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REVIEW

Involvement of cannabinoid receptors in gut motility and visceral perception

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From a historical perspective to the present day, all the evidence suggests that activation of cannabinoid receptors (CBRs) is beneficial for gut discomfort and pain, which are symptoms related to dysmotility and visceral perception. CBRs comprise G-protein coupled receptors that are predominantly in enteric and central neurones (CB1R) and immune cells (CB2R). In the last decade, evidence obtained from the use of selective agonists and inverse agonists/antagonists indicates that manipulation of CB1R can alter (1) sensory processing from the gut, (2) brain integration of brain-gut axis, (3) extrinsic control of the gut and (4) intrinsic control by the enteric nervous system. The extent to which activation of CB1R is most critical at these different levels is related to the region of the GI tract. The upper GI tract is strongly influenced by CB1R activation on central vagal pathways, whereas intestinal peristalsis can be modified by CB1R activation in the absence of extrinsic input. Actions at multiple levels make the CB1R a target for the treatment of functional bowel disorders, such as IBS. Since low-grade inflammation may act as a trigger for occurrence of IBS, CB2R modulation could be beneficial, but there is little supporting evidence for this yet. The challenge is to accomplish CBR activation while minimizing adverse effects and abuse liabilities. Potential therapeutic strategies involve increasing signaling by endocannabinoids (EC). The pathways involved in the biosynthesis, uptake and degradation of EC provide opportunities for modulation of CB1R and some recent evidence with inhibitors of EC uptake and metabolism suggest that these could be exploited for therapeutic gain.
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Introduction

A growing body of literature indicates that substances which act on cannabinoid receptors (CBR) alter secretion and motility of the gastrointestinal (GI) tract (reviewed in Pinto *et al*., 2002a; Di Carlo & Izzo, 2003) and have antinociceptive or antihyperalgesic properties (reviewed in Rice *et al*., 2002). This makes them an attractive target for GI functional disorders, such as Irritable Bowel Syndrome (IBS). In the introduction, we will review the background on the cannabinoids and their receptors, and then we will focus on the GI effects of drugs that interact with the CBR.

The evidence that cannabinoids exert beneficial effects on the GI tract has a long history. Reportedly, the Greek doctor Galen used marijuana to treat pain and flatulence, and its first reported use as an antiemetic is by Li Shih-Chen in 1578 (Earleywine, 2002). In the 1840s in India, where marijuana was a commonly used remedy, O'Shaughnessy reported his observations that, although it did not cure disorders, it eased the pain and nausea associated with them (O'Shaughnessy, 1842). By the early 1900s Squibb Company offered a mixture of cannabis and morphine (Chlorodyne) for stomach problems (Roffman, 1982), and other companies followed suit. However, subsequent restrictions on the use of cannabis, culminating in prohibition in the United States, dampened research and medicinal interest in cannabis in the first half of the 20th century.

Cannabis contains 66 cannabinoids of which delta^9-tetrahydrocannibol (delta^9-THC) and delta^8-THC appear to account for the majority of effects. Cannabis also contains high amounts of a nonpsychoactive constituent cannabidiol. In the 1960s and 1970s a majority of studies described the effects of delta^9-THC in experimental models. Several pharmaceutical companies developed cannabinoid analogues that went into clinical trials. This led to two marketed products, Nabilone, a chemical derivative of delta^9-THC (developed by Eli Lilly and Company but only available in the U.K.), and dronabinol (Marinol), a synthetic delta^9-THC (approved by the FDA in 1985 for cancer patients and in 1992 for AIDS patients). In 1999, the United States Drug Enforcement Agency reduced dronabinol's classification from schedule II to III. Marijuana is classified as Schedule I and its medicinal use is prohibited at the federal level, although an increasing number of states have passed referendums that allow its use ingested or inhaled. The relatively better apparent effect of smoked marijuana for its orexigenic and antiemetic effects than dronabinol are presumably due to better absorption and rapid onset of effect, which enables the effective dose to be more easily titrated.

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Delta^9-THC inhibits adenylyl cyclase and reduces cellular cAMP levels, which identified its receptor as a G-protein coupled receptor (GPCR). Sequence similarity to known GPCRs lead to cloning of CB1R (Matsuda *et al*., 1990), and soon after the CB2R (Munro *et al*., 1993). The CB1R has been localized in neural tissue throughout the body and described most thoroughly in central (Tsou *et al*., 1998; Fride, 2002) and enteric (Kulkarni-Narla & Brown, 2000; Coutts *et al*., 2002; MacNaughton *et al*., 2004) neurons. The CB2R is primarily expressed in the immune system (reviewed in Parolaro *et al*., 2002). Both receptors are G protein coupled *via* Gi/o. CB1R is highly conserved in rodent and human (Gerard *et al*., 1991) and found in a wide variety of species. This conservation is somewhat unusual among GPCRs, and has enabled much progress in the understanding of the site and potential roles of CBR in physiological and pathophysiological systems relevant for human.

The identification of the first reported endogenous ligand was a fatty acid amide, arachidonoylethanolamide (AEA), also termed, anandamide (Devane *et al*., 1992). Subsequently, a number of related endocannabinoids (EC) that bind to CBRs have been identified. The monoglyceride, 2-arachidonoyl-glycerol (2-AG) is even more abundant in brain tissue than AEA, and may be more potent at CB2R than AEA (Mechoulam *et al*., 1995; Sugiura *et al*., 2000). Both AEA and 2-AG are present in the mouse small intestine, with 2-AG being approximately 1000-fold higher than AEA (Izzo *et al*., 2001; Pinto *et al*., 2002a). The fact that 2-AG acts at CB2R on immune cells (Parolaro *et al*., 2002) has implications for inflammatory-related GI diseases, such as postinfectious IBS, but there are no data to demonstrate potential therapeutic benefits of this at present.

Another ether-type EC, 2-arachidonyl glyceryl ether (noladin ether) has also been identified in the porcine brain (Hanus *et al*., 2001), but negligible levels were found in the rat brain by using gas chromatography–mass spectrometry analysis and fluorometric high-performance liquid chromatography analysis (Oka *et al*., 2003). *cis*-9-Octadecenoamide (oleamide) has been recently proposed to be a selective endogenous agonist for CB1R (Leggett *et al*., 2004). Palmitoylethanolamide is another proposed EC, but it does not bind to CBR (Sugiura *et al*., 2000). Finally, virodhamine has been identified as a CB1R partial agonist *in vitro*, with antagonist activity *in vivo*, and as a full agonist activity of CB2R (Porter *et al*., 2002). Virodhamine is arachidonic acid and ethanolamine joined by an ester linkage, and although the initial results are intriguing, more studies are needed to determine the role of this novel EC.

