Luminal CCK-releasing factor stimulates CCK release from human intestinal endocrine and STC-1 cells

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1Department of Medicine, Duke University Medical Center, Durham 27710; Durham Veterans Affairs Medical Center, Durham, North Carolina 27705; 2Department of Physiology, University of Texas, San Antonio, Texas 78284; 3CURE, University of California at Los Angeles, and 4Department of Medicine, Wadsworth Veterans Affairs Medical Center, Los Angeles, California 90073

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Wang, Yu, Vera Prpic, Gary M. Green, Joseph R. Reeve Jr., and Rodger A. Liddle. Luminal CCK-releasing factor stimulates CCK release from human intestinal endocrine and STC-1 cells. Am J Physiol Gastrointest Liver Physiol 282: G16–G22, 2002.—CCK is secreted into the blood from intestinal endocrine cells following ingestion of a meal. Recently, it has been demonstrated that the ability of certain foods to stimulate CCK release is mediated by endogenously produced CCK-releasing factors. A newly discovered luminal CCK-releasing factor (LCRF) is secreted into the intestine, where it stimulates CCK secretion. However, the mechanism whereby LCRF affects intestinal epithelial cells is unknown. The current study was designed to determine whether LCRF has a direct effect on CCK cells to stimulate hormone secretion. In dispersed human intestinal mucosal cells, LCRF (5–200 nM) significantly stimulated CCK release in a concentration-dependent manner. This stimulatory effect was absent in calcium-free media and was inhibited by the L-type calcium-channel blockers diltiazem and nifedipine. To examine direct cellular effects of LCRF on CCK cells, further studies were conducted in the CCK-containing enteroendocrine cell line STC-1. As in native cells, LCRF significantly stimulated CCK release from STC-1 cells in a calcium-dependent manner. In cells loaded with a calcium-sensitive dye, LCRF stimulation produced a rapid increase in intracellular calcium. To examine the electrophysiological basis for this stimulation, whole cell recordings were made from STC-1 cells. Whole cell calcium currents were identified under basal conditions; moreover, calcium-channel activity was increased by LCRF. These studies demonstrate that 1) LCRF has a direct effect on human intestinal cells to stimulate CCK secretion, 2) stimulated hormone release is calcium dependent, and 3) LCRF activates calcium currents in CCK cells, which leads to CCK secretion.

CCK is secreted into the blood following ingestion of a meal and has been shown to play a critical role in the ingestion, absorption, and digestion of food. In experimental animals and humans, ingested protein and fat are potent stimulants of CCK secretion (22, 35). Due to difficulties in purifying native CCK cells, little information is known about CCK secretion at the cellular level. However, recently, a variety of complementary methods has been applied to this problem, and several important observations have been made regarding regulation of CCK release in vivo and in vitro.

It has been demonstrated that endogenously produced releasing factors are secreted into the gut lumen and stimulate CCK secretion (27, 33). These releasing factors appear to mediate negative-feedback regulation of CCK secretion (7, 13, 17, 18, 34, 43). Luminal CCK-releasing factor (LCRF) was purified from rat intestinal washings and has been shown to stimulate CCK secretion in rats when introduced into the duodenum (43). Furthermore, immunoabsorption of LCRF with specific LCRF antiserum eliminated CCK-releasing activity from intestinal preparations. However, it is not known whether LCRF has a direct effect on CCK cells, nor is the mechanism by which LCRF affects CCK release understood. It is also unknown whether LCRF can stimulate CCK release in humans.

In a limited number of studies, the release of CCK has been examined in freshly isolated, partially enriched intestinal CCK cells. In canine jejunal cells, secretion of CCK was stimulated by depolarization of the plasma membrane by high potassium solution or following elevations in cAMP levels (3). Additionally, in cells in short-term culture, CCK release was stimulated by increases in protein kinase C (19). We have previously observed that stimulation of CCK secretion by potassium depolarization was inhibited by removal of extracellular calcium or addition of the calcium-entry blocker magnesium chloride (29, 30, 42).

A major limitation in studying the regulation of enteric endocrine cells is the lack of suitable in vitro...
models. In the current study, we used a recently identified intestinal CCK-secreting cell line for studies of CCK regulation (39). The STC-1 cell line was derived from an intestinal tumor arising in double transgenic mice carrying the rat insulin II promoter cloned upstream to the coding region of the SV 40 T antigen and the polyoma small t antigen genes. STC-1 cells synthesize and secrete high amounts of CCK, which is greater than any other hormone produced by these cells (30).

