 nm) was accomplished through the use of specific filter combinations. Samples were scanned sequentially to avoid any potential for bleed through of fluorophores. Images of 1,024 × 1,024 pixels were obtained under identical exposure conditions (pinhole aperture, laser strength, scan speed, Kalman averaging ×2) and were processed identically using Adobe Photoshop. Confocal micrographs are digital composites of Z-stack scans through 1-μm optical sections, as detailed in the figure legends. In some cases, differential interference contrast microscopy, with the use of Wollaston prisms on the Olympus BX50 microscope, was used to determine a three-dimensional shadowlike image of blood vessels in the tissue.

Electrolyte transport studies. Guinea pigs were killed by an overdose of pentobarbital sodium. The presence of this anesthetic does not adversely affect neurally regulated ion transport in isolated segments of ileum (unpublished observation). Segments of distal ileum, taken 4 cm proximal to the cecum, were rinsed with Krebs buffer (4°C) to remove feces and were stripped of external muscle by blunt dissection. Segments of stripped ileum were opened along the mesenteric border and mounted between halves of Ussing-type diffusion chambers (Harvard Apparatus, Saint Laurent, QC) to provide an exposed mucosal surface area of 0.64 cm². The serosal side of the preparation was bathed with Krebs buffer containing 10 mM glucose, whereas the mucosal side was bathed with Krebs buffer containing 10 mM mannitol. Tissues were voltage clamped to 0 V by applying a Isc delivered with a voltage-clamp apparatus (EVC-4000; World Precision Instruments, Sarasota, FL). Potential difference was recorded, and Isc was delivered via pairs of glass-barreled, ceramic-tipped Ag-AgCl electrodes (Harvard Apparatus) containing 3 M KCl and placed on either side of the tissue. Net changes in Isc were measured as indicators of changes in electrogenic ion transport.

For studies using agonists or antagonists, tissues (n = 4–5) were paired on the basis of basal electrical conductance. Pairs of tissues having electrical conductances that differed by >20% were not included in the study. Drugs were added to the serosal side of the tissue. Each tissue received only one concentration of a drug. For VIP receptor desensitization, tissues were exposed serially to 100 nM VIP and Isc was allowed to return to baseline. Twenty minutes later, the tissues were again exposed to 100 nM VIP to ensure desensitization, and 10 min later, they were exposed to capsaicin. To ensure that any decrease in response was not due to a general reduction in the responsiveness of the tissue, preparations were subsequently exposed to 1 μM carbachol and 10 μM forskolin, and changes in Isc were determined. For studies in which EFS was used to assess neurally

Table 1. Primary antibodies

<table>
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<tr>
<th>Antigen</th>
<th>Species</th>
<th>Dilution</th>
<th>Product No.</th>
<th>Source</th>
</tr>
</thead>
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<tr>
<td>Transient receptor potential vanilloid-1</td>
<td>rabbit</td>
<td>1:2,000</td>
<td>AB5370</td>
<td>Chemicon International, Temecula, CA</td>
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<tr>
<td>Calcitonin gene related peptide</td>
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<td>IHC6006</td>
<td>Peninsula Laboratories, Belmont, CA</td>
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<tr>
<td>Vasoactive intestinal polypeptide</td>
<td>mouse</td>
<td>1:500</td>
<td>VIP31</td>
<td>Regulatory Peptide Research Group, University of British Columbia, BC, Canada</td>
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<tr>
<td>Neuropeptide Y</td>
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<td>1:500</td>
<td>NT115</td>
<td>Prots Biotech, New York, NY</td>
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<tr>
<td>Substance P</td>
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<td>1:500</td>
<td>NC1 1021</td>
<td>Medicorp, Montreal, PQ, Canada</td>
</tr>
<tr>
<td>Cannabinoid-1 receptor</td>
<td>goat</td>
<td>1:100</td>
<td></td>
<td>K. Mackie, University of Washington, Seattle, WA</td>
</tr>
</tbody>
</table>
evoked secretion, the following stimulation parameters were used as previously described (42): stimulus strength, 50 V; frequency, 10 Hz; pulse duration, 500 μs; total stimulus duration, 3 s.