GI effects of cannabinoids

Characterization of the effects of CBR stimulation comes from administration of selective agonists, such as analogs of delta^9-THC, and inverse agonists/antagonists. Since there have been several reviews on this subject in the last couple of years, we have focused on the most recent data and organized the known effects of cannabinoids on different regions of the upper to lower GI tract. In the subsequent section, we have reviewed the evidence for the potential sites of action of CBRs mediating these effects.

Upper GI tract and CBR

Early studies showed that delta^9-THC slowed the rate of gastric emptying and small intestinal transit in mice and in rats (Shook & Burks, 1989). The ability of cannabinoids to decrease motor activity in the stomach (Krowicki *et al*., 1999) and decrease gastric emptying (Izzo *et al*., 1999a) were confirmed. In both studies, the effects were reversible by the selective CB1R antagonist, *N*-piperidino-5-(4-chlorophenyl)-l-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide (SR14 1716A or rimonibant, Sanofi Recherche) indicating that the effects were mediated *via* CB1R. Similar findings have also been reported in healthy volunteers given delta^9-THC, confirming that the drug delays gastric emptying of a radiolabeled solid food (McCallum *et al*., 1999). Delta^9-THC also decreased intragastric pressure in rats (Krowicki *et al*., 1999) and, by using a miniaturized rigid cylinder barostat, it was shown that this resulted in an increase in intragastric volume (Ball *et al*., 2001). Whether these effects could be beneficial in patients with functional dyspepsia that have impaired fundic relaxation is unknown. However, since basal gastric tone and compliance is related in some way to nausea and feeling of fullness, these effects could contribute to the antiemetic and orexigenic effects of delta^9-THC. In this regard is somewhat puzzling that CB1R activation delays gastric emptying (Izzo *et al*., 1999a; Krowicki *et al*., 1999) since this can be associated with nauseogenic stimuli. The dissociation of delayed gastric emptying and gastric stasis from the sensation of nausea may be an example of the way in which CB1R can alter visceral perception. Certainly, antinausea and antiemetic effects of cannabinoids have been well characterized.

The antiemetic effect of delta^9-THC and related compounds has been confirmed clinically (Tramer *et al*., 2001). In animal studies, activation of CBR1 has dose-related antiemetic effects in experimental models of emesis (Darmani, 2001; Simoneau *et al*., 2001; Van Sickle *et al*., 2001; 2003; Darmani *et al*., 2003b; Parker *et al*., 2004). In non-humans, nausea is hard to measure, however, conditioned rejection reactions in rats may reflect a sensation of nausea (Parker & Kemp, 2001). Delta^9-THC and CB1R agonists interfere with nausea elicited by lithium chloride and with conditioned nausea elicited by a flavor paired with lithium chloride (Parker *et al*., 2002; 2003). The same group also present evidence that CB1R activation may be effective to prevent an animal model of anticipatory nausea and vomiting. In *Suncus murinus* (musk shrew) the investigators paired a novel contextual cue with an emetogenic injection of lithium chloride. After training, the context alone could elicit retching in the absence of the toxin. This conditioned response was completely suppressed by pretreatment with delta^9-THC, at a dose that did not suppress general activity (Parker & Kemp, 2001). A more detailed discussion of the site of action for the antiemetic effects of cannabinoids is discussed later, but multiple lines of evidence suggest that it is on CB1R vagal pathways both centrally and peripherally (see below).

Activation of CBRs also has effects on the lower oesophageal sphincter (LOS) that may be beneficial in gastro-oesophageal reflux disease (GORD). Although the majority of GORD patients are well controlled by antacids, proton pump inhibitors, and histamine2 receptor antagonists, there remains a population of less well-defined patients who do not respond to these treatments and have symptoms of pain and

discomfort associated with reflux. It is now generally accepted that neural control of LOS pressure may provide a different mechanism for reducing acid reflux. Specifically, LOS tone is generally maintained except during transient lower oesophageal sphincter relaxations (TLOSRs), swallowing, and prior to emesis. TLOSRs are defined as rapid, sustained reductions in pressure, which are not associated with a swallow (Holloway & Dent, 1990). They increase in frequency after a meal, are associated with reflux events, and may promote the development of GORD (Holloway & Dent, 1990). Therefore, reducing the incidence of TLOSRs could remove an underlying cause of reflux, and several substances (e.g. $GABA_B$ receptor agonist, baclofen) have been shown to be effective to prevent TLOSRs in human (Lidums *et al*., 2000).

The CBR agonist, WIN 55,212-2, attenuated TLOSRs evoked by gastric distention in conscious dogs (Lehmann *et al*., 2002). Specifically, WIN 55,212-2 reduced by 80% the incidence of TLOSRs and increased the latency of the first TLOSR, as well as reduced swallowing (Lehmann *et al*., 2002). Both drugs also reduced gastric distention-evoked LOS relaxation in decerebrate and unanesthetized ferrets (Partosoedarso *et al*., 2003b). These effects are *via* CBIR activation in both of these studies since they were prevented by the selective CB1R antagonist, rimonibant. The site of action for CB1R to inhibit TLOSRs is *via* modulation of vagal pathways at peripheral and central levels (see below). However, despite the encouraging preclinical data, it is unknown whether CBRs mediate the same effect in humans. In addition, it unclear the extent to which reduction of TLOSRs alone would be effective in GORD patients. This is partly because inhibition of TLOSRs is not always associated with reduced acid exposure in the oesophagus, for example, Zhang *et al*. (2002). A two pronged approach – to inhibit TLOSRs and reduce gastric acid secretion could potentially be more effective.

