Cholecystokinin Increases Cytosolic Calcium
in a Subpopulation of Cultured Vagal Afferent Neurons.

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ABSTRACT

Imaging fluorescent measurements with fura-2 were used to examine cytosolic calcium signals induced by sulfated cholecystokinin octapeptide (CCK-8) in dissociated vagal afferent neurons from adult rat nodose ganglia. We found that 40% (184/465) of the neurons responded to CCK-8 with a transient increase in cytosolic calcium. The threshold concentration of CCK-8 for inducing the response varied from 0.01 nM to 100 nM. In most neurons (13/16) the response was eliminated by removing extracellular calcium. Depleting intracellular calcium stores with thapsigargin slightly augmented the response. Most neurons were unresponsive to nonsulfated CCK-8. The response was eliminated by the CCK-A receptor antagonist lorglumide. Low concentrations of JMV-180 had no effect, however high concentrations of JMV-180 reduced responses to CCK-8. These results demonstrate that CCK acts at the low affinity site of the CCK-A receptor to trigger the entry of extracellular calcium into vagal afferent neurons. Increased cytosolic calcium may participate in acute activation of vagal afferent neurons, or it may initiate long-term changes, which modulate future neuronal responses to sensory stimuli.

Key words: nodose ganglia

JMV-180

satiation

lorglumide
INTRODUCTION

Cholecystokinin (CCK) is a peptide, which is released into extracellular space by I-cells of the small intestinal mucosa. Although it is the product of a single gene, CCK is released in forms ranging from 8 to 58 amino acids in length (Deschenes et al., 1984; Gubler et al., 1984; Eysselein et al., 1990). CCK promotes the delivery of bile into the small intestine, stimulates pancreatic enzyme secretion, inhibits gastric emptying, promotes intestinal peristalsis, and induces satiation (Liddle, 1997). Many of these effects of CCK, including stimulation of pancreatic exocrine secretion (Li et al., 1997), inhibition of gastric emptying (Raybould and Tache, 1988; Holzer et al., 1994; Moran et al., 1994), and satiation (Smith et al., 1985; Ritter and Landenheim, 1985), are mediated by activation of CCK-A receptors on vagal afferent fibers (Asin and Bednarz, 1992; Yox et al., 1992; Weatherford et al., 1993; Moran et al., 1994; Brenner and Ritter, 1996; Li et al., 1997). Although plasma CCK concentrations are elevated following entry of fats and proteins into the small intestine (Liddle, 1997; Brenner et al., 1993), mounting evidence suggests that CCK mediates some responses to intestinal stimulation without entering into the circulation. Hence, several investigators have proposed that some responses to CCK are mediated via a paracrine action of the peptide to stimulate vagal afferent nerve endings in the intestinal mucosa (Greenberg et al., 1987; Brenner et al., 1993; Schwartz and Moran, 1994; Dockray et al., 1996).

Vagal responsiveness to CCK has been demonstrated by recordings from vagal afferent fibers (Davison and Clarke, 1988; Blackshaw and Grundy, 1990; Schwartz and Moran, 1994; Cox and Randich, 1997) and nodose ganglion cells (Li et al., 1999) in vivo. In addition to these direct actions, CCK has been shown to enhance vagal responses to mechanical stimuli in vivo (Davison and Clarke, 1988; Schwartz and Moran, 1994). Finally, several reports indicate that chronic elevation of CCK levels reduces sensitivity to acute elevation of CCK (Crawley and Beinfeld,
1983; Covasa et al., 2001), and that chronic exposure to high fat or high protein diets, which release CCK, also reduce sensitivity to the satiation effects of acute CCK (Covasa and Ritter, 1998). Unfortunately, the cellular and biochemical basis for CCK-induced activation of vagal afferent fibers remain unappreciated, and thus the cellular basis for these interactions and adaptive responses are unknown.

A CCK-induced increase in cytosolic calcium could play a major role in the vagal afferent response to CCK, and in adaptive and synergistic actions of CCK with other vagal stimuli. Therefore, in the present report we have used cultured nodose ganglion neurons to test the hypothesis that increases in intracellular calcium constitute a part of the vagal afferent response to CCK. We found that sulfated CCK-8 induced an increase in cytosolic calcium in 38% of the vagal afferent neurons tested. This increase in calcium was triggered by CCK action at the low affinity site of the CCK-A receptor, and was dependent on extracellular calcium. Finally, a few cells were found to respond to nonsulfated CCK-8 indicating that CCK-B receptors also can trigger increases in cytosolic calcium in a small subpopulation of vagal afferent neurons.

**METHODS**

*Cell isolation and culture.* Nodose ganglia were excised from anesthetized male Sprague-Dawley rats (ketamine 25 mg/100 g, xylazine 2.5 mg/100 g) under aseptic conditions and placed in ice-cold Ca\(^{2+}\)- and Mg\(^{2+}\)-free Hank's balanced salt solution (HBSS) supplemented with antibiotic (penicillin/streptomycin, 100 units/ml and 100 µg/ml, respectively). The ganglia were then cleaned of connective tissue and desheathed. For each rat both left and right ganglia were combined into a single isolation. Nodose cells were dissociated according to a procedure described by Lancaster et al. (2001). Briefly, the desheathed ganglia were washed once in HBSS, placed in ~3 mls of diges-
tion buffer (1 mg/ml dispase II and 1 mg/ml collagenase type Ia in HBSS), sliced into ~1-mm³ fragments using a sterile scalpel blade, and then placed in an incubator at 37°C to digest for 90 min. At the end of the digestion the cells were dispersed by gentle trituration through Pasteur pipets coated with Sigmacote. The dispersed cells were then washed 2 times in Hepes-buffered Dulbecco's modified Eagle's medium (HDMEM), supplemented with 10% fetal calf serum and antibiotic (penicillin/streptomycin). After the final resuspension the cells were plated onto poly-l-lysine coated coverslips (100 µg/ml poly-lysine for 30 min) and grown in HDMEM with 10% fetal calf serum at 37°C in an atmosphere of 5% CO₂/95% air. In some isolations the procedure of Lankisch et al. (2002) was followed. Briefly, dispase II was replaced with trypsin II, the digest proceeded for only 30 min, and the digest reaction terminated with soybean trypsin inhibitor. All experimental procedures were conducted within 2 days of collection of the nodose ganglia.

