Expression of cannabinoid CB1 receptors by vagal afferent neurons: kinetics and role in influencing neurochemical phenotype

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Expression of cannabinoid CB1 receptors by vagal afferent neurons: kinetics and role in influencing neurochemical phenotype. Am J Physiol Gastrointest Liver Physiol 299: G63–G69, 2010. First published April 29, 2010; doi:10.1152/ajpgi.00059.2010.—The intestinal hormone cholecystokinin (CCK) inhibits food intake via stimulation of vagal afferent neurons (VAN). Recent studies suggest that CCK also regulates the expression of some G protein-coupled receptors and neuropeptide transmitters in these neurons. The aim of the present study was to characterize the expression of cannabinoid (CB1) receptors in VAN and to determine whether stimulation of these receptors plays a role in regulating neurochemical phenotype. Expression of CB1 in rat VAN was detectable by in situ hybridization or immunochemistry after 6 h of fasting and increased to a maximum after 24 h when ~50% of neurons in the mid and caudal regions expressed the receptor. Melanin-concentrating hormone (MCH)1 receptors also increased with fasting, but the changes were delayed compared with CB1; in contrast Y2 receptors (Y2R) exhibited reciprocal changes in expression to CB1. Administration of CCK8s (10 nmol ip) to fasted rats decreased expression of CB1 with a t1/2 of ~1 h compared with 3 h for MCH1. The action of CCK8s was inhibited by ghrelin and orexin-A. The CB1 agonist anandamide (intraperitoneally) reversed the effect of CCK8s on CB1, MCH1, and Y2 receptor expression. In contrast, in rats fasted for 18 h, administration of a CB1 antagonist/inverse agonist (AM281 ip) downregulated CB1 expression and increased Y2 receptor expression. Activation of vagal CB1 receptors therefore influences the neurochemical phenotype of these neurons, indicating a new and hitherto unrecognized role for endocannabinoids in gut-brain signaling.

Stimulation of the Vagal Afferent Neurons (VAN) serving the gastrointestinal tract inhibits food intake and activates a variety of autonomic reflexes including those regulating gastric and pancreatic secretion, gut motility, intestinal immune responses, and gastric cytoprotection (14, 24). These neurons respond to gastric distension and to a variety of neurohumoral agents (19, 25). It has been known for many years that luminal nutrients in the intestine may stimulate VAN, and it now seems likely that these effects are mediated, at least in part, by the release of gut hormones such as cholecystokinin (CCK), glucagon-like peptide-1, and PYY3–36 (14, 24). In addition, there is growing evidence that gut-derived factors released during fasting also act on VAN, notably the gastric orexigenic hormone ghrelin, which is reported to inhibit these neurons (6, 9).

Until recently, it was tacitly assumed that the expression of receptors and neurotransmitters by VAN serving the gut was essentially constitutive and did not vary with food intake. However, it has recently been shown that the expression of cannabinoid (CB1) receptors and melanin-concentrating hormone (MCH)1 receptors (MCH1R) is increased by food deprivation, whereas that of the Y2 receptor (Y2R) (at which PYY3–36 acts) is depressed by food deprivation (2, 3, 5). Moreover, the orexigenic peptide MCH increases in fasted rats, whereas that of the anorexigenic peptide cocaine- and amphetamine-regulated transcript (CART) decreases (5, 10). Refeeding of fasted rats reverses these changes through the action of endogenous CCK. In addition, receptor expression by these neurons, as revealed by qPCR, may be changed in response to high-fat diets (20, 21).

The role of the central nervous system (CNS) endocannabinoid system in control of food intake has attracted considerable attention and has been the focus for the development of therapeutic approaches to obesity (12, 13). In addition, however, there is a body of evidence that points to peripheral mechanisms controlling food intake mediated by the endocannabinoids (16). Thus CB1 agonists such as anandamide (arachidonylethanolamide, AEA) and 2-arachidonoylglycerol (2-AG) increase not only in CNS with fasting (18) but also in small intestine, and, when administered peripherally, AEA stimulates food intake by a mechanism dependent on intact VAN, presumably mediated by CB1 receptors expressed by these neurons (7, 16, 17). In the present study, we have sought to define more precisely the pattern of expression of CB1 receptors by VAN and their functional significance; we report here evidence that expression of CB1 receptors by VAN plays a role in determining neurochemical phenotype.