The experimental data in animals suggests that CB1R activation also decreases gastric acid secretion. In anesthetized rats, although CB1R activation does not alter unstimulated basal (low level) secretion (Coruzzi *et al*., 1999), it attenuates gastric acid secretion induced by both pentagastrin and 2-deoxy-D-glucose (Adami *et al*., 2002). In contrast, CB1R agonists have no effect on histamine-stimulated gastric acid secretion (Adami *et al*., 2002). Because pentagastrin and 2-deoxyglucose both stimulate vagally mediated acid secretion, these data implicate CB1R on vagal efferent control of parietal cell secretion, rather than directly on parietal cells. However, it also should be noted that since the receptor is present on enterochromaffin-like cells in the stomach, activation of CB1R could also reduce endogenous histamine release and thereby reduce acid secretion (Adami *et al*., 2002). CB2R-immunoreactivity was not visualized in the rat stomach by using a human CB2R antibody (Adami *et al*., 2002) and activation of CB2R selectively did not attenuate basal or stimulated gastric acid secretion (Coruzzi *et al*., 1999; Adami *et al*., 2002). WIN 55,212-2 acting on CB1R also reduces gastric ulceration in cold-restraint stress paradigm (Germano *et al*., 2001), but the site of action of this effect is not yet known. Consistent with the experimental studies, an early study showed that human volunteers who smoked cannabis more than twice a week had low gastric acid output (Nalin *et al*., 1978).

In summary, experimental animal data support the notion that CB1R activation both reduces TLOSRs and gastric acid output. However, to our knowledge, it is not known whether delta^9-THC or its analogues are effective in humans to reduce TLOSRs and gastric acid secretion or what their potential therapeutic utility may be in GORD patients.

Lower GI tract and CBR

Low-frequency electrical field stimulation (EFS) results in contraction of muscle in a longitudinal muscle-myenteric plexus-preparation of the guinea-pig small intestine due to acetylcholine (ACh) release (Pertwee *et al*., 1996; Coutts & Pertwee, 1997). CBR agonists inhibit the EFS-evoked response in a rimonibant reversible manner, but have no effect on responses to exogenous Ach. These data indicate a presynaptic site of action of CB1R to reduce excitatory (cholinergic) neurotransmission to the smooth muscle (Pertwee *et al*., 1996; Coutts & Pertwee, 1997; Pertwee, 2001). Indeed, myogenic contractions of the guinea pig ileum induced by indomethacin are not inhibited by CBR agonists, suggesting that there are minimal effects directly on smooth muscle (Heinemann *et al*., 1999). Similar to the situation in guinea-pig, WIN 55,212-2 prevented contractions elicited by EFS in human ileum (and colon), but not carbachol (Manara *et al*., 2002), suggesting that functional receptors are present in human.

CB1R are involved in regulation of small intestinal water and electrolyte transport. In guinea-pig (MacNaughton *et al*., 2004) and rat (Tyler *et al*., 2000) ileum, WIN 55,212-2 reduces the EFS-evoked increases in short-circuit current (I_{sc}), which is an indicator of net electrogenic ion transport. Both studies showed that the effect of the agonist to inhibit neurogenically mediated increases in I_{sc} was reversible by rimonibant. CB1R are visualized on both noncholinergic (VIP) and cholinergic (NPY + ve) submucosal secretomotor neurones (MacNaughton *et al*., 2004). It should be noted that, in contrast to this study, VIP neurons did not contain CB1R in the pig (Kulkarni-Narla & Brown, 2000).

Capsaicin can be used to activate extrinsic primary afferents resulting in an increase in I_{sc} (MacNaughton *et al*., 2004). CB1R activation inhibits the responses to capsaicin (by just under 50%) and EFS (by about 30%). WIN 55,212-2 did not alter I_{sc} responses to forskolin or carbachol (MacNaughton *et al*., 2004). Therefore, these studies clearly indicate that the agonists act on nerves rather than directly on the epithelium to attenuate stimulated ion transport. They further present evidence that suggests that CB1R on extrinsic nerves may be important for this response (see below).

Increased accumulation of fluid in the *ex vivo* small intestine in response to cholera toxin is significantly reduced by administration of CB1R agonists, in an rimonibant reversible manner (Izzo *et al*., 2003). In an *in vivo* model of diarrhea, oral administration of croton oil induces diarrhea and increased GI transit, which are inhibited in a dose-related fashion by WIN55,212-2 and cannabinol (Izzo *et al*., 2000). Furthermore, croton oil administration induced levels of inflammation that were correlated with increased CB1R expression, and CBR agonists more effectively decreased intestinal motility in inflamed than in control animals (Izzo *et al*., 2001). This evidence points to a protective role of CB1R in inflammation-mediated motility changes. Other models also demonstrate efficacy of CB1R activation to reduce diarrhea. An early study showed that delta^9-THC (5–10 mg kg) reduces diarrhea associated with naloxone-precipitated withdrawal from morphine in rats (Hine *et al*., 1975). Additionally, blockade of

endogenous EC with rimonibant precipitates diarrhea in morphine-dependent rats (Navarro *et al*., 1998). However, a low oral dose of 20 mg^{-1} kg delta^9-THC in mice was not able to reduce diarrhea precipitated by naloxone in morphine-tolerant animals, although other aspects of withdrawal could be attenuated (Cichewicz & Welch, 2003). Therefore, this effect was only evident at doses that were associated with other CNS effects. Despite these data, the overall effect of CB1R activation on secretion and absorption in humans is not established. In one early study, it was noted that human volunteers who smoked cannabis developed more copious diarrhea when exposed to *Vibrio cholerae* (Nalin *et al*., 1978); however, no clinical studies using orally administered CB1R agonists are available to our knowledge.

Peristalsis can be initiated by radial extension of the intestines and results in oral ascending excitatory and descending inhibitory activity in the intrinsic myenteric nerves. Methanandamide inhibited both the cholinergic component of the ascending excitatory reflex, as well as the hexamethonium-resistant portion, which is thought to be due to tachykinin release (Heinemann *et al*., 1999). Since the ascending reflex initiates peristalsis, the effect of CBR would be to raise the threshold for peristalsis and reduce propulsive activity.