Because STC-1 cells are enteric endocrine in origin, we hypothesized that they would behave in a manner similar to native CCK cells. The present study was designed to determine at the cellular level the critical secretory and electrophysiological events that are important for understanding how CCK secretion is regulated by LCRF.

METHODS

Preparation of human intestinal mucosal cells. Intestinal mucosal cells were prepared from human jejunum collected at the time of surgery in which tissue was removed for other reasons (e.g., pancreatic or small bowel resection). The intestine was everted and placed in PBS at pH 7.4. The mucosal surface was rinsed twice with PBS and placed in trypsin-free dissociation media (Sigma Chemical, St. Louis, MO) at 37°C with gentle agitation for 2 min. The solution was removed and replaced with 10 ml of fresh dissociation media and incubated for 10 min more with gentle agitation. The remaining suspension was filtered (450-μm mesh), and the effluent was centrifuged at 1,000 rpm for 3 min. The pellet was resuspended in Hank’s balanced salt solution with HEPES (HHBSS; 10 mM; pH 7.4) and centrifuged as above. The pellet was resuspended in 5 ml HHBSS and divided into equal aliquots for analysis. The total CCK content of 1-ml tissue aliquots averaged 75 ± 14 fmol (n = 5).

Culture of STC-1 cells. STC-1 cells were maintained in DMEM with 4.5% glucose, 15% horse serum, and 2.5% fetal bovine serum and were typically studied between passages 30 and 60. Cells were cultured at 37°C in 95% air/5% CO2 and were passaged every 2–3 days. Cells were plated on 24-well culture dishes and were used for secretion studies when they were 90–95% confluent. Cells plated onto 35-mm tissues were used for microelectrode studies. These cells were studied 1–2 days after plating when they were ~80% confluent. Incubation wells contained on average 622 ± 60 (n = 5) fmol CCK.

Radioimmunoassay of CCK. CCK release was measured by a sensitive and specific radioimmunoassay as previously described using antiserum #9322 (28, 29, 40).

Source of LCRF. LCRF (1–35) was synthesized by Dr. J. R. Reeves, Jr. at the UCLA peptide synthesis facility. Synthesis, purification, and chemical characterization of LCRF (1–35) are described in detail elsewhere (44). We have previously demonstrated that LCRF (1–35) has similar bioactivity to the native peptide (44).

Measurements of intracellular calcium in STC-1 cells. Microspectrofluorometric techniques were adapted to STC-1 cells for the measurement of changes in intracellular calcium concentration. Cells were grown in monolayer culture on microscope slides. On the day of study, cells were loaded with the acetoxyethyl ester of the calcium-sensitive fluorochrome Calcium Green-1 (Molecular Probes), dispersed in 10% Pluronic 147, and diluted to 10 μM (31). Cells on microscope coverslips were incubated at 37°C for 30 min in the dark with the Calcium Green solution. Before the experiment, cells were washed three times and maintained in the dark for 15 min. The coverslips were mounted into a perfusion chamber, and changes in intracellular Ca2+ were measured using an Olympus epifluorescence microscope equipped with a 40× objective fluorescence Sensys KAF 1400 CCD camera (Photometrics, Tucson, AZ) and Lamba 10–2 optical filter (Sutter Instruments, Novato, CA) (2). Fluorescence signals were digitized and stored on computer disk using an integrated image processor controlled by Axon Imaging 2.2 workbench software (Axon Instruments, Foster City, CA). Calcium Green was excited at 488 nm, and the fluorescence was measured at emission wavelength 510 nm. Images were acquired every 1–2 s. Because Calcium Green is a nonratiometric Ca2+ indicator, the changes in intracellular Ca2+ were depicted as fluorescence intensity ratio (F/F0), where F0, the resting fluorescence, was determined at the beginning of each experiment by averaging 10 images before the addition of test reagents. To exclude possible influences associated with changes in cell volume or movement, these parameters were monitored throughout each experiment by encircling the area of each cell (25). Studies were performed at room temperature.