Materials and Methods

Animals. Adult male Wistar rats (250–350 g, ~10 wk old) were housed at 22°C with a 12-h:12-h light/dark cycle (lights on at 0800 h) and given ad libitum access to water. Rats were killed by a rising concentration of CO2, and the mid and caudal regions of nodose ganglia were either fixed in 4% paraformaldehyde (PFA) for immunocytochemistry or immersed in liquid nitrogen for in situ hybridization. Experiments were conducted under appropriate UK Home Office authority and local ethics committee approval.

Chemicals and peptides. CCK8s was obtained from Bachem (St. Helens, Merseyside, UK), AEA from Sigma-Aldrich (Dorset, UK), and ghrelin, orexin-A, and AM281 from Tocris Bioscience (Bristol, UK); peptides were dissolved in saline, AEA in ethanol, and AM281 in 100% DMSO.

Fasting-refeeding experiments. Rats were fasted for up to 48 h with water ad libitum. Typically the fasting period started in the first relevant dark cycle, but in some experiments rats were fasted during
were washed in 1 and 20 mM DDT]. After hybridization (overnight, 42°C) sections
salmon sperm DNA, 0.1 mg after 6 ho of fasting, there was detectable CB1 expression with
revealed virtually no detectable expression of CB1. However, in situ hybridization of nodose ganglia from rats fed ad libitum
indicated in the text.

In situ hybridization. Cryostat sections (10–15 μm) of quickly frozen (~70°C) rat nodose ganglia were thaw mounted on UV-treated
poly-L-lysine-coated slides, fixed in 4% PFA in 1× PBS, and acetylated in 0.25 M acetic anhydride-0.1 M triethanolamine (10 min) as
described previously (2). Oligonucleotide probes (in ratios of 1:1:1) complementary to bases 4–51, 349–396, and 952–999 of rat CB1
cDNA or to bases 7–32, 451–478, and 702–734 of rat MCH1R cDNA (Sigma-Genosys) were 3′-end labeled with [35S]dATP (10
mCi·ml−1·ml−1; Amersham Biosciences, Buckinghamshire, UK) and used at concentrations of 3,000 cpm·1·μl−1 in hybridization buffer
(50% formamide, 10% dextran sulphate, 4× sodium chloride-sodium phosphate-EDTA (SSPE) (1× SSPE contained 3 M NaCl, 0.2 M
NaH2PO4 ·2H2O, and 0.2 M EDTA; pH 7.4), 0.2 mg·1·ml−1·ml−1 sheared salmon sperm DNA, 0.1 mg·1·ml−1·ml−1 polyA RNA, 5× Denhardt’s,
and 20 mM DTT]. After hybridization (overnight, 42°C) sections were washed in 1× SSC (150 mM NaCl and 15 mM sodium citrate;
pH 7.0) at 55°C (30 min), washed again in 0.1× SSC (30 s), dehydrated through ethanol, air dried, coated with autoradiography emulsion (LM-1; Amersham Biosciences), and exposed for 4–6 wk before development. Sections were developed, counterstained with
ehematoxylin-eosin or toluidine blue, dehydrated, and mounted in Histomount (Oncogene Research, Boston, MA). Silver grains were
visualized using an Axioplan Universal microscope. Images were processed using the AxioVision 3.0 Imaging system (Carl Zeiss
Vision, Jena, Germany) combined with dark- and bright-field illumination. Control slides were hybridized with probes together with a
100-fold excess of unlabeled oligonucleotides, which virtually abolished the localization of silver grains.

Immunohistochemistry. Frozen cryostat sections of rat nodose ganglia were rinsed in PBS, permeabilized in ethanol, and processed for
single- or double-labeling immunofluorescence as described previously (4). For detection of CB1 both affinity-purified goat and rabbit
polyclonal antibodies were used (1:90; Santa Cruz Biotechnology, Santa Cruz, CA). For detection of MCH1R, we used affinity-purified
goat polyclonal antibody (1:50; Santa Cruz Biotechnology) or chicken polyclonal antibody (1:600; Acris Antibodies; Hiddenhausen, Ger-
many) raised against a conserved peptide at the COOH terminus of MCH1R. Y2R expression was localized using a rabbit polyclonal
Y2R antibody (1:200; Neuromics Antibodies, Edina, MN). Secondary antibodies were used as appropriate and included FITC-conjugated
(Jackson ImmunoResearch Laboratories, West Grove, PA) donkey anti-rabbit, anti-goat, or anti-chicken IgG, and Texas red-conjugated
(Jackson) donkey anti-rabbit or anti-goat IgG (Jackson). Specificity of immunostaining was determined by preincubation with an excess of
appropriate peptide where available (SC-10068P and SC-14513P from Santa Cruz; P14112 from Neuromics), or by omitting the primary antibody. Sections were examined using an Axioplan Universal microscope, and images were processed using the Axi Vision
3.0 Imaging system with deconvolution options (Carl Zeiss Vision). The quantification of neurons expressing CB1, MCH1R, or Y2R was
made by counting 400–600 immunoreactive cell profiles in five sections per ganglion, selecting sections separated by 90 μm that
passed through the full length of the caudal and mid regions of the ganglia. Results are expressed as immunopositive cells as a proportion
of all neurons in the relevant region.