The antipropulsive effects of CB1R should reduce transit time in the large intestine, and this appears to be the case. Delta^9-THC reduces fecal pellet output in open field behavior of rats, and this was prevented by rimonibant, suggesting that stress-evoked changes in transit can be reversed by CB1R activation (Jarbe *et al*., 1998). In the mouse colon, the expression of CB1R in myenteric neurones combined with functional data in myenteric neurone/smooth muscle preparation indicate that CB1R are expressed in cholinergic neurones (Storr *et al*., 2004). For example, CB1R agonists reduced the excitatory junctional potential evoked by focal stimulation and consistent with this, the evoked response is significantly higher in CB1R-deficient mice than in wild-type littermate controls (Storr *et al*., 2004). Consistent with this, rimonibant increased excitatory junctional potentials in wild type but not in CB1R-deficient mice. Interestingly, CB1R is not visualized in nitrergic myenteric neurones (Storr *et al*., 2004) which include descending inhibitory motor neurons. These investigators confirmed that in mouse colon the CB1R is colocalized in a subpopulation of choline acetyltransferase-immunoreactive neurones and fiber bundles in the myenteric plexus. In mouse colon, CB1R agonists slowed the expulsion of a glass bead inserted into the distal colon, whereas rimonibant alone increased motility (Pinto *et al*., 2002b). All these data suggest that CB1R inhibits excitatory cholinergic neurotransmission in mouse colon similar to other rodents.

There are no studies in human volunteers on the colonic effects of CB1R activation; however, in one study AEA did not modify the relaxant effect of capsaicin on mucosa-free circular strips of the human sigmoid colon *in vitro* (Bartho *et al*., 2002).

CBR sites of action mediating GI effects of cannabinoids

What is clear from the experimental data is that CB1R agonists act at multiple sites to mediate their GI effects. Most of the evidence points to the central vagal site of action of cannabinoids to modulate vomiting (Van Sickle *et al*., 2001; Darmani *et al*., 2003b; Van Sickle *et al*., 2003) gastric motility (Krowicki *et al*., 1999), gastric volume/pressure (Ball *et al*., 2001), lower esophageal sphincter pressure (Partosoedarso *et al*., 2003b) and gastric acid secretion. In the hindbrain medulla, CB1R immunoreactivity is visualized with varying intensity within the area postrema, subnuclei of the nucleus tractus solitarius and dorsal motor nucleus of the vagus nuclei (Figure 1), which altogether form the dorsal vagal complex (Van Sickle *et al*., 2001; 2003; Partosoedarso *et al*., 2003b). Clear evidence of the central vagal site of action comes from experiments where local application of CB1R agonists to the surface of the medulla above the dorsal vagal complex mimicked the antiemetic (Van Sickle *et al*., 2003) and LOS (Partosoedarso *et al*., 2003b) effects of i.v. delta^9-THC, at a dose 100-fold lower than the lowest effective intravenous dose. Maybe this is not surprising that CB1R activation inhibits emesis centrally, since both LOS relaxation and emesis are part of central motor pattern generation involving the dorsal vagal complex and other nuclei in the hindbrain (reviewed in Hornby, 2001; Hornby *et al*., 2002). Functional activation of emetic neuronal pathways induced by cisplatin results in Fos expression in the area postrema, dorsal motor nucleus of the vagus, and the medial and dorsal subnuclei of the nucleus tractus solitarius (Van Sickle *et al*., 2003). In all these regions, Fos expression was significantly reduced by delta^9-THC (Van Sickle *et al*., 2003). Within the nucleus tractus solitarius, cannabinoids may act on the central terminals of vagal primary afferents or on interneurones synapsing with vagal motor neurones. In the CNS it is well known that ECs are released from the nerve terminal and presynaptically inhibit the release of excitatory and inhibitory neurotransmitters (reviewed in Freund *et al*., 2003). In the dorsal vagal complex both GABAergic and glutamatergic input to vagal motor

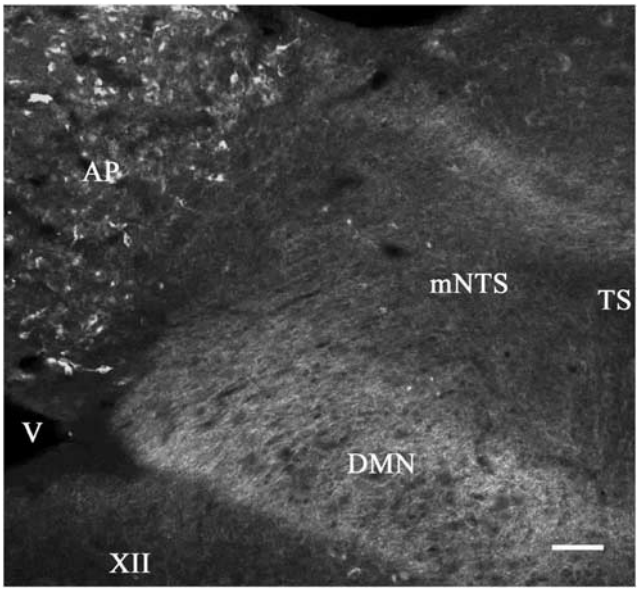

Figure 1 Photomicrograph of CB1R-immunostaining in the ferret hindbrain dorsal vagal complex. Intense staining in cell bodies is noted in the area postrema (AP) and within the medial nucleus tractus solatarius (mNTS). Punctate terminal field like staining is intense in the dorsal motor nucleus of the vagus, but not within vagal motor neurones. Abbreviations: TS, tractus solitarius; V, fourth ventricle XII hypoglossal nucleus. Bar = 100 μm. Reproduced with permission from Partosoedarso *et al.* (2003b).

neurones are present (Travagli *et al*., 1991). Preliminary data in whole-cell patch-clamp recordings from a hindbrain slice preparation showed that CB1R agonists inhibited spontaneous and evoked excitatory and inhibitory postsynaptic currents in vagal motor neurones (Derbenev *et al*., 2002; Derbenev & Smith, 2003). These effects were sensitive to synaptic transmission blockade by tetrodotoxin, which suggests that CB1R agonists reduce the synaptic activity during transfer of information from the nucleus tractus solitarius to the vagal motor neurones.