**Calcium measurements.** Intracellular calcium levels were determined by ratiometric measurements with fura-2. All manipulations and experiments were performed at room temperature in a physiological salt solution (in mM: 140 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 6 glucose, and 10 Hepes with the pH adjusted to 7.4 with NaOH) (standard bath) unless noted. Any ionic adjustments to the standard bath where made such that the adjusted solution remained iso-osmotic (i.e., Ca²⁺ replaced with Mg²⁺ and Na⁺ replaced with K⁺). Cells on coverslips were washed in standard bath and then allowed to take up fura-2-AM for 30 min (1 µM with 0.05% pluronic acid), followed by a 30 min wash period. Coverslips were then mounted in a closed chamber (~0.3 ml volume) through which solutions were perfused by gravity (~3 ml/min). Solution changes were made by switching inflow lines through a common manifold (new solutions reach cells ~15 sec after switch). Chambers were then mounted on the stage of a Nikon Diaphot inverted microscope and examined with a 40x oil immersion lens. Cells were alternatively exposed to 340 nm and 380 nm light (800 and 400 msec
exposures, respectively) and images collected through a 510 nm filter with a CCD camera (SenSys camera, Photometrics, Tucson, AZ). Image pairs were captured every 6 seconds and ratios of fluorescent intensities at the two excitation wavelengths obtained from regions over individual cells. Data collection and manipulations were performed with MetaFluor Software (Universal Imaging Inc., West Chester, PA). Calcium concentrations were then determined by comparing ratio values to a standard curve obtained in a bath containing 10 μM fura-2, 130 mM KCl, 10 mM MOPS buffered to pH 7.4 with KOH, 10 mM EGTA, and various concentrations of CaCl₂ (0 to 10 mM). Free calcium concentrations were calculated using the computer program EQCAL (Biosoft, Ferguson, MO).

**Cell selection.** Nodose neurons were easily identified and selected based on their large, round cell bodies. Non-neuronal cells had spindle or filamentous shapes. In most cases basal calcium levels were below 200 nM, however, in some neurons a challenge with CCK resulted in a large calcium increase that did not completely recover. Whether this lack of recovery was due to the neuron entering a prolonged activated state or was due to its inability to regulate calcium because of cellular damage could not be determined. Thus we only analyzed those neurons in which calcium increases reversed and that maintained basal calcium levels of less than 400 nM throughout the experiment. Finally, at the end of every experiment, cells were depolarized with a saline containing 55 mM K⁺. Any cells that did not exhibit a calcium increase of at least 40 nM in response to K⁺ were excluded from further analysis. A total of 465 neurons studied from 35 separate isolations met these criteria.

**Experimental protocols.** Challenge solutions were applied for 1 min or until cytosolic calcium signal stopped increasing, whichever was longer. Challenges to 55 mM KCl were for 30 sec or until the calcium response stopped increasing, whichever was longer. Repeated challenges
were typically separated by 2-3 min unless recovery from a previous response required more time.
Administration of CCK in combination with CCK-A receptor antagonist was preceded and
followed by administration of CCK alone in standard bath. This approach assured that attenuation
of calcium signal by the presence of the antagonist was not the result of desensitization. In general,
a similar design was used to examine the effect of calcium-free bath on CCK-induced responses.
However, for some of the calcium-free bath experiments the first CCK exposure was made in the
absence of bath calcium, and then followed by a 2nd and 3rd challenge with CCK after the return of
bath calcium. Once again, we took this approach in order to be sure that any attenuation of the
CCK-response observed under calcium-free conditions could not be ascribed to desensitization.
Accordingly, with the exception of the experiments employing thapsigargin (see below), results
from experiments involving multiple CCK exposures were analyzed only when neurons responded
to CCK alone after all other experimental manipulations were complete. Thapsigargin was used to
test the possibility that intracellular stores of calcium were involved in the CCK induced calcium
response. Because thapsigargin is irreversible, a challenge after washout of thapsigargin could not
be performed. In experiments comparing the sensitivity of the neurons to nonsulfated CCK versus
sulfated CCK-8, or CCK-8 and JMV-180, the order of exposure to the various agents was
randomized to ensure that the exposure to the first challenge was not responsible for the lack of
response in to the second challenge. For each experimental protocol, nodose neurons from at least
three separate isolations were used.

Data analysis. Calcium responses to CCK and/or other challenges are expressed as the
change in cytosolic calcium from a basal value (Δcalcium). The basal value was sampled over the
30 seconds just prior to exposure to the challenge solution. Summarized results are expressed as
the average Δcalcium ± S.E. For experimental protocols that involved three challenges (control,
experimental condition, recovery) a repeated-measures two-factor analysis of variance was performed. One factor was the experimental condition and the other was the individual neuron. This design was selected because the absolute magnitude of the control responses could vary from one neuron to the next. As mentioned above, for some of the calcium-free bath experiments, the calcium-free condition was tested first, followed by two control challenges under standard conditions. In these cases, the first response to CCK in the presence of standard calcium condition was used for the control condition. If analysis of variance revealed a significant effect, subsequent multiple comparisons were made using a Newman-Keuls procedure. For thapsigargin experiments a paired t-test was used to evaluate statistical significance of the results. Comparisons of cell size, basal calcium levels, and KCl-induced calcium responses between populations (CCK-responsive vs. CCK-non-responsive) were performed by an unpaired t-test. In all statistical tests p<0.05 was considered significant.