Microelectrode recordings. For microelectrode studies, STC-1 cells were plated onto 5 × 5-mm polystyrene coverslips placed in 35-mm culture dishes. Before an experiment, a coverslip was placed in a recording chamber (1-ml volume) with control and drug-containing buffer (see below). The bath was grounded with the Ag-AgCl electrode separated from the bathing solution by a short Ringer-agar bridge. Cells were viewed at a magnification of ×600 with an Olympus inverted phase-contrast microscope. Recording pipettes were made from Corning 7052 glass and had resistances of 3 MΩ (Sutter P-87). Studies were performed at room temperature. Data were filtered at 1 kHz and sampled at 5 kHz (6-pole Bessel filter, 3 dB down). The potential inside the pipette was clamped at the required level. Voltage commands and current measurements were performed using pCLAMP 6.3 software (Axon Instruments), a Digidata 1200 interface (Axon Instruments), and a 300-MHz computer (8, 11, 15).

Whole cell recording. For whole cell voltage-clamp studies, the standard extracellular solution contained (in mM): 30 BaCl2, 10 KCl, 5 NaCl, 90 choline-Cl, 1 MgCl2, 10 glucose, 10 HEPES (pH 7.4 with CsOH), and 0.0025 tetrodotoxin. Low-resistance patch electrodes (2–5 MΩ) were filled with (in mM): 120 CsCl, 26 TEA-Cl, 0.1 CaCl2, 11 EGTA, 3 MgATP, and HEPES (pH 7.4 with CsOH). With these solutions, only movement of barium would represent an inward current, which is shown as downward deflections on the current traces. Both series resistance (~70%) and capacitance compensation were used.

Data analysis. The peak conductance (G) of whole cell calcium currents (Icap) at each potential was calculated from the corresponding peak current (32). All data were expressed as means ± SE (n = number of observations for at least 3 measurements, unless otherwise stated). * Indicates a significance level of P < 0.05 as determined by Student’s t-test.

RESULTS

The effects of LCRF on dispersed human intestinal mucosal cells were tested in vitro by incubating cells with various concentrations of LCRF (1–35) (Fig. 1). Within 15 min, at concentrations >5 nM, LCRF caused a significant elevation in CCK release. Basal CCK release ranged from 10 to 30 fmol/ml, and maximal CCK release averaged 40% of CCK content from representative aliquots. The stimulatory effect of LCRF...
was inhibited by addition of the L-type calcium-channel blocker diltiazem (Fig. 2). These findings indicated that LCRF has a direct effect on cells of the intestinal mucosa.

We and others have used STC-1 cells as a model for intestinal CCK-containing cells (6, 28, 30). To determine whether LCRF has a direct effect on this model for enteric endocrine cells, we incubated various concentrations of LCRF (1–35) on STC-1 cells. As shown in Fig. 3, at doses similar to those used on human intestinal cells, LCRF significantly increased CCK release. To determine the importance of extracellular calcium in LCRF-stimulated CCK release, STC-1 cells were incubated in the presence or absence of calcium in the culture media. LCRF did not increase CCK release in the absence of added extracellular calcium (Fig. 4). In calcium-containing media, the stimulatory effects of LCRF on CCK secretion were blocked by addition of either of the L-type calcium-channel blockers diltiazem and nifedipine (Fig. 5).

It therefore appeared that calcium was a possible trigger for intestinal CCK release. To determine the effects of LCRF on intracellular calcium ([Ca$$^{2+}$$]i) in CCK-containing cells, STC-1 cells were loaded with the calcium-sensitive dye Calcium Green-1-AM. With the use of single-cell fluorescence imaging, a rapid increase in [Ca$$^{2+}$$]i was detected after LCRF (200 nM) treatment (Fig. 6). However, elevation of [Ca$$^{2+}$$]i was not observed in calcium-free media. Moreover, pretreatment of cells with diltiazem or nifedipine completely eliminated the increase in [Ca$$^{2+}$$]i after LCRF administration. In Fig. 6, right, are shown cells retreated with 200 nM LCRF ~10 min after the initial LCRF exposure. Interestingly, the LCRF-induced calcium response was not seen on restimulation with LCRF.

These secretion and fluorescence studies suggested that elevation in fluorescence was perhaps due to involvement of a dihydropyridine-sensitive pathway in LCRF-mediated secretion. We next sought to determine whether LCRF exposure modified calcium conductance in CCK-secreting cells. With the use of whole cell recording techniques, we initially identified calcium ion-channel activity in STC-1 cells.