Cannabinoids could also act at the peripheral terminals of vagal afferents to alter visceral perception. CB1R is highly expressed in the nodose ganglion (the location of vagal sensory cell bodies (Partosoedarso *et al*., 2003b) and denervation of c-fiber afferents by perivagal capsaicin treatment abolished the increase in gastric volume evoked by i.v. delta^9-THC (Ball *et al*., 2001). Consistent with this site of action, delta^9-THC also inhibited the cisplatin-induced emesis (Van Sickle *et al*., 2003), and cisplatin induces an early emesis *via* serotonin release from gut enterochromaffin cells that acts on vagal afferents. Interestingly, CB1R and cholecystokinin1 receptors are co-expressed within vagal afferents that project to the stomach and duodenum, and the data suggest that these interact to modulate food intake and satiety (Burdyga *et al*., 2004). In this regard it is perplexing that, in a relatively small number of fibers tested, WIN 55,212-2 did not alter firing of gastric vagal mechanoreceptors in response to gastric distention (Lehmann *et al*., 2002).

Although the above evidence strongly supports a primary site of action of CB1R in the brain to mediate upper GI effects, CB1R immunoreactivity is also present in choline acetyltransferase-positive neurones innervating the gastric muscle and mucosa (Adami *et al*., 2002; Casu *et al*., 2003). The role of CB1R at these peripheral gastric sites has not been established yet since vagotomy prevents the antisecretory effect of CB1R on acid release stimulated by pentagastrin and 2-deoxy D-glucose (Adami *et al*., 2002). Although preliminary data suggest that intracerebroventricular administration of CBR agonists, HU210 and WIN55,212-2, did not attenuate pentagastrin-stimulated acid secretion (Adami *et al*., 2001), this has not been confirmed after administration directly to the dorsal vagal complex, where the agonists would be expected to be effective. Further studies on the site of action for the gastric antisecretory effects on CB1R agonists are needed.

Upper GI transit is increased within 3 h after administration of croton oil. The GI transit effects of croton oil were assessed after intraperitoneal and intracerebroventricular administration of CB1R agonists, in an attempt to ascertain their most likely site of action (Izzo *et al*., 2000). In these mice, the ED50 values for WIN55,212-2 for inhibition of upper GI transit were lower for intracerebroventricular compared to intraperitoneal administration. In addition, the GI transit effects of WIN55,212-2 given centrally were reversed by hexamethonium (given i.p.). However, a 10-fold higher dose of the CB1R agonist, given intraperitoneally, resulted in reduced GI transit that was not altered by hexamethonium. This suggests that at higher doses there may be direct effects of CB1R activation on nonextrinsic nerves controlling the upper GI tract, that is, on the enteric nervous system. The overall data are consistent with the central site of action of cannabinoids being critical for regulation of upper GI transit, at least when the doses of exogenous agonist are low.

Whereas for the upper GI tract, the CNS is the primary site for many of the motility affects of CB1R agonists, the picture is somewhat less clear for the intestines. Colocalization studies of CB1R and neurotransmitters in the guinea-pig and rat enteric neurones showed that CB1R is expressed on cholinergic sensory, interneuronal, and motor neurones in myenteric ganglia (Coutts *et al*., 2002). Indeed, the majority of choline acetyltransferase-positive neurones expressed CB1R and myenteric neurones responded to cannabinoids in the presence of hexamethonium suggesting that functional CB1R exist on excitatory intrinsic motoneurones (Coutts *et al*., 2002). CB1R is also present within intrinsic neurons in the submucosal plexus of the ileum, and is colocalized with vanilloid receptors are paravascular nerves and fibers suggesting that there is also an extrinsic source of CB1R (MacNaughton *et al*., 2004). It is interesting that vanilloid receptors and CB1R are colocalized because vanilloid receptors are expressed in extrinsic primary afferents and it has been suggested that vanilloid receptors functions as a cannabinoid-gated channel in the CNS (Roberts *et al*., 2004). The extrinsic source of CB1R was confirmed to be on the peripheral terminals of primary afferents to the submucosal plexus because when the segments of ileum were extrinsically denervated the inhibitory effect of WIN 55,212-2 on EFS evoked I_{sc} was abolished (MacNaughton *et al*., 2004). It is been demonstrated that CB1R are synthesized in cells of the dorsal root ganglia and inserted on terminals in the periphery (Hohmann & Herkenham, 1999), which could account for the presence of CB1R in submucosal extrinsic afferents. These data collectively show that an important site of action for CB1R agonists to reduce I_{sc} is *via* the extrinsic primary afferents that act on cholinergic secretomotor pathways. Therefore, CB1R in extrinsic afferents may be important for controlling water balance in the intestines. The role of CB1R localized in intrinsic submucosal neurons (Izzo *et al*., 2003; Kulkarni-Narla & Brown, 2000) remains to be clarified. However, CB1R may have pivotal actions on intrinsic submucosal secretomotor neurons in pathophysiological states; for example, CB1R agonists inhibit cholera toxin induced fluid accumulation in mice after ganglionic blockade with chlorisondamine (Izzo *et al*., 2003).

Cannabinoids can also mediate their effects directly by acting on the intrinsic neurones in the absence of extrinsic input. For example, in the isolated mouse distal colon, WIN 55,212-2 attenuated peristaltic activity and decreased contractile activity and volume of fluid ejected during peristalsis (Mancinelli *et al*., 2001). Rimonibant alone enhanced both tonic and phasic motor activities in the colonic longitudinal smooth muscle (Mancinelli *et al*., 2001). However, an interesting observation was made *in vivo* that delta^9-THC produced relatively less inhibition of large bowel transit than gastric emptying and small intestinal transit (Shook & Burks, 1989). If one assumes that the vagal modulation of the lower GI tract by the CNS is relatively less than the upper GI tract then the implication is that although CB1R activation can inhibit peristalsis on isolated intestine, overall the effects on the GI tract are more profound when the receptor is acting centrally. However, it is not clear how this situation may be changed in pathophysiological states, which may also involve spinal and sympathetic pathways rather than vagal pathways. For example, it has been shown in other systems, such as a neuropathic pain model, that WIN 55,212-2 reduces the hyperalgesia that develops after sciatic nerve ligation and

reduces the levels of pronociceptive substances, such as prostaglandin E2 (Costa *et al*., 2004). In addition, since the CB1R and vanilloid receptor (Roberts *et al*., 2004) are colocalized in dorsal root ganglion, CB1R modulation may have benefit in pain-related states. Finally, inflammation induced by croton oil increased CB1R expression, and CBR agonists were more effective at reducing intestinal motility in inflamed than in control animals (Izzo *et al*., 2001).