Chemicals. In all experiments the sulfated form of CCK-8 was used unless indicated. Culture media and fetal calf serum were obtained from Life Technologies (Grand Island, NY). Lorglumide (L-109) was obtained from Research Biochemicals International (Natick, MA). Dispase II was obtained from Boehringer Mannheim (Indianapolis, IN). Fura-2 pentapotassium salt and fura-2-AM were obtained from Molecular Probes (Eugene, OR). JMV-180 was obtained from Research Plus, Inc (Bayonne, NJ). All other chemicals were obtained from Sigma (St. Louis, MO).

RESULTS

General characteristics of responses to CCK. Of the 465 cultured nodose neurons challenged with CCK-8 (10 or 100 nM), 184 (or 40%) responded with a transient increase in cytosolic calcium. Most neurons exhibited a relatively stable baseline calcium level with
fluctuations no greater than 5 nM in amplitude. For these neurons to be categorized as responding to CCK-8, we required that CCK-8 induce a calcium increase greater than 20 nM above baseline. On the other hand, a few neurons exhibited spontaneous calcium oscillations. For these neurons to be categorized as responding to CCK-8, we required that the CCK-8-induced calcium response be at least twice the magnitude of any spontaneous oscillation observed prior to the CCK-8 challenge. When the maximal calcium response to CCK-8 was considered, regardless of concentration of CCK-8, we found that the average ± SE increase in intracellular calcium was 131 ± 9 nM (n = 184), the largest increase we observed was 865 nM, and the median increase was 94 nM.

Concentration dependence of responses to CCK. We performed a series of studies to examine the dependence of the calcium responses on CCK-8 concentration (10 pM to 300 nM). Some neurons exhibited a monotonically increasing calcium response to increasing concentrations of CCK-8 (Fig. 1A). However, summarizing concentration-response characteristics was complicated by several problems. First, in many neurons, but not all, the response to CCK-8 exhibited desensitization following repeated challenges with CCK-8 (Fig. 1B). This desensitization could be striking in that about 20% of the neurons tested (24 of 116 given repeated challenges) responded to CCK-8 only one time (for example, Fig. 2A). Furthermore, some neurons responded repeatedly to CCK-8, but their responses were small making expression of the responses as a percentage of a standard response susceptible to large errors (Fig. 2B). We also observed wide variation in the threshold for responses to CCK-8. In a series of concentration-response experiments, 6 of 41 neurons that eventually responded to 10 nM CCK-8, responded to 10 pM CCK-8 (15%), and in a study on a separate group of neurons, 6 of 44 neurons that eventually responded to 10 or 100 nM of CCK-8, responded to 100 pM CCK-8 (14%). On the other hand, of 30 responsive neurons tested with a series of CCK-8 concentrations up to or greater than 100 nM, 6 (20%) required at least 100
nM CCK-8 before they responded. Thus, not only was there a large variation in the magnitude of responses, but the threshold CCK-8 concentration necessary to induce a response varied over 10,000 fold.

**Role of bath calcium in responses to CCK.** We next examined whether calcium responses to CCK-8 were dependent on extracellular calcium. When calcium was removed from the bathing media, the basal cytosolic calcium concentration dropped slightly (Fig. 3). In 13 of 16 neurons challenged in the absence of calcium, the response to CCK-8 was completely eliminated (for example, Fig. 3A). In the remaining 3 neurons, the increase in cytosolic calcium was greatly reduced, but a small residual calcium response could still be detected (for example, Fig. 3B). In these three neurons the calcium response in the absence of bath calcium was 48 ± 13% of the control response (average ± SE). When all 16 neurons tested were considered, the average calcium response after removal of bath calcium was statistically significant (Fig. 3C). After return of calcium to the bathing media, the CCK-8-induced calcium response was significantly greater than the response in the absence of bath calcium (Fig. 3C). These results show that in most, if not all, nodose neurons the calcium response to CCK-8 is due entirely to the influx of calcium from the extracellular milieu, but that recruitment of calcium from intracellular stores might occur in a few neurons.

**Role of intracellular calcium stores in responses to CCK.** The conclusion that extracellular calcium is the main source of calcium for CCK-8 induced calcium responses is further supported by the observation that thapsigargin, an irreversible inhibitor of the smooth endoplasmic reticular Ca-ATPase (Thastrup et al., 1989), failed to abolish the calcium response to CCK-8. Thapsigargin by itself caused a slow increase in calcium levels in all 9 neurons tested (Fig. 4A; average increase ± SE in response to thapsigargin alone was 84 ± 20 nM, p<0.005), indicating that the Ca-ATPase was
functional in these neurons. In 8 of the 9 neurons, CCK-8 still induced a calcium response after thapsigargin. Indeed the calcium response following thapsigargin was 108 ± 23% of the pre-thapsigargin response (minimum 63%, maximum 244%; summarized in Fig. 4B).