Statistics. Results are expressed as means ± SE, and comparisons were made by ANOVA.

RESULTS

Differential expression of CB1 and MCH1R with fasting. In situ hybridization of nodose ganglia from rats fed ad libitum
revealed virtually no detectable expression of CB1. However, after 6 h of fasting, there was detectable CB1 expression with
a further increase after 12-h fasting (Fig. 1A). In contrast, MCH1R expression was virtually undetectable both in rats fed
ad libitum and fasted for 6 h (Fig. 1B) but was detectable after fasting for 12 h or longer.
Immunocytochemical localization of CB1 and MCH1R in nodose ganglia neurons also revealed differential responses to fasting. Thus CB1-immunoreactive neurons were virtually undetectable in rats fed ad libitum but were found after 6-h fasting and increased progressively thereafter (Figs. 2A and 3). In contrast, MCH1R-immunoreactive neurons were virtually undetectable in rats fed ad libitum or fasted up to 12 h. Thereafter there was a progressive increase in MCH1R-immunoreactive neurons (Figs. 2B and 3). Both CB1 and MCH1R could be localized to the same neurons (Fig. 2C); after 24-h fasting virtually all MCH1R-expressing neurons also expressed CB1, but ~50% of CB1-expressing neurons did not have detectable MCH1R (Supplemental Fig. S1; supplemental material for this article is available online at the American Journal of Physiology Gastrointestinal and Liver Physiology website). Moreover, whereas CB1 was found in vesicles throughout the cell soma in rats fasted 6 h or longer, MCH1R immunoreactivity was typically localized in perinuclear vesicles up to 24 h of fasting and only thereafter was found in vesicles throughout the cell soma. The changes in CB1 and MCH1R immunoreactivity with fasting do not reflect a non-specific change in expression of all G protein-coupled receptors in these neurons because there were reciprocal changes in Y2R expression, i.e., strong expression in nodose ganglion neurons in rats fed ad libitum and a progressive decrease after fasting for 6 h or longer (Fig. 3; Supplemental Fig. S2).

The increase in CB1 expression with fasting for 12 h was found regardless of whether food withdrawal occurred during the light or dark cycles. Food intake during the light cycle was ~3 g or about 10% of total daily food intake. In rats fed ad libitum, CB1 expression remained low at the end of this period (2000 h), whereas there were abundant CB1-expressing neurons at the end of the light cycle when food was withheld during this period (Fig. 4). The very modest changes in MCH1R expression with 12-h fasting were similar in rats deprived of food during either the light or dark cycles (Figs. 3 and 4). Interestingly, there was a small but not significant decrease in the number of nodose neurons expressing Y2R at the end of the light cycle in rats fed ad libitum, and there was a significant decrease following withdrawal of food over the same period (Fig. 4).

**Differential effects of CCK on CB1 and MCH1R expression.**

In view of the different time courses of CB1 and MCH1R expression, we then examined the kinetics of decrease in CB1 and MCH1R following administration of CCK8s (10 nmol ip) to rats fasted for 24 h. There was rapid loss of CB1-positive neurons with a t_{1/2} of about 1 h, whereas loss of MCH1R was slower with a t_{1/2} of 3 h (Fig. 5A, Supplemental Fig. S1). Moreover, in fasted rats treated with CCK8s, CB1 expression returned to control within 8 h, whereas MCH1R only returned to control after 15 h (results not shown).

**Ghrelin inhibits the action of CCK8s on CB1, MCH1R, and Y2R expression.**

We then asked whether CB1 and MCH1R showed similar responses to CCK in the presence of orexigenic factors. Administration of ghrelin just before CCK8s dose dependently inhibited the action of CCK on both CB1 and MCH1R expression (Fig. 6, A and B; Supplemental Figs. S2 and S3). However, reversal of CCK8s-inhibited CB1 expression was found at a dose of 1 nmol (ip) ghrelin with a further increase at 4 nmol, whereas inhibition of MCH1R expression required 4 nmol. Administration of ghrelin alone had no effect on CB1 and MCH1R expression. Ghrelin also reversed the action of CCK8s on Y2R expression: there was inhibition at 1 nmol and a further significant decrease at 4 nmol (Fig. 6C; Supplemental Fig. S4).