Materials. Krebs buffer contained (in mM) 115.0 NaCl, 2.0 KH₂PO₄, 2.4 MgCl₂, 1.3 CaCl₂, 25.0 NaHCO₃, and 8.0 KCl. Routine buffer reagents were from BDH (Toronto, ON). WIN 55,212–2 was obtained from Tocris (Ellisville, MO). SR 141716A was a generous gift from Sanofi Research (Montpellier, France). Both WIN 55,212–2 and SR 141716A were dissolved in DMSO. The final concentration of DMSO in the Ussing chamber baths was 0.1%, which did not affect basal or evoked transport parameters. All other chemicals were purchased from Sigma (Mississauga, ON).

Statistics. Data are expressed as means ± SE. Counts were obtained from 10–20 submucosal ganglia from each of four guinea pigs. Total numbers of neurons labeled with specific neuronal markers and the percentage overlap of those markers were determined. For Ussing chamber experiments, pairs of tissues from the same animal were compared using Student’s t-test for paired data. Comparisons of more than two groups was made using ANOVA with a post hoc Tukey’s test. A probability (P value) of <0.05 was considered significant.

RESULTS

Immunohistochemistry. CB₁ receptors were localized by indirect immunofluorescence techniques in the submucosal plexus and on paravascular nerve fibers of the mucosa. The neuronal labeling was cytoplasmic, and the majority of neurons was labeled. With the use of double-labeling techniques, CB₁ receptor colocalized with 58 ± 13% (n = 4 animals) of neuropeptide Y (NPY) neurons (Fig. 1, A–C) and 100% of VIP neurons (Fig. 1, D–F), unique markers for the noncholinergic and cholinergic secretomotor neuronal populations, respectively (5). In addition, 80 ± 7% of calretinin neurons also

Fig. 1. Confocal fluorescence micrographs of cannabinoid (CB₁) receptor immunoreactivity in whole mount preparations of guinea pig ileal submucosal plexus. Single labels for each marker are shown in the first 2 columns, and the overlaid (O/L) double-labeled image is shown in the 3rd column. A, D, and G: CB₁ receptor (CB1). B: neuropeptide Y (NPY). E: VIP. H: transient receptor potential vanilloid-1 (TRPV1; VR1). CB₁-receptor immunoreactive neurons colocalized with some NPY neurons (A–C: 3 optical sections) and with all VIP neurons (D–F: 3 optical sections). CB₁ receptor also colocalized with TRPV1 receptors found on nerve fibers and paravascular nerves of the submucosa (G–I: 23 optical sections). Scale bar: 100 μm.
expressed CB₁-receptor immunoreactivity. CB₁ receptors were also colocalized with TRPV1 (VR1) receptors found on paravascular nerves and nerve fibers in submucosal ganglia (Fig. 1, G–I).

Because the distribution of TRPV1 and its relationship to submucosal neurons has not been thoroughly documented in the guinea pig ileal submucosal plexus, we investigated this further. In normal animals, TRPV1 colocalized with substance P immunoreactivity in paravascular nerves and on some varicose immunoreactive fibers in the submucosal plexus (Fig. 2, A–C). In general, the larger varicosities were those that were double-labeled, whereas the smaller varicosities were only immunoreactive for substance P. After extrinsic denervation, only fine varicose substance P-immunoreactive fibers derived from intrinsic submucosal neurons were apparent. TRPV1 immunoreactivity was abolished (Fig. 2, D–F).