Probably all levels of the brain-gut axis can be modulated by exogenous and endogenous CBR agonists (Figure 2), and so far, the evidence indicates that the ultimate effect on the organ is similar, no matter where the specific site of action(s) are. This makes the cannabinoid system quite attractive to target since a drug should have a similar effect whether it modulates the EC system locally in the gut or more remotely in the brain. The case can be made for beneficial effects of agonists (antiemetic and antimotility) and antagonists (anorexic and to prevent GI stasis/hypomotility) in this regard.

Endocannabinoids and the gut

The dose-limiting psychotropic adverse effects of potent CB1R agonists restrict their therapeutic utility. In addition, most

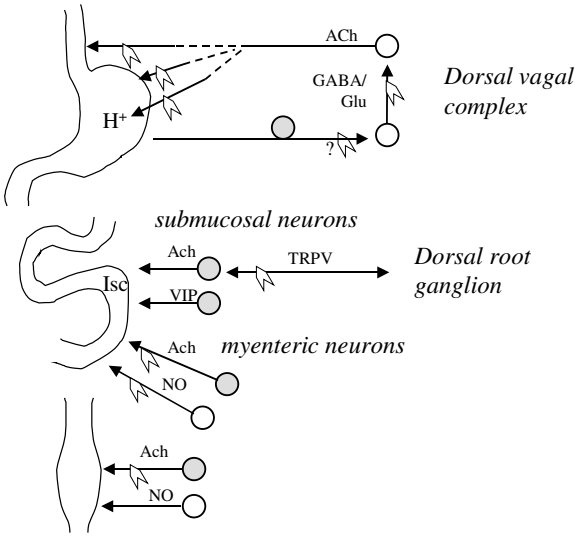

Figure 2 Schema that summarizes the main site(s) of action of cannabinoids on CB1R (open arrowhead) and immunocytochemical localization of CBR1 (stipple). Arrows indicate motility effects, except H^+ refers to gastric acid secretion and I_{sc} intestinal ion transport. For the upper GI tract, some evidence suggests that CBR activation could inhibit vagal afferents centrally, excitatory (Glu) and inhibitory (GABA) interneurones in the nucleus tractus solitarius and cholinergic (Ach) motor output peripherally at the cholinergic postganglionic/enteric neurons level to reduce gastric motility, acid secretion, increase volume, delay gastric emptying, and to reduce lower oesophageal sphincter relaxation. In the submucosal plexus of the small intestine, CB1R is expressed in ACh and vasoactive intestinal peptide (VIP) neurones but functionally inhibits I_{sc} through extrinsic primary afferents, some of which which contain the vanilloid receptor (TRPV), and act *via* excitatory ACh neurons. Within the myenteric plexus, CB1R inhibits contractile activity and peristalsis of the intestines *via* inhibition of excitatory ACh. It does not appear to act on inhibitory nitric oxide (NO) containing neurons in the myenteric plexus of the large intesting but inhibits NANC small intestinal relaxation. CB1R distribution and effects in different species may vary. See text for further details.

available agents are highly lipophilic and therefore penetrate centrally, even though the sites of action for their therapeutic effects are less protected by the blood-brain barrier, such as within the dorsal vagal complex, or peripherally on extrinsic nerves or on intrinsic enteric neurones. Therefore, recent efforts towards modulation of the CB1R have focused on the EC system.

The data reviewed above indicate that selective antagonists/inverse agonists of CB1R, such as rimonibant, have been critical not only for demonstrating CB1R selectivity of the observed effects, but also to demonstrate endogenous tone of bound CB1R, or a tonic spontaneous level of activity of unbound CB1R. A neutral antagonist will only prevent the activation of the receptor in the presence of the agonist. Inverse agonists will decrease the tonic level of activation of the receptor in the absence of the agonist. In general, inverse agonism in GPCRs has only unequivocally been demonstrated in cell systems where there is overexpression of the receptor or the mutated active receptor. Therefore, in functional studies where rimonibant alone is effective, it is not certain that it is acting as an inverse agonist of the CB1R in the absence of EC. For the purposes of this review, we will take the most conservative interpretation of effects of rimonibant *in vivo*, that tonic EC release is being antagonized at the CB1R.

In the majority of studies reviewed above, rimonibant given alone has effects opposite to that evoked by CB1R agonists (with a few exceptions, where there is no effect, such as found in Partosoedarso *et al*., 2003b). This suggests that throughout the GI tract CB1R are tonically active due to underlying EC release. However, what is not clear is whether EC are released constitutively or only in response to perturbation of the physiological system. For example, large doses of rimonibant cause vomiting in the least shrew (*Cryptotis parva*) with maximal response at $20\,mg^{-1}\,kg$ (Darmani *et al*., 2003a). In other species, CB1R antagonists alone have no effect (Darmani, 2001; Van Sickle *et al*., 2001; 2003; Darmani *et al*., 2003b). These data can be reconciled if one speculates that there is a high degree of ongoing emetic signalling in the least shrew, and that this stimulus is being attenuated by a relatively high level of EC release. In ferret, CB1R antagonists given alone are not emetogenic in the absence of emetic stimulation, but potentiate the emesis in response to 6 morphine-glucuronide (Van Sickle *et al*., 2001). In this species, maybe emetic stimulation is required to increase the level of EC release such that the antagonist increases emesis. However, it is not known whether there is constitutive EC release within these pathways, or whether neuronal activation is essential for EC release in most cases. In isolated muscle preparations where CB1R antagonists have effects, it is often in the presence of chemical or electrical field stimulation. The presynaptic anterograde action of CB1R activation suggests that EC release would be quiescent until the neuronal circuitry is recruited. This would appear to be the case in several animal models of diarrhea. For example, an acute model of cholera toxin increased fluid accumulation in the small intestine was correlated with an increase in AEA released and the expression of CB1R mRNA locally (Izzo *et al*., 2003). The extent to which EC system is engaged in experimental models seems to depend on the pathophysiological condition. For example, when upper GI transit was enhanced after oral administration of the inflammation-evoking irritant croton oil, the ED50 for WIN 55,212-2 was more than two fold less than in control mice,

suggesting a robust increase in CB1R efficiency or number (Izzo *et al*., 2000). However, after castor oil administration there was little antidiarrheal effect of CB1R agonists (Izzo *et al*., 1999b).