**Anandamide inhibits the action of CCK8s on CB1 and MCH1R expression.**

Because there is evidence that AEA and ghrelin both increase food intake via vagal mechanisms (8, 9, 16), we examined whether AEA replicated the action of ghrelin in inhibiting the effect of CCK8s on nodose ganglion neurons. In response to increasing concentrations of AEA there was progressive attenuation of the CB1 and MCH1R responses to CCK (Fig. 7, A and B; Supplemental Figs. S5 and S6). However, in response to administration of AEA alone, there were no dramatic changes in CB1 and MCH1R expression. Doses of AEA that inhibited that action of CCK on CB1 and MCH1R also had modest effects on CCK8-stimulated Y2R expression, but the effect was only significant at high doses (Fig. 7C; Supplemental Fig. S7). Moreover, at a high dose AEA stimulated Y2R expression in the absence of CCK.

We wondered whether the similarity in action of AEA and ghrelin was also shared by other orexigenic agents acting on VAN, and indeed orexin A, which is known to act at vagal Ox-R1 receptors, also reversed CCK8s inhibition of CB1 and MCH1R expression (Supplementary Fig. S8).

A CB1 antagonist/inverse agonist suppresses fasting-induced changes in CB1 and Y2R expression.**

On the basis of the data described above, we hypothesized that activation of the endocannabinoid system might act to maintain CB1 and MCH1R expression in VAN in fasted rats. We therefore examined the action of the CB1 antagonist/inverse agonist, AM281 (15). When given to rats fasted for 18 h, AM281 depressed the expression of CB1 and increased expression of Y2R at euthanasia 3 h later (Fig. 8; Supplemental Figs. S9 and S10). Only modest changes in MCH1R expression were expected using this protocol (i.e., before fasting-induced expression of MCH1R is maximal), but, interestingly, there was no change in MCH1R-expressing neurons with administration of AM281.

**DISCUSSION**

The present data show that quite modest food deprivation leads to expression of CB1 receptors by VAN. In contrast, relatively prolonged periods of fasting are required for expression of MCH1R. Although CCK downregulates both CB1 and MCH1R, the response of the former is more prompt than the latter. Interestingly, the action of CCK on CB1 expression is reversed by the CB1 agonist AEA, and in this sense the activation of CB1 receptors resembles that of ghrelin (GHS1) receptors. Importantly, we also show that a CB1 antagonist/inverse agonist, AM281, has similar actions to CCK8s with respect to expression of CB1 and Y2R, suggesting an autoregulatory maintenance of CB1 receptor expression. The data provide new insight into the control of vagal afferent signaling in different physiological states.

The work of several groups has now contributed to the idea that the neurochemical phenotype of VAN is dependent on nutrient status (2, 3, 5, 10, 20, 21). Thus in addition to changes in receptor expression with food withdrawal it has also been reported that feeding high-fat diets may change receptor expression although there are apparently species differences in the pattern of responses (20, 21). The changes in CB1 expres-
Fig. 2. Immunohistochemical localization of CB1 and MCH1 receptors in vagal afferent neurons of fasted rats. A: immunohistochemistry shows virtually undetectable expression of CB1 in nodose ganglion neurons of rats fed ad libitum (0 h) but shows progressive increases with fasting from 6–24 h. B: there is virtually undetectable expression of MCH1R in nodose ganglion neurons of rats fed ad libitum (0 h) or fasted up to 12 h, but thereafter there are progressive increases with fasting to 48 h. C: overlay of A and B showing coexpression of CB1 and MCH1 in the same neurons particularly from 18-h fasting. Scale bars = 30 μm.
sion appear to be relatively sensitive to food withdrawal. For example, during the daylight period rats consumed ~10% (about 3 g) of their total daily food intake, and prevention of day-time feeding was sufficient to increase CB1 expression. During the night-time phase, withdrawal of food for a period of 6 h was sufficient. The same stimuli are also sufficient to inhibit Y2R expression. We suggest, therefore, that these are relatively rapid and sensitive responses to food withdrawal. In contrast, expression of MCH1R occurs after prolonged fasting. Apparently, then, there are graded changes in the phenotype of VAN that allow a neurochemical coding of the duration of fasting.