Currents were measured with barium as the charge carrier in STC-1 cells during application of depolariz-
ing steps from a holding potential \( V_h \) of −80 or −40 mV. From a \( V_h \) of −40 mV, a voltage-activated calcium current was identified that activated near −20 mV with a maximal peak current amplitude between −10 and +10 mV. The current demonstrated slow activation over time (Fig. 7). Current amplitude substantially increased with addition of LCRF. In five cells examined, addition of LCRF (200 nM) to the media increased the peak current \( I_{Ba} \) from 889 ± 163 to 1,191 ± 225 pA \( (P < 0.05) \). After addition of LCRF, the mean peak amplitude increased from 1,230 ± 257 to 1,361 ± 276 pA \( (P < 0.05) \). In data not shown, this current was blocked by diltiazem.

**DISCUSSION**

A fundamental problem in gastrointestinal endocrinology is that it remains unknown how foods interact with enteric endocrine cells to stimulate secretion. However, it has recently been shown that intestinal

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**Fig. 5.** Effect of calcium-channel blockade on CCK release. Cultured STC-1 cells were incubated with LCRF (100 nM) in the presence or absence of diltiazem (10 μM) or nifedipine (100 nM) added 10 min before the addition of LCRF. CCK released into the media was measured 15 min after LCRF exposure. Neither diltiazem nor nifedipine alone had an effect on basal CCK secretion \( (n = 4 \text{ experiments}) \).

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**Fig. 6.** Effect of LCRF on intracellular calcium concentration in STC-1 cells. Calcium fluorescence was measured in cells loaded with Calcium Green-1-AM. Fluorescence recordings were made from 21 single cells as described in METHODS. Addition of LCRF (200 nM) caused an increase in calcium fluorescence. After the elevation in intracellular fluorescence, the media were changed and 10 min later LCRF (200 nM) was readministered. Studies are representative of at least 5 experiments.

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**Fig. 7.** Effects of LCRF on calcium currents in STC-1 cells. With pipette solutions containing 110 mM Ba\(^{2+}\), recordings were made in the whole cell configuration with step increases in membrane potential from −60 to +50 mV. The current-voltage relationship \( (I-V) \) is shown by the current chart \( (A) \) and \( I-V \) plot \( (B) \). Whole cell recordings showed channel activation after addition of LCRF. This recording is representative of 5 cells studied.
releasing factors that are secreted into the lumen of the upper small intestine stimulate CCK release, and it is believed that the effects of dietary nutrients, such as protein, on CCK release are mediated by releasing factors. In the current study, we sought to determine whether the releasing factor LCRF has direct effects on CCK cells to stimulate secretion. Moreover, we wanted to determine whether LCRF could stimulate CCK release from human intestinal cells. In dispersed intestinal mucosal cells, LCRF, in a concentration-dependent manner, significantly stimulated CCK secretion. This finding was important because it had not been demonstrated previously that LCRF could stimulate hormone secretion in vitro.

All prior studies of LCRF have characterized its ability to stimulate CCK release in vivo. CCK release has been shown to be under negative-feedback regulation, whereby active proteases in the lumen of the gut inhibit CCK release and diversion or inhibition of pancreatic enzymes stimulates CCK release (20, 21, 26, 36). The concept of negative-feedback regulation of pancreatic secretion and later CCK release was based on studies first conducted in rats (9, 10, 23). This general observation was later extended to other species, and it is now accepted that negative-feedback regulation of CCK release exists in humans (16, 24, 36).

LCRF was purified from rat intestinal washings, but a human counterpart is unknown. Therefore, it was important to determine whether LCRF could affect human CCK release. To address this issue, the effects of LCRF were specifically tested on human intestinal cells. The finding that LCRF potently stimulated CCK secretion indicates that human cells share a common mechanism with murine cells and raises the possibility that LCRF may exist in humans. At the very least, LCRF appears to represent a novel method for stimulating CCK secretion in humans.

Because LCRF is active in the intestinal lumen, it is hypothesized that it interacts with the apical surfaces of intestinal cells rather than the basolateral surfaces (43). However, neither dispersed human cells nor STC-1 cells in culture maintain their apical-basal polarity, thus additional studies are necessary to fully explore the site of LCRF action in the intestine.