It has previously been shown that CGRP colocalizes with substance P in primary afferent nerves in the small intestine (13). We have repeated these findings (Fig. 3, A–D) and now show that, after extrinsic denervation, the colocalized intraganglionic and paravascular labeling but not the immunoreactivity due to intrinsic nerves is completely abolished (Fig. 3, E–H). We have also extended these observations to show that these colocalized fibers are not distributed homogeneously in submucosal ganglia but, rather, selectively innervate the cholinergic secretomotoneurons that are uniquely labeled by NPY and are located at the poles of submucosal ganglia (Fig. 3, I–L) and fibers that traverse through the ganglia.

**Electrolyte transport.** In stripped segments of ileum mounted in Ussing chambers, serosal application of capsaicin evoked a rapid and transient increase in \( I_{sc} \), similar to that previously observed. This increase was partially (but significantly) reduced by pretreatment of the tissue with atropine but not by VIP receptor desensitization, hexamethonium, \( \alpha \)-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid, or \( N \)-methyl-\( \alpha \)-aspartate glutamate receptor antagonists (Fig. 4).

The responses to capsaicin and to EFS were also reduced by the CB₁ agonist WIN 55,212–2 in a concentration-dependent manner (Figs. 5 and 6). The concentration of WIN 55,212–2 (1,000 nM), which inhibited the responses to capsaicin (by 47 ± 12%, \( n = 5 \)) and EFS (by 30 ± 14%, \( n = 5 \)), did not affect the increase in \( I_{sc} \) evoked by serosal application of the cAMP-dependent secretagogue, forskolin, or the \( \mathrm{Ca}^{2+} \)-dependent secretagogue carbachol (Fig. 6). The inhibitory effect of the CB₁ agonist on capsaicin- and EFS-evoked secretion was completely reversed by pretreatment of the tissue with the selective CB₁ antagonist SR 141716A before application of WIN 55,212–2 (Fig. 5).

Additional studies were conducted to determine the role of extrinsic primary afferents on the inhibitory effects of the CB₁ agonist. Segments of denervated and innervated regions of ileum were mounted in Ussing chambers. Exposure of denervated segments to capsaicin did not elicit a change in \( I_{sc} \). Furthermore, in denervated segments of ileum, there were no differences in the response to EFS between tissues exposed to WIN 55,212–2 (1,000 nM) or the vehicle (vehicle, \( n = 9 \), 10).
DISCUSSION

In the present study, we have shown that CB1 receptors are present on enteric neurons and extrinsic primary afferent nerves in the submucosa of the guinea pig ileum. Furthermore, primary afferent nerves that express the CB1 receptor and contain substance P and CGRP predominantly innervate regions of the submucosal ganglia where cholinergic secretomotor neurons are located. This finding was supported by studies in Ussing chambers that revealed a cholinergic component of the response to capsaicin but not a response sensitive to VIP receptor desensitization. Despite the presence of CB1 receptors on submucosal neurons, a CB1 agonist acted primarily on the extrinsic primary afferent nerves to inhibit transmitter release, because the response to stimulation in the presence of this agonist was lost after extrinsic denervation.