Higher concentrations of AEA can also mediate effects that are not reversed by CB1R antagonist, rimonibant but which may be due to actions on the vanilloid receptor. For example, in the guinea-pig myenteric plexus-longitudinal muscle preparation, AEA inhibited electrically evoked contractions with a pEC_{50} of 5.2, and acetylcholine release with a pEC_{50} of 5.8 (Mang *et al*., 2001) and addition of rimonibant resulted in the expected rightward shift in the dose – response curves. In contrast, AEA increased basal acetylcholine release and resting longitundial muscle tone with a pEC_{50} of 6.3 for both responses (Mang *et al*., 2001). These effects of AEA on basal tone and acetylcholine release were reversed by capsazepine, which is a vanilloid receptor antagonist, and tachykinin (NK1 and 3) receptor antagonists, but not by rimonibant (Mang *et al*., 2001). The effects of AEA on evoked muscle contraction were not reversed by capsezipine. The investigators conclude that AEA increases basal acetylcholine release *via* a non-CB1R-mediated mechanism that could involve vanilloid receptors on primary afferent nerves (Mang *et al*., 2001). Additional support for a role of AEA on extrinsic primary afferents comes from a study using an acute model of ileitis (McVey *et al*., 2003). Both AEA (30 and 100 μg) and 2-AG (10 and 100 μg) given intraluminally increased myeloperoxidase levels and fluid accumulation and both these effects were reversed by pretreatment of rats with subcutaneous capsezepine (McVey *et al*., 2003). Intraluminal CB1R agonists did not mimic the effects of the ECs. They further showed that intraluminal AEA induced neurokinin receptor internalization in myenteric neurons (used as a measure of substance P release) and they conclude that a likely scenario is that at the concentration of AEA and 2-AG applied intraluminal activated vanilloid receptors resulting in SP release and subsequent neurogenic inflammation.

Antagonists have demonstrated the presence of the EC system and its role in GI functions, however, therapeutic benefits of EC that have been discussed here involve increasing their levels, rather than antagonizing the CB1R. Therefore, in the last section we will discuss the various metabolic pathways that govern synthesis, release, uptake, and degradation that regulate the activity of the EC system. The proteins involved in these pathways, as well as their regulation, provide possible targets for diseases in which EC signaling is implicated.

Synthesis of AEA and 2-AG

Two pathways have been shown biochemically to produce AEA: (1) condensation of ethanolamine with arachidonic acid by fatty acid amide hydrolase (FAAH), and (2) transacylation by *N*-acyltransferase to form *N*-arachidonoyl phosphatidylethanolamine (PE), which is followed by phospholipase D (PLD)-catalyzed release of AEA. Although the former reaction has been demonstrated in testis membranes (Schmid *et al*., 1998) and has been confirmed with recombinant FAAH enzyme (Kurahashi *et al*., 1997), the high K_m for ethanolamine *in vitro* suggests that it is the latter reaction that is physiologically relevant. With regard to the transferase-phosphodiesterase reaction pathway, relevant substrates, enzyme activities, and products have been demonstrated in various tissues, including brain (Cadas *et al*., 1997) and testis (Sugiura *et al*., 2002). Consistent with the idea that the EC system is activated in response to neural activity, *N*-arachidonoyl PE accumulates during neural injury (Hansen *et al*., 2001) and *N*-acyltransferase enzyme activity is regulated by calcium (Cadas *et al*., 1997), as well as by activation of neurotransmitter receptors (Stella & Piomelli, 2001).

In contrast to AEA, 2-AG has more synthetic pathways, and these are dependent on the type of cell and tissue (Sugiura *et al*., 2002). Levels of 2-AG are elevated by increased calcium (Bisogno *et al*., 1997), activation of NMDA receptors (Stella & Piomelli, 2001), and in response to lipopolysaccharide (Di Marzo *et al*., 1999).

Little is known about the mechanism whereby ECs are released from the cell. In the section below, we will discuss uptake of EC's through an anandamide membrane transporter. This transporter may be bidirectional (Figure 3) and facilitate release of AEA (Hillard *et al*., 1997; Hillard & Jarrahian, 2003), however this mechanism is controversial. Once released, EC's appear to remain localized at the site since there is synapse-specific inhibition of neurotransmitter release in cerebellar slices (Brown *et al*., 2003).

Uptake and breakdown of AEA and 2-AG

The mechanisms involved in EC catabolism are of great interest due to the possibility of interfering with these functions in order to enhance levels of extracellular EC, for treatment of

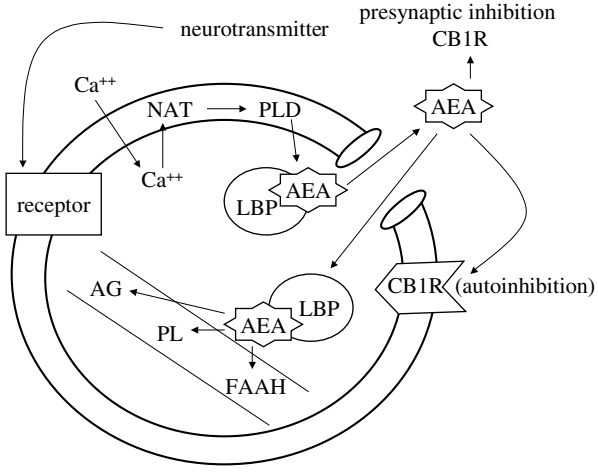

Figure 3 Activity-dependent depolarization of a presynaptic neurone causes neurotransmitter release, which after binding to its receptor on the postsynaptic neurone, causes calcium influx. Increased calcium activates *N*-acyltransferase (NAT), which results in production of *N*-arachidonoyl phosphatidylethanolamine. Phospholipase D (PLD) liberates anandamide (AEA) from *N*-arachidonoyl phosphatidylethanolamine, which then could associate with a lipid binding protein (LBP), and be transported to the anandamide membrane transporter (illustrated as a pore in the plasma membrane of the postsynaptic neurone). AEA can then bind to CB1 receptors (CB1R) on the presynaptic neurone, resulting in decreased intracellular calcium and presynaptic inhibition. AEA signaling could be inactivated by reuptake through the anandamide membrane transporter, where it could bind a LBP and be transported to microsomal membranes (illustrated as parallel lines) for degradation by fatty acid amide hydrolase (FAAH) or esterification into phospholipids (PL) and/or acyglycerols (AG).