Responses to sulfated vs. nonsulfated CCK-8. Sulfated CCK-8 activates both CCK-A and CCK-B receptors (Noble and Roques, 1999), while nonsulfated CCK-8 activates CCK-B receptors but not CCK-A receptors (Dunlop, 1998). Overall, only 5% of neurons (3/62) challenged with nonsulfated CCK-8 responded. Because so few neurons responded to nonsulfated CCK-8, these responses were not further characterized. However, in 16 neurons that had positive responses to 10 or 100 nM sulfated CCK-8, 14 had no response to an equal concentration of nonsulfated CCK-8 (for examples, see Fig. 5A and 5B) while two neurons responded to both forms of CCK-8. In these two neurons, the calcium response to nonsulfated CCK-8 averaged just 34% of the response to sulfated CCK-8 (data not shown). Finally, one additional neuron exhibited a large robust response to nonsulfated CCK-8, but did not respond at all to sulfated CCK-8 (Fig. 5B).

Effect of CCK-A receptor antagonist on responses to CCK. In 14 of 14 neurons tested, lorglumide, a potent and selective CCK-A receptor antagonist (Makovec et al., 1992), completely abolished responses to CCK-8 (for example, Fig. 6A, results summarized in Fig. 6C). The average response in the presence of lorglumide was just 2 ± 1% of the response prior to lorglumide. After lorglumide was washed from the neurons the response to CCK returned to 53 ± 7% of the response before lorglumide. This represented a statistically significant recovery of the response (Fig. 6C).

Effect of JMV-180 on responses to CCK. We next used the CCK analog JMV-180 to investigate the role of the high and low affinity sites of the CCK-A receptor in the calcium response. In the rat JMV-180 is an agonist for the high affinity site (Kd ~70 pM) and an antagonist for the low affinity site (Kd ~10 nM) (Stark et al., 1989; Yule and Williams, 1994). In a first set of
experiments, we first challenged neurons with CCK-8 to determine if a neuron would respond to CCK. If a neuron responded to CCK-8 it was then challenged with a concentration of JMV-180 (100 pM) that would be adequate to activate the high affinity site but would have minimal actions on the low affinity site of the CCK-A receptor. In some neurons the order of the first challenge was reversed to ensure that prior CCK-8 exposure was not interfering with any JMV-180 responses. Neurons were then challenged with a higher concentration of JMV-180 (100 nM) that would block most low affinity sites. In the continued presence of JMV-180, the neurons were then re-challenged with CCK-8 (10 or 100 nM, concentration matching the first CCK-8 challenge). After washing JMV-180 from the neurons, the neurons were then challenged a final time to CCK-8 alone. In 16 neurons identified as CCK-8 responsive, neither 100 pM nor 100 nM JMV-180 induced any response by itself (example in Fig. 6B). In 8 of these neurons we could obtain no calcium response to CCK-8 in the presence of JMV-180 or after JMV-180 was washed from the neurons (data not shown). In the other 8 neurons, the calcium response to CCK-8 in the presence of JMV-180 (100 nM) was only $52 \pm 12\%$ of the first response to CCK-8 (for example, Fig. 6B, summarized results Fig. 6D), a statistically significant decrease. In these cells the response to the final challenge with CCK-8 alone returned to $79 \pm 9\%$ of the first response, which represented significant recovery of the response from that in the presence of 100 nM JMV-100 (Fig. 6D). Using 100 nM or 10 nM CCK-8 for the standard challenge did not produce significantly different results (data not shown). These results suggest that the low affinity site of the CCK-A receptor is responsible for the calcium signal induced by CCK-8 in cultured nodose neurons.

In contrast to our findings, a recent publication (Lankisch et al., 2002) reported evidence for a unique oscillatory calcium response induced by low concentrations of CCK-8 (10 pM) and the high affinity CCK-A receptor agonist CCK-OPE. Differences between this study and our study were
slight variations in the neuron isolation procedure and that neurons were challenged with CCK-8 on
the same day as they were isolated, whereas we typically used them the day after isolation. We
thus reexamined the effects of low concentrations of CCK-8 and JMV-180 on neurons isolated by
the procedure of Lankisch et al., and used the neurons on the same day as they were isolated. Using
this procedure we found 6 of 44 neurons tested responded to 10 pM CCK-8 (for example, Fig. 7A).
However, whereas the neuron illustrated in Fig. 7A had response characteristics similar to those
reported by Lankisch et al. (oscillatory response at 10 pM, spike and plateau at 10 nM CCK-8), this
was the only neuron that we found that responded in such a manner. Other neurons that responded
to 10 pM CCK-8 did not have distinct oscillatory responses. Furthermore, some of the neurons that
responded to CCK-8 at 10 nM did have oscillatory responses (for examples, Fig. 7B and 7C).
Overall, we found that 34 of 98 neurons that responded to 10 nM CCK-8 exhibited some evidence
of an oscillatory response regardless of whether we used our original isolation and testing paradigm
or the one reported by Lakeisch et al. The character of the oscillations we observed were either
distinct oscillations as in Fig. 7C or oscillations on top of a large increase in cytosolic calcium as in
Fig. 7B. Further, in this second series we did find a few neurons (4 of 41) that may have had very
small responses to JMV-180 as well as to 10 nM CCK-8, but these potential responses to JMV-180
only occurred after exposure to 10 nM CCK-8 and they were never mimicked by 10 pM CCK-8
(see Fig. 7A and 7D for examples). Thus it was never clear that these were responses mediated by
the high affinity state of the CCK-A receptor or were long-lasting responses activated by prior
exposure to 10 nM CCK-8. Finally, we did not find any particular aspect of the CCK-8-induced
calcium responses were different (percent responding, oscillatory or non-oscillatory) when neurons
were isolated by the procedure of Lankisch et al. (2002) versus when the neurons were isolated by
the procedure of Lancaster et al. (2001). Also, it made no difference whether the measurements
were made at room temperature vs. 36°C, or on the same day of isolation versus on the day after isolation.