Exposure to capsaicin elicited an increase in $I_{sc}$ as previously described (25, 42). There was some intergroup variation in these experiments. To account for this, we conducted experiments in paired tissues (i.e., vehicle control and drug-treated segments of ileum from the same animal) from animals of the same age, weight, and housing conditions and compared the data using paired $t$-tests. We have previously shown that the secretory response to capsaicin in guinea pig ileum is dependent on extrinsic primary afferent nerves and the activa-
tion of NK1 receptors on submucosal secretomotoneurons (25, 42). However, the population of secretomotoneurons activated after exposure to capsaicin has not been described. VIP-containing noncholinergic and cholinergic neurons (that also express NPY as a unique marker) make up the bulk of the secretomotoneurons controlling chloride secretion (5) and do so through the activation of cAMP- and Ca\(^{2+}\)-dependent pathways, respectively. Interestingly, VIP receptor desensitization did not affect the \(I_{sc}\) response to capsaicin. Similarly, experiments with the VIP receptor antagonists VIP\(^{10-28}\) (16) and [Acetyl-His\(^1\), d-Phe\(^2\), Lys\(^15\), Arg\(^{16}\), Leu\(^{17}\)] VIP(3-7)/GRF(8-27) (14) did not affect the response to capsaicin. Indeed, in our preparations, these antagonists failed even to block the secretory response to serosally applied VIP (data not shown). The muscarinic cholinergic antagonist atropine only reduced the response to capsaicin by \(\sim 35\%\). What the other component of the response is due to has yet to be determined. It is likely not due to a nicotinic receptor or an ionotropic glutamate receptor, as far as we have shown in this study, and neither is it likely due to GABA release, because MacNaughton et al. (26) have previously shown that the response to capsaicin is largely resistant to blockade of GABA receptors. The cholinergic submucosal neurons also contain other prosecretory transmitters including CGRP and cholecystokinin (5, 6, 11, 21). CGRP may be involved because this is both coreleased from capsaicin-sensitive extrinsic primary afferents (41) and is contained in the cholinergic secretomotoneurons (5, 6), but the CGRP antagonist hCGRP\(^{8-37}\) does not alter the response to capsaicin (25). However, we have previously shown that there may be a novel CGRP receptor on intestinal epithelial cells that is not blocked by that receptor antagonist (11). The effects of CCK receptor blockade on the response to capsaicin have not yet been investigated. Another possibility is that the response to capsaicin is through antidromic activation of intrinsic primary afferent neurons that contain substance P, which can act on NK1 receptors on the epithelia (12, 31). These neurons are also located at the poles of the submucosal ganglia and so would also be expected to be a target of extrinsic primary afferent nerves. Along with the cholinergic secretomotor nerves, they are the only neurons in the submucosal plexus to express NK1 receptors (24). Thus one could interpret the results of NK1 receptor blockade to suggest that capsaicin activates intrinsic primary afferent neurons that, along with extrinsic primary afferents, also release substance P. However, it is not possible to separate these effects at the current time.

The presence of extrinsic primary afferent nerves containing substance P in the submucosal plexus of the guinea pig ileum was first described by Costa et al. (8), who also made the observation that the varicosities of the extrinsic nerves were larger than those of the intrinsic nerve fibers. We have extended these observations to show that these nerves express CB\(_1\) receptors and, as mentioned above, that they appear to be preferentially localized at the poles of the submucosal ganglia. CB\(_1\) receptors have previously been shown (19) to be localized to a subpopulation of primary afferent neurons that express...
response to EFS, in agreement with previous findings in the rat (40). Furthermore, an incubation time of 20 min was required to achieve activation of the receptor. Nevertheless, evidence that the effect of WIN 55,212–2 was acting specifically at the CB1 receptor was obtained by completely reversing the inhibitory effect with the selective CB1 antagonist SR 141716A. Furthermore, we showed that the inhibitory effect of the CB1 agonist was on the enteric nerves and not on the epithelial cells, because the I_{sc} responses to forskolin and carbachol, which act directly on the epithelium to elicit secretion, were unaffected by WIN 55,212–2 pretreatment. Because we showed colocalization of the TRPV1 and CB1 receptors on extrinsic primary afferent nerves, we repeated experiments in extrinsically denervated segments of guinea pig ileum. As in our previous studies (42), extrinsic denervation rendered the preparations unresponsive to capsaicin and abolished TRPV1 immunoreactivity on paravascular nerve fibers and nerves in the submucosal plexus. Under these conditions, the inhibitory effect of WIN 55,212–2 on the response to EFS was completely lost, suggesting that the capsaicin-sensitive extrinsic primary afferent nerves are responsible for the CB1-receptor sensitivity of the response to EFS. This leaves open for further study the role of the CB1 receptors localized to the submucosal neurons. Possibly, this is just pool of receptors that is being trafficked to presynaptic sites in the mucosa or elsewhere and is not an active pool of receptors in enteric (postsynaptic) neurons. This requires further electrophysiological investigations.