Because of the technical difficulties associated with isolation of a homogeneous population of CCK (I) cells together with the inability to maintain the cells in culture, it has not been possible to sufficiently examine the cellular mechanisms of hormone secretion. Although dispersed intestinal mucosal cells are free of neural or hormonal stimuli that are present in whole animal or organ preparations, they are still not pure CCK cells. To determine whether LCRF directly regulates CCK-containing cells, we used the CCK-containing cell line STC-1. By immunofluorescent staining, using CCK-specific antisera, over 95% of cultured STC-1 cells stained positively for CCK (28). STC-1 cells are intestinal in origin, contain high amounts of immunoreactive and bioactive CCK, and have many properties of native CCK cells including responsiveness to secretagogues such as bombesin, β-agonists, cAMP analogs, calcium ionophores, and membrane-depolarizing concentrations of KCl (6, 29, 40–42). Therefore, STC-1 cells demonstrate regulated secretion that appears analogous to I cells and thus provide a unique model for studies of CCK secretion. In STC-1 cells, LCRF also stimulated CCK release, indicating that this releasing factor acts directly on intestinal endocrine cell types. Secretion was also calcium dependent, and inhibition of secretion by diltiazem and nifedipine indicated that calcium influx through L type calcium channels was critical for LCRF-stimulated hormone release.

It is clear from other endocrine systems that hormone secretion can be intimately coupled with membrane ion-channel activity (1, 12, 37, 38). Secretagogue-stimulated hormone secretion can occur through activation of classic second-messenger pathways such as cAMP and the phospholipase C cascade (with generation of inositol trisphosphate and diacylglycerol). Either of these major signaling pathways can increase intracellular calcium concentrations in CCK-containing cells by opening membrane calcium channels, allowing an influx of calcium from the outside to the inside of the cell, or by causing the release of intracellular calcium stores (40, 42). Alternatively, signaling molecules may activate calcium currents directly through ligand-gated channels (4). In STC-1 cells, LCRF induced a rapid rise in [Ca^{2+}]i. However, this elevation was not sustained in most cells, and desensitization appeared to develop because repeat stimulation did not generate a Ca^{2+} response. Secretagogue-induced changes in [Ca^{2+}]i were completely absent in calcium-free media and following exposure to L type calcium channel blockers, suggesting that a calcium influx pathway is critical for the increase in [Ca^{2+}]. Moreover, it appears that influx of calcium is necessary for secretion of CCK, at least to the level that is measurable in our assay (42).

Electrophysiological data from the present study indicate that LCRF activates membrane calcium channels, although it remains to be determined whether LCRF is signaling through a receptor-mediated mechanism or it interacts directly with calcium channels. Nevertheless, the observed dependence of LCRF’s secretory effect on [Ca^{2+}]i suggests that intracellular calcium has a critical role in signaling secretion, and it is possible that [Ca^{2+}]i serves as a second messenger for LCRF-stimulated secretion.

Calcium channels represent a diverse class of molecules that traditionally have been grouped into two major categories according to their kinetics and voltage-dependent properties (5, 14, 45). The calcium-channel activity activated by LCRF in STC-1 cells has voltage dependence, kinetics, and pharmacological characteristics typical of high voltage-activated Ca^{2+} channels. Specifically, these channels activate on depolarization to relatively positive potential and display diverse kinetics and sensitivities to voltage. To distinguish between high voltage-activated calcium channels such as N, P, and L types, their relatively large channel
size and sensitivity to pharmacological agents such as diltiazem indicate that these channels are L type.

Regulation of CCK release by releasing factors such as LCRF, which are active within the lumen of the intestine, represents a new concept in gastrointestinal physiology. CCK is produced by discrete endocrine cells whose apical surfaces are exposed to luminal contents in the small intestine and is secreted in response to ingestion of a meal. Although it is not known how foods interact with the CCK cell to stimulate hormone secretion, it is likely that releasing factors such as LCRF mediate some component of this response. The present studies help elucidate the mechanism by which LCRF affects CCK cell function. By directly interacting with CCK cells, LCRF activates calcium ion-channel activity to increase [Ca2+]i, and stimulate hormone release. It is conceivable that other secretagouges in the lumen of the gut activate similar signaling mechanisms to regulate endocrine cell function essential for coordinating the vast complexity of processes associated with digestion.

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