**CCK-responsive versus CCK-insensitive neurons.** A final issue we addressed was whether some property of the neurons that responded to CCK-8 was different from CCK-8-insensitive neurons. We examined neuron size, basal calcium levels, and responses to KCl. Neuron size, estimated from phase contrast pictures of neurons, varied more than 10-fold (smallest 79, largest 908; reported in arbitrary units based on number of pixels in the image covered by the neuron). The size of CCK-8-responsive neurons was not significantly different from CCK-8-insensitive neurons (responsive: 349 ± 23 (n = 41), insensitive: 360 ± 25 (n = 48); averages ± SE, p = 0.76, two-tailed t-test). Basal calcium levels were also not significantly different (responsive: 166 ± 4 nM (n = 184), insensitive: 169 ± 3 nM (n = 281); averages ± SE, p = 0.58, two-tailed t-test), nor were responses to KCl (responsive: 412 ± 22 nM (n = 184), insensitive: 406 ± 18 nM (n = 281); averages ± SE, p = 0.83, two-tailed t-test).

**DISCUSSION**

*General characteristics of the CCK-8-induced calcium response in nodose neurons.* The results presented in this report demonstrate that CCK-8 triggers an increase in cytosolic calcium in neurons cultured from adult rat nodose ganglia. In some neurons calcium responses could be observed with concentrations as low as 10 pM but some neurons required at least 100 nM CCK-8 to elicit a response. This signal depends on the presence of extracellular calcium. In contrast to the study by Lankisch et al. (2002), our results suggest that this response is mediated primarily by activation of the low affinity site of the CCK-A receptor. In a few neurons we found evidence that CCK-B receptors also are capable of inducing a calcium response. Overall we found that about
40% of the cultured nodose neurons tested responded to CCK-8. This percentage is consistent with the 33% of nodose neurons reported to express CCK-A receptor mRNA (Broberger et al. (2001), and the percentage of nodose neurons reported to express a calcium response to 1 nM CCK-8 (45%, Lankisch et al., 2002). Broberger et al. (2002) also reported that just 9% of nodose neurons expressed mRNA coding for the CCK-B receptor, a number in close agreement with the 5% of the cultured neurons we observed that responded to nonsulfated CCK-8.

Validity of cultured nodose neuronal model. Although it would be ideal to examine changes in cytosolic calcium in the peripheral terminals of vagal afferent neurons, these structures are too small to allow for convenient biochemical analysis of their responses. Furthermore, examination of vagal afferent fibers themselves necessitates inclusion of end organ tissues that they innervate. These tissues may release neuroactive substances in response to CCK, making it difficult to distinguish direct vagal responses to CCK from responses to CCK-induced release of other vagal stimulants. Since the cell bodies for all vagal afferent fibers reside within the nodose ganglia, a method that removes these limitations is to examine the actions of CCK on neurons dissociated from the nodose ganglia and maintained in short-term culture. Previous studies have established that vagal afferent neurons from the nodose ganglia retain their electrophysiological characteristics in short-term culture (Oyama, 1987; Leal-Cardoso et al., 1993). Furthermore, receptor autoradiographic studies indicate that cell bodies of neurons within the nodose ganglia normally express CCK receptors (Ghilardi et al., 1994; Widdop et al., 1994; Moriarty et al., 1997). Therefore, it is likely that CCK-induced responses in cultured nodose neurons reflect the nature of responses of vagal afferent neurons in vivo.

Calcium responses in nodose neurons vs. other cell types. CCK induces an increase in cytosolic calcium in a variety of different cells, however the nature of the CCK-induced calcium
signal varies depending on the preparation under study and concentration of CCK used. These CCK-induced calcium signals range from an oscillating signal independent of extracellular calcium (Yule et al., 1991; Thorn et al., 1993), to a single spike of calcium from intracellular sources (Fridof et al., 1992), to a spike and plateau pattern with the spike from intracellular sources and the plateau from extracellular sources (Staley et al., 1989; Matozaki et al., 1990; Saluja et al., 1992; Kuwahara et al., 1993; Smith et al., 1993; Lignon et al., 1993; Pinnock et al., 1994; Del Valle et al., 1996), to a single spike of calcium that is totally dependent on extracellular calcium (Miyoshi et al., 1991).

The preparation best studied regarding CCK-induced calcium responses is the pancreatic acinar cell of rat and guinea pig. In these cells, activation of the high affinity site of the CCK-A receptor with either low concentrations of CCK or JMV-180 results in an oscillating calcium signal that is not dependent on extracellular calcium and is independent of IP3 generation (Matozaki et al., 1990; Yule et al., 1991; Saluja et al., 1992; Thorn et al., 1993). It is unlikely that this signaling mechanism accounts for CCK-induced increases in cytosolic calcium in vagal afferent neurons. We, as well as Lankisch et al. (2002), found that CCK-induced calcium signals in nodose neurons were dependent on extracellular calcium. Further, we found that depletion of intracellular calcium pools with thapsigargin did not prevent CCK-induced calcium responses. Consistent with our finding, Lankisch et al. found the response to be blocked by nicardipine, a blocker of L-type calcium channels. Taken together these results indicate that CCK-induced depolarization and activation of calcium influx through voltage-dependent calcium channels is likely to be the mechanism responsible for the calcium signals induced by CCK in nodose neurons. Dependence on extracellular calcium appears to be associated with activation of the low affinity receptor site in cells that exhibit both low and high affinity sites (Matozaki et al., 1990; Saluja et al., 1992).
When pancreatic acinar cells are challenged with higher concentrations of CCK that activate the low affinity site of the CCK-A receptor, a spike of calcium followed by a sustained plateau is observed (Matozaki et al., 1990; Saluja et al., 1992). This spike and plateau pattern also is produced by activation of CCK-B receptors in a variety of other preparations including pituitary cells (Kuwahara et al., 1993), pancreatic duct cells (Smith et al., 1993), lymphocytes (Lignon et al., 1993), pancreatic tumor cells (Pinnock et al., 1994), small cell lung cancer cells (Staley et al., 1989), and gastric D cells (Del Valle et al., 1996). In these responses the spike of calcium arises from intracellular stores and the plateau comes from extracellular sources. In one other preparation, pancreatic islet cells, activation of CCK-A receptors triggers only a spike of calcium that is dependent on intracellular stores (Fridof et al., 1992). The lack of participation of intracellular calcium stores that we observed in nodose neurons suggests that the signaling pathway activated by CCK-8 in nodose neurons is different from that activated in non-neuronal cells.