CB1 receptors have been described previously in the porcine and murine submucosal plexus (20, 23) as they have in the myenteric plexus (9). In the pig, CB1 receptor immunoreactivity was found colocalized with cholineacetyltransferase and substance P (23), which is consistent with observations we have made. It was similarly colocalized in the mouse (20). In the pig, it was not found in VIP neurons, which is different from the guinea pig (23). CB1 receptor immunoreactivity was also found in association with blood vessels as we have observed (23), suggesting that it may be in primary afferent nerves in this species. The function of CB1 receptors in the pig submucosal plexus has not been thoroughly investigated, but addition of the potent CB1-receptor agonist CP-55,940 at 10 nM did not alter baseline I_{sc} or EFS-induced increases in I_{sc} across porcine ileal mucosal sheets mounted in Ussing chambers (D. R. Brown, personal communication).

TRPV1 receptors are expressed in the porcine small intestine in the inner and outer submucosal plexuses as well as the myenteric plexus (34). We did not observe neurons expressing TRPV1 in the submucosal or myenteric plexus (data not shown) of the guinea pig. This may be due to a difference in species reactivity or tissue-fixation techniques. Anavi-Goffer and Coutts (3) report TRPV1 receptors on all types of neurons of the myenteric plexus in the ileum and colon of the guinea pig, although they did not examine the submucosal plexus. It may be that neuronal cell bodies did not express sufficient TRPV1 for us to detect or that our antibody detected only receptors that are functionally incorporated into the membrane after posttranslational modification. However, denervated animals also showed a lack of fiber staining. Our functional evidence would also support the hypothesis that these undetected neurons are not a part of the secretory response and are

![Graph](http://ajpgi.physiology.org/00000000/00000000/00000000)
therefore unlikely to be intrinsic primary afferent neurons of the submucosal plexus.

We have localized CB1 and TRPV1 receptors on extrinsic primary afferent nerve fibers in the ileal submucosal plexus. It is not clear whether these are vagal or spinal afferent fibers. Vagal afferent neurons in the nodose ganglion have been reported to express CB1 receptors (29), as they do TRPV1 receptors, which are known to project to the gastrointestinal tract (30). However, using anterograde tracing techniques in combination with immunohistochemistry, Ward et al. (43) found no evidence of colocalization of TRPV1 receptors with vagal afferents, suggesting that the majority, if not all, TRPV1 (and presumably the colocalizing CB1 receptor) is derived from spinal afferents whose cell bodies are in the dorsal root ganglia.

In summary, CB1 receptors are present on submucosal neurons and extrinsic primary afferent nerves in the submucosa of the guinea pig small intestine. CB1-receptor activation in vitro inhibits intestinal secretion, primarily through receptors localized on extrinsic primary afferent nerves that innervate submucosal secretomotor neurons. Studies of the physiological role of cannabinoids in the gastrointestinal tract are becoming increasingly important because of the finding that endocannabinoids are present in the gut (32). Endocannabinoids have been shown to be physiological regulators of gastrointestinal motor functions (27, 33), but their role in the modulation of intestinal secretion under normal conditions has not been extensively investigated. However, there are recent data to show that an endogenous cannabinoid tone is important in regulating the extent of the secretory response to cholera toxin (20). This suggests that CB1 receptors may have important roles in pathophysiological conditions in the gut and that drugs interfering with endocannabinoid metabolism may be useful therapeutically in a variety of diseases of the gastrointestinal tract (10).

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REFERENCES


35. Poonyachoti S, Portughesa PS, and Brown DR. Pharmacological evidence for a 7-benzylidenealtrexone-prefering opioid receptor mediating the inhibitory actions of peptidic \( \delta \)- and \( \mu \)-agonists on neurogenic ion transport in porcine ileal mucosa. J Pharmacol Exp Ther 297: 672–679, 2001.