diseases. Inactivation of EC signaling is dependent on cellular uptake, localization to appropriate intracellular compartments, and FAAH-mediated hydrolysis. This latter reaction produces arachidonic acid, and either ethanolamine (from AEA) or glycerol (from 2-AG). ECs can also be metabolized by fatty acid oxygenases (Kozak & Marnett, 2002). Although it is generally recognized that there is uptake, intracellular transport, and hydrolysis of ECs, it is only the hydrolysis step that has a definitively assigned protein (FAAH). The mechanisms underlying the movement of AEA across plasma membranes is highly controversial and is the subject of several recent reviews (Hillard & Jarrahian, 2000; 2003; Fowler & Jacobsson, 2002). Several proteins, acting alone or in conjunction, could account for AEA uptake, these being a membrane transporter, intracellular binding proteins, and FAAH (Figure 3). Consistent with the presence of a membrane transporter is the reduction of AEA uptake with selective transport inhibitors (Lopez-Rodriguez *et al*., 2003). Intracellular binding proteins are supported by the role of fatty acyl Co-A synthase in uptake of long chain fatty acids (Schaffer & Lodish, 1994). Finally, FAAH can be a driving force for uptake of AEA since there is increased AEA uptake in cell lines that have been transfected with FAAH (Day *et al*., 2001). Other possible intracellular 'sinks' for ECs are enzymes involved in esterification of fatty acids into acylglycerols and phospholipids. Consistent with this idea is the finding that the arachidonic acid moiety of 2-AG is hydrolyzed and incorporated into phospholipids and monoacylglycerols of astrocytoma cells. This process can be blocked with triacsin C, an acyl Co-A synthetase inhibitor, suggesting that esterification of arachidonic acid is a driving force for 2-AG uptake (Beltramo & Piomelli, 2000). Of all the potential ways of increasing EC in the cell discussed above, research interest has focused on FAAH (Bisogno *et al*., 2002). Briefly, FAAH is a membrane-associated protein that is localized to internal membranes, such as the endoplasmic reticulum, where it is active (Figure 3). FAAHs broad substrate specificity allows it to hydrolyze AEA, 2-AG, *N*-palmitoylethanolamine, and oleamide (Bisogno *et al*., 2002). Although 2-AG can be hydrolyzed by FAAH, it is largely metabolized by monoacylglycerol lipase (Goparaju *et al*., 1999; Dinh *et al*., 2002). Small molecule inhibitors of FAAH have a wide variety of mechanisms, with the most potent compounds binding irreversibly to serine 241 (Deutsch *et al*., 2002). Although these compounds hold much promise for therapeutic strategies aimed at elevating EC levels, the caveat is that the enzyme is widely distributed and it may be involved in more than just catabolism of EC.

GI effects of modulation of uptake, synthesis/degradation of EC

Emerging data do support the idea that agents which alter the synthesis and uptake of EC have efficacy in models of GI dysfunction. For example, cholera toxin induced fluid accumulation in the small intestine was prevented by VDM11, a selective inhibitor of AEA uptake, and this effect was reversed by rimonibant (Izzo *et al*., 2003). This suggests that the stimulus was associated with an increase of EC signaling which has an antisecretory role in the small intestine. Preliminary data suggest that VDM11 given alone increased gastric volume in anesthetized rats and that this effect was also dependent on CB1R activation since it was reversed by rimonibant (Partosoedarso *et al*., 2003a). This result is interesting since it provides evidence for EC constitutive release to promote increased gastric volume in a normal nonpathological system. Another example of this is that the rate of expulsion of a glass bead inserted into the distal colon seems to be under EC control because the rate was decreased by VDM11 (Pinto *et al*., 2002b). In addition, there are high amounts of 2-AG and AEA in the colon, as well as a high level of activity of FAAH (Pinto *et al*., 2002b). The small intestine in rats has also been demonstrated to have a high level of mRNA for FAAH (Katayama *et al*., 1997). Thus, the components are in place within the intestines to suggest that constitutive release of EC tonically inhibits propulsive activity. But this may be a disadvantage if a pathophysiological state involves stasis of the GI tract. For example, acetic acid-induced intestinal hypomotility, which is a model for paralytic ileus, is worsened by VDM-11 (Mascolo *et al*., 2002). One final caveat is that EC may act on non-CBR in order to mediate GI effects. For example, palmitoylethanolamide decreases intestinal transit in mice (Capasso *et al*., 2001), and 2-AG acts on myenteric cholinergic neurones to produce contract longitudinal muscle of the guinea-pig distal colon (Kojima *et al*., 2002). However, these effects were not reversed by selective antagonists, therefore modulation of the pathways for uptake and degradation of EC may mediate effects that are not related to CBR.

Conclusion

In conclusion, tremendous progress has been made in the last decade to demonstrate the role and site of action of CBR agonists in many aspects of GI function. The beneficial effects of CB1R activation in animal models include reduction of transient lower esophageal sphincter relaxations, increased compliance of the proximal stomach, reduced acid secretion, reduction of GI transit, reduced intestinal fluid secretion in response to secretogogues and reduced large intestinal propulsive activity are all aspects that could be beneficial in functional bowel disorders such as IBS. However, administration of CB1R agonists to patients would be associated with CBS adverse effects due to the psychotropic actions. It is not clear to what extent increasing the release, or reducing the uptake of ECs would be beneficial for treatment of GI disorders. However, there is emerging evidence for tonic EC release in both physiological and pathophysiological systems suggests that these are important molecules in control of the GI tract. Newer approaches to their modulation by inhibition of FAAH or the uptake mechanisms hold promise for future therapeutic avenues. However, whether these approaches can be successful depends on minimizing CNS adverse effects, and it is not known whether such manipulation would also evoke psychotropic central effects associated with CB1R activation. The ideal approach would be to increase the levels of ECs within the dorsal vagal complex (an region that is less protected by the blood–brain- barrier), the vagal pathways, the dorsal root ganglion and within the enteric nervous system, without affecting higher brain function. This would hold the greatest promise for minimizing risks while treating or ameliorating the symptoms of complicated disorders with unclear etiology such as IBS.

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