A CCK-induced calcium response similar to the one we and Lankisch et al. (2002) observed in nodose neurons was observed in striatal neurons of the central nervous system by Miyoshi et al. (1991). These investigators found that the CCK-induced calcium response was completely dependent on extracellular calcium, and that sulfated CCK-8 was more potent for triggering the response than nonsulfated CCK-8. Whether CCK-induced calcium responses in central striatal neurons versus peripheral nodose neurons are mediated by identical pathways will require additional work on both preparations. However, the similarity of these responses, and the apparent differences observed in non-neuronal cells, suggest that there may be a neuron-type specific calcium response to CCK that differs from CCK-induced calcium responses in non-neuronal cells.

*Lack of role for the high affinity site of the CCK-A receptor in nodose calcium responses.* An apparent discrepancy between our results and previous studies (Li et al., 1999; Lankisch et al.,
is that we did not observe convincing responses to JMV-180, an agonist at the high affinity site of the CCK-A receptor. An explanation for this discrepancy is not immediately obvious. We replicated the conditions of Lankisch et al. during the process of revising this report. However, we could find little evidence for a unique oscillatory response to low concentrations of CCK-8 or the high affinity site agonist JMV-180. We did observe that concentrations of CCK-8 as low as 10 pM could activate a small minority of nodose neurons, but these responses were not qualitatively different from those activated at higher concentrations. Even Lankisch et al. found that the their responses to low concentrations of CCK-8 or CCK-OPE were qualitatively similar to those they observed with higher concentrations of CCK-8 in that all responses were dependent of extracellular calcium. This would suggest that these high and low dose responses are fundamentally similar rather than a result of activating a unique oscillatory mechanism as occurs in pancreatic acinar cells. Furthermore, we would submit that while nodose neurons exhibit a wide range in sensitivity to CCK, most data are consistent with the interpretation that vagal afferent calcium responses are all dependent upon activation of a low affinity site. Finally, a speculation that is consistent with all observations is the oscillatory nature of the CCK-8-induced calcium signal reported by us and by Lankisch et al. is that the nodose neurons are oscillating into and out of spiking behavior with resulting rounds of calcium influx through voltage-dependent calcium channels. The weaker depolarizing effect of low concentrations of CCK-8 may make it more likely that oscillations are observed. Additional work is needed to test this hypothesis.

While vagally-mediated CCK-induced stimulation of pancreatic secretion appears to be mediated by the high affinity site of the CCK-A receptor (Li et al., 1997), other vagally-mediated actions of CCK, such as suppression of feeding and gastric emptying are mediated by the low affinity site (Asin and Bednarz, 1992; Yox et al., 1992; Weatherford et al., 1993; Moran et al.,
Thus we suggest that the calcium responses we observe to CCK-8 represent a step in the pathway that underlies vagally-mediated CCK-induced satiation and inhibition of gastric emptying.

**Role of CCK-B receptor in CCK-8 induced responses.** While sulfated CCK-8 exhibits much higher affinity for the CCK-A receptor than nonsulfated CCK-8, both the sulfated and nonsulfated peptides bind with equal affinity at the CCK-B receptor. Thus, responses to nonsulfated CCK-8 usually are indicative of CCK-B receptor activation. Although we did observe a few calcium responses to nonsulfated CCK-8, these responses occurred so infrequently that it was not possible to characterize them pharmacologically. CCK-B receptors reportedly are expressed by nodose neurons (Lin and Miller, 1992; Corp et al., 1993; Moriarty et al., 1997; Broberger et al., 2001), however, to date no physiological functions have been linked to CCK-induced activation of CCK-B receptors on vagal afferent fibers.

**Variation in threshold response to CCK.** We observed that the threshold response to CCK-8 in cultured vagal afferent neurons could vary as much as a 10,000 fold between individual neurons. In vivo electrophysiological experiments also have revealed variations in CCK-sensitivity in vagal afferent fibers innervating the gastrointestinal tract. For example, Blackshaw and Grundy (1990) concluded that most vagal afferents innervating the gastric wall were only indirectly sensitive to CCK by virtue of its effects on gastric smooth muscle (100-200 pmol, by near arterial infusion). On the other hand vagal afferents that innervated the antral or duodenal mucosa were directly excited by CCK at near arterial doses ranging from 3 to 200 pmol. Data such as these suggest that vagal afferent sensitivity to CCK varies widely, depending on the target of vagal afferent innervation. In addition they suggest that even neurons innervating the same organ exhibit a range of CCK sensitivities. In spite of this range, when one considers the relatively small volume of distribution for in vivo near arterially administered CCK, even low pmol doses are likely to result in
local concentrations in the nM range. Such concentrations are likely to be similar to those that produced calcium responses in our cultured nodose neurons.