CCK reverses the effects of fasting on VAN relatively rapidly. It is known that the acute action of CCK on VAN discharge or increased intracellular calcium is potentiated by leptin (23, 29). These neurons express the putative neuropeptide transmitter CART, and there is evidence that CCK releases CART, which in turn augments the action of CCK in stimulating Y2R expression, but not in inhibiting expression of CB1, MCH1R, or MCH (10, 11). However, there has been some controversy over the role of ghrelin (GHS1) receptors on VAN (1, 9); our own findings suggest that both in vivo and in vitro ghrelin acts to inhibit the action of CCK in depressing expression of CB1, MCH1R, or MCH and increasing expression of Y2R and CART in these neurons (2, 6, 10, 11). The present findings indicate that activation of CB1 and GHS1 receptors on VAN produces similar effects on CCK-stimulated changes in receptor expression.

The CB1 agonist AEA is also a transient receptor potential vanilloid (TRPV1) agonist in certain conditions (27, 30), and VAN express TRPV1. We found that at low concentrations AEA reversed the action of exogenous CCK in downregulating expression of CB1 and MCH1R. However, at high concentrations it stimulated Y2R expression, which might be attributable to TRPV1 activation. Importantly, however, the CB1 antagonist/inverse agonist AM281 had CCK-like actions in fasted rats with respect to CB1 and Y2R expression. Because AM281 is both an antagonist and an inverse agonist (22), it is not in a formal sense possible to say whether its actions in the present study are attributable to inhibition of the action of endocannabinoids or to suppression of the constitutive activation of CB1 receptors (or a combination of both). It should be noted, however, that endocannabinoid agonists such as AEA and 2-AG are increased in intestine during fasting (16, 17), thereby providing a specific mechanism to increase endogenous stimulation of CB1 receptors against which AM281 might act. Either way, the observations suggest that CB1 receptors play a role in regulating their own expression in VAN as well as that of Y2R receptors. Interestingly, we did not see an action of AM281 on MCH1R expression. Further work will be required to determine the significance of this observation; nevertheless
the findings contribute to the idea that, although CB1 and MCH1R expression often change in parallel, this is not always the case.

The present data provide further evidence of the neurochemical plasticity of vagal afferent neurons. These neurons appear to have a simple form of memory whereby nutritional status over the previous 6–48 h determines the expression of some G protein-coupled receptors and neuropeptide transmitters. During food withdrawal, there is increased capacity for orexigenic signaling through expression of CB1 and MCH1R (and loss of Y2R), whereas in the postprandial state there is increased capacity for satiety signaling through selective increase of Y2R (and loss of CB1 and MCH1R). Previously, the role of endogenous CCK in switching neuronal phenotype was documented, and the action of exogenous ghrelin in inhibiting this was noted (14). The present data now provide evidence of a role for CB1 receptor activation in maintaining the neurochemical phenotype seen during fasting and presumably therefore enhancing the capacity for orexigenic signaling to the CNS during the fasted state and perhaps early after a following meal.

VAN expressing CB1 receptors project to both thoracic and subdiaphragmatic structures. The latter neurons tend to lie in

Fig. 6. Ghrelin reverses the action of CCK. Rats were fasted 24 h and then received ghrelin (0.25–4.00 nmol ip) followed after 5 min by CCK8s (10 nmol ip). Ghrelin dose dependently reversed the action of CCK8s on Y2R, CB1 and MCH1R expression. Data are expressed as the proportion of neurons expressing CB1 or MCH1. 2.5 h following administration of CCK8s. Means ± SE, n = 4 rats; *P < 0.05, **P < 0.01, ***P < 0.001 compared with expression in the absence of ghrelin.

Fig. 7. Anandamide (AEA) inhibits the effect of CCK on vagal afferent neurons. Rats fasted for 24 h received AEA (0.1–10.0 mg ip) followed after 5 min by CCK8s (10 nmol ip). Data are expressed as the proportion of neurons expressing CB1, MCH1R, or Y2R 2.5 h after CCK8s. Means ± SE, n = 6; **P < 0.1, ***P < 0.001 compared with expression in the absence of AEA.

Fig. 8. The CB1 receptor antagonist/inverse agonist, AM281, reverses expression of CB1 and Y2R in fasted rats. In rats fasted for 18 h, AM281 (0.33 mg ip) depressed expression of CB1 and increased expression of Y2R in nodose neurons in rats killed 3 h later. Note there was no change in MCH1R expression. Means ± SE, n = 5 rats.
the mid and caudal regions of the nodose ganglion (3), and our work sampled this population of neurons. A role for vagal CB1 receptors in both emesis and food intake has been documented (16, 26, 28). The present data indicate that the endocannabinoid system should now be included in the range of factors that influence the neurochemical phenotype of VAN. The integrative role of these neurons is becoming increasing appreciated, and with recognition of their different neurohumoral inputs it should become possible to develop novel therapeutic strategies not least for conditions such as obesity.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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