Another possible explanation for the relatively high CCK concentrations required for activation of cultured vagal afferent neurons could be that disconnecting the afferent neuron cell body from its axon might reduce the overall responsiveness of the cell to various stimuli, including CCK. In support of this possibility, Lancaster et al. (2001) recently reported that the excitability of isolated vagal afferent neurons was reduced if cells were isolated 5 days after cutting the vagus. On the other hand, they found that there was no decrease in excitability if neurons were isolated within 17 hrs of the vagotomy and recorded within 9 hrs of isolation. Because of this we recorded from our neurons within 2 days of isolation, and neurons sensitive to 100 pM of CCK-8 were observed in both 1-day and 2-day old cultures (unpublished observation). Further, the fact that the proportion of nodose neurons in culture that responded to CCK-8 with increased cytosolic calcium that we observed agrees quite well with the proportion of nodose neurons expressing message for the CCK-A receptor (Broberger et al., 2001) would seem to support that the cultured neurons were not losing their responsiveness to CCK within this time frame.

A possible adaptive function for the wide range of threshold responses might be that it provides a mechanisms for coding the intensity of CCK release. We found that CCK responses rapidly fade in the continued presence of CCK and can also show a profound degree of desensitization. Thus the magnitude of the response in any individual neuron may not be a reliable indicator of the magnitude of CCK release. Rather the magnitude of the CCK release, which presumably reflects the magnitude of nutrients entering into the small intestines, may be coded by the recruitment of progressively less sensitive afferent fibers. The cellular basis for this large range in the threshold response remains to be determined. Threshold sensitivity could be related to the density
of CCK-A receptors on individual cells, or it could be related to the expression of other downstream effectors activated by CCK-A receptor stimulation.

**Paracrine actions of CCK.** Because systemic plasma levels of CCK are reported to be in the low pM range (Brenner et al., 1993; Liddle 1997), one could conclude that only responses we observed at subnanomolar concentrations are physiologically relevant. On the other hand, vagal afferent nerve endings terminate in the small intestinal lamina propria, in close proximity to the basal pole of the enterocytes and CCK-secreting I cells (Berthoud et al., 1995; Wang and Powley, 2000). The concentrations of CCK that are achieved in this interstitial area are not known, but they are likely to be much higher than either portal venous or systemic venous levels. In support of this idea Brenner et al. (1993) found inhibition of gastric emptying and reduction of food intake following intestinal infusion of some nutrients produces little or no elevations of plasma CCK, yet these phenomena are abolished by CCK-A receptor antagonists, suggesting that plasma CCK may not be an appropriate indicator of CCK acting on vagal afferent neurons. Indeed, several investigators have suggested that some of the effects of CCK may be paracrine in nature (Greenberg et al., 1987; Brenner et al., 1993; Schwartz and Moran, 1994; Dockray et al., 1996). On the other hand, two recent studies of the immunohistochemical localization of CCK-A receptor were unable to find labeling in vagal afferent nerve endings (Sternini et al., 1999; Patterson et al., 2002). Whether the failure to find CCK-A receptors in these terminals is due to the absence of the receptors, or that the antigenic regions of the receptor were somehow masked in these structures, is unknown. The fact remains that reduction of food intake and inhibition of gastric emptying by exogenous CCK are mediated by the low affinity CCK-A receptor site and involves vagal activation. This observation supports the notion that vagal afferent processes may be adapted to respond to relatively high local concentrations of paracrine CCK *in vivo*. In this regard, it seems
appropriate that afferent neuron cell bodies whose terminals have adapted to respond to high local concentrations of a peptide might not respond to low circulating concentrations of the peptide.

Conclusions. In conclusion, we have found that nodose neurons in culture respond to stimulation with CCK-8 with an increase in intracellular calcium. This response is dependent primarily on extracellular calcium and is mediated by the low affinity site of the CCK-A receptor. We propose that this response is part of the signaling pathway that mediates the activation of vagal afferent neurons by CCK, and that ultimately results in vagally mediated responses such as satiation and inhibition of gastric emptying.

PERSPECTIVE

We contend that cultured vagal afferent neurons are a useful preparation with which to examine the cellular mechanisms by which CCK activates visceral afferent neurons that inform the brain of conditions in the gastrointestinal tract. While it is possible that cultured neuronal cell bodies may not reflect the physiology of the afferent terminals, studies in these afferent nerve endings are complicated by their small size. Therefore, cultured neuronal cell bodies provide the best available preparation in which to examine cellular mechanisms underlying vagal afferent responses. Therefore it is important to demonstrate that the characteristics of the CCK response in the cultured neurons resembles that observed in vivo.

Both reduction of food intake (Smith et al., 1985; Ritter and Landenheim, 1985) and inhibition of gastric emptying (Raybould and Tache, 1988; Holzer et al., 1994; Moran et al., 1994) by CCK are mediated by vagal afferent neurons. These actions of CCK are abolished by antagonists for the low affinity site of the CCK-A receptor (Asin and Bednarz, 1992; Yox et al., 1992; Weatherford et al., 1993; Moran et al., 1994; Brenner and Ritter, 1996), as are the calcium
responses that we observed in cultured neurons. Likewise, antagonists for the low affinity site of the CCK-A receptor abolish activation of mesenteric afferent fibers, which are probably vagal, by exogenous CCK-8 (Eastwood et al., 1998). Thus, the specific site on the receptor mediating vagal afferent responses in vivo is the same as that which mediates the calcium responses in cultured nodose cells.

While we can not rule out the possibility that cellular processes involved in the response to CCK differ qualitatively between the afferent terminals and their cell bodies, our results to date are consistent with the supposition that cultured nodose neurons represent a valid model to gain cellular insights into how vagal afferent fibers are activated. As a better understanding of the mechanisms by which CCK activates vagal afferent neurons is developed, it will not only enhance our understanding of how vagal afferent neurons monitor the internal environment of the gut, but it will also provide the insights necessary to determine whether transduction processes in afferent neuron terminals differ from those currently observed in their cell bodies.
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REFERENCES


FIGURE LEGENDS

Fig. 1. **Panel A:** Example of concentration-response characteristics of calcium responses induced by CCK-8 in an isolated nodose neuron. The trace represents the calcium signal from a single nodose neuron (experiment ID no. in lower right corner). Bars over trace represent when inflow line was switched to a test solution. In this, and subsequent figures, when a bar is labeled with a number, it indicates the concentration of CCK-8 (in nM) in the test solution. Bars labeled with K indicates when cell was challenged with 55 mM K\(^+\). **Panel B:** Example of modest desensitization of the calcium response to repeated CCK-8 challenges.

Fig. 2. **Panel A:** Example of a neuron showing complete desensitization after a single CCK-8 challenge. **Panel B:** Example of a neuron showing small but reproducible calcium responses to CCK-8.

Fig. 3. Effect of removing bath calcium on the calcium response to CCK-8. **Panel A:** Example of a neuron that demonstrated a complete lack of calcium response to CCK-8 when bath calcium was removed (indicated by bar labeled -Ca). Arrow on figure indicates the lack of response. **Panel B:** Example of a neuron that could still partially respond even though bath calcium was removed. **Panel C:** Summary of results from removing bath calcium during the CCK exposure. Results are expressed as change in calcium (\(\Delta\)calcium) from basal levels (averages ± SE, n=16). Bar labeled Con indicates response to first control CCK exposure. Bar labeled no Ca indicates response to CCK in the absence of bath calcium. Bar labeled Rec indicates response to a second exposure of
CCK in the presence of bath calcium. *p<0.001 compared to first CCK response. **p<0.001 compared to response in absence of bath calcium and p<0.05 compared to first CCK challenge.

**Fig. 4.** Panel A: Effect of thapsigargin (50 nM; bar labeled Tg) on basal calcium levels and the calcium response to CCK-8. Panel B: Summary of results from thapsigargin experiments. Results are expressed as the change in cytoplasmic calcium (\(\Delta\)calcium) from basal levels (averages ± SE, n = 9). Bar labeled Con indicates response to CCK during the first CCK exposure. Bar labeled +Tg indicates response to CCK after thapsigargin exposure. Treatment with thapsigargin had no effect on CCK-induced calcium responses (p = 0.845).

**Fig 5.** Examples of responses to sulfated and non-sulfated CCK-8 (CCK-8 analog indicated on figure). Panel A: Responses from two neurons within the same image are illustrated. CCK-8 concentration was 100 nM for both analogs. Panel B: CCK-8 concentration was 10 nM for both analogs.

**Fig 6.** Panel A: Example of effect of the CCK-A antagonist lorglumide (10 µM, applied as indicated on figure) on CCK-8 response. Panel B: Effects of JMV-180 on CCK-8 responses. Cells were exposed to JMV-180 as indicated on figure (JMV-0.1 is when 0.1 nM JMV-180 was applied; JMV-100 was when 100 nM JMV-180 was applied). Break in X-axis was when data was not collected. Panel C: Summary of results from experiments with lorglumide. Results are expressed as the change in cytoplasmic calcium (\(\Delta\)calcium) from basal levels (averages ± SE, n = 14). Bar labeled Con indicates the response to the first exposure to CCK. Bar labeled +Lorg indicates the response to CCK in the presence of lorglumide. Bar labeled Rec indicates the
response to CCK after lorglumide was washed from the neurons. *p<0.001 compared to first CCK exposure. **p<0.005 compared to the response in the presence of lorglumide and p<0.001 compared to first control response. Panel D: Summary of results from experiments with JMV-180. Results are expressed as the change in cytoplasmic calcium (Δcalcium) from basal levels (averages ± SE, n = 8). Bar labeled Con indicates the response to the first exposure to CCK. Bar labeled +JMV indicates the response to CCK in the presence of 100 nM JMV-180. Bar labeled Rec indicates the response to CCK after JMV-180 was washed from the neurons. *p<0.001 compared to the first CCK exposure. **p<0.025 compared to CCK response in the presence of JMV-180 and p<0.05 compared to the first CCK response.

Fig 7. Examples of responses to CCK-8 and JMV-180 in cells isolated by a trypsin digest rather than with dispase (concentrations in nM are indicated on figure). In all panels the thin line indicates when 10 pM CCK-8 was applied and the thick bar indicates when the bathing solution was switched to 10 nM CCK, or other indicated agents.
Fig. 1

A

![Graph A](image)

B

![Graph B](image)
Fig. 2

A

B

- The diagrams show the calcium signal responses to different concentrations of K. The x-axis represents time (min) and the y-axis represents calcium concentration (nM).

- Graph A shows a pronounced calcium signal with a peak at 8 minutes for the 300 nM concentration of K.

- Graph B displays a more gradual response with multiple smaller peaks across the concentration range.

- Both graphs indicate an increase in calcium levels following the application of K at 0 minutes.
Fig. 3
Fig. 4

A

B

NOD927E

Δcalcium (nM)

0

50

100

150

0

50

100

150

Con + Tg
Fig. 5
Fig. 6

A

B

C

D

Δcalcium (nM)

Δcalcium (nM)
Fig. 7

A

B

C

D

calcium (nM)

calcium (nM)

calcium (nM)

calcium (nM)

time (min)

time (min)

time (min)

time (min)