Cholecystokinin cells purified by fluorescence-activated cell sorting respond to monitor peptide with an increase in intracellular calcium

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ABSTRACT Cholecystokinin (CCK) is secreted from specific enteroendocrine cells of the upper small intestine upon ingestion of a meal. In addition to nutrients, endogenously produced factors appear to act within the gut lumen to stimulate CCK release. One such factor is a tryptic-sensitive CCK-releasing peptide found in pancreatic juice, known as monitor peptide. This peptide is active within the intestinal lumen and is hypothesized to stimulate CCK secretion by interacting directly with the CCK cell. We have found that monitor peptide releases CCK from isolated rat intestinal mucosal cells and that this effect is dependent upon extracellular calcium. In the present study, we used monitor peptide as a tool for isolating CCK cells from a population of small intestinal mucosal cells. Dispersed rat intestinal mucosal cells were loaded with the calcium-sensitive fluorochrome Indo-1, and CCK secretory cells were identified spectrofluorometrically by their change in fluorescence when stimulated with monitor peptide. Cells demonstrating a change in their emission fluorescence ratio were sorted using a fluorescence-activated cell sorter. More than 90% of the sorted cells stained positively for CCK with immunohistochemical staining. Furthermore, sorted cells secreted CCK when stimulated with calcium-depolarizing concentrations of potassium chloride, dibutyryl cAMP, calcium ionophore, and monitor peptide. These findings indicate that functional intestinal CCK cells can be highly enriched using fluorescence-activated cell sorting. Furthermore, monitor peptide appears to interact directly with CCK cells to signal CCK release through an increase in intracellular calcium.

Cholecystokinin (CCK) is an established gastrointestinal hormone that is localized in discrete mucosal endocrine cells of the upper small intestine (1). CCK is secreted from the intestine in response to eating and has effects on many digestive processes including gallbladder contraction, pancreatic secretion, bowel motility, and perhaps satiety (2). Although much is known about the actions of CCK, many aspects regarding the cellular mechanisms regulating its secretion have not been characterized. CCK release can be regulated in several different ways. It is hypothesized that foods in the intestinal lumen that come in contact with the apical surface of the CCK cell may affect CCK release directly. In addition, it is likely that neural and hormonal factors also modulate CCK secretion (3–9). CCK secretion is also under negative feedback regulation by pancreatic secretion whereby active proteases in the intestinal lumen inhibit CCK secretion, and inactivation of intestinal proteases or binding of proteases by substrates such as protein or trypsin inhibitor stimulates CCK secretion (10–12).

Recently, it has been proposed (13–16) that CCK secretion is regulated by two types of trypsin-sensitive “CCK releasing factors.” One type of releasing factor is produced in the upper small intestine and appears to be responsible for the pronounced increase in CCK secretion that occurs with diversion of bile and pancreatic juice (13, 14). Therefore, it is likely that this intestinal CCK releasing factor is physiologically important in the regulation of CCK secretion. However, this factor has not yet been isolated and its chemical structure is not known. A second releasing factor named monitor peptide has been identified in high amounts in pancreatic juice (15–17). Monitor peptide is a 61-amino acid peptide that is structurally related to a 56-amino acid protein that possesses trypsin inhibitor-like activity—hence, the name pancreatic secretory trypsin inhibitor (PSTI). However, the ability of monitor peptide to stimulate CCK release is independent of its trypsin inhibitor activity and the 56-amino acid PSTI itself does not stimulate CCK secretion. Therefore, monitor peptide appears to directly stimulate the CCK cell by interacting with the apical surface possibly through a membrane receptor.

Most studies of CCK secretion have been conducted in whole animals, intestinal segments, or only partially enriched preparations of CCK cells (2, 18–20). Accordingly, it has not been possible to study intracellular mechanisms regulating CCK release without possible interference from other hormones, neurotransmitters, or paracrine agents. We have recently developed a method for studying hormone secretion from rat intestinal mucosal cells in a perfusion apparatus. In this preparation, CCK cells represent <1% of the mucosal cell population. Nevertheless, cells release CCK in response to calcium ionophores and monitor peptide. Moreover, the ability of monitor peptide to stimulate CCK secretion was dependent upon extracellular calcium. Therefore, we postulated that monitor peptide is a specific secretagogue for the CCK cell and that one mechanism by which monitor peptide affects the CCK cell is by increasing intracellular calcium ([Ca2+]i).

Calcium-sensitive fluorochromes that accumulate intracellularly are sensitive to changes in [Ca2+]i (21, 22). We have utilized these properties to identify CCK cells among a mixture of rat intestinal mucosal cells loaded with a calcium-sensitive dye, the acetoxyethyl ester of Indo-1 (Indo-1 AM), by measuring the change in calcium fluorescence of CCK cells when stimulated with monitor peptide. By using fluorescence-activated cell sorting, it was then possible to isolate a highly enriched population of biologically responsive CCK cells that could be used for studying CCK secretion in vitro.

MATERIALS AND METHODS

Materials. The following materials were purchased. Hepes, EDTA, EGTA, and goat serum were from Sigma; minimal Eagle's medium amino acid supplement and Dulbecco's modified Eagle's medium (DMEM) were from Gibco; bo-

Abbreviations: CCK, cholecystokinin; [Ca2+]i, intracellular calcium ion concentration; Indo-1 AM, the acetoxyethyl ester of Indo-1. §To whom reprint requests should be addressed at: Department of Medicine, P.O. Box 3083, Duke University Medical Center, Durham, NC 27710.
vine serum albumin, fraction V, was from Miles; octadecyl-
silylsilica (C<sub>18</sub> Sep-Pak) cartridges were from Waters; Seph-
adex G-50 medium resin was from Pharmacia; collagenase was from Worthington; Indo-1 AM and Pluronic F-127 were from Molecular Probes; CCK antibody AB 1972 was from Chemicon; goat anti-rabbit antibody fluorescein-conjugate was from Tago; A23187 and ionomycin were from Calbio-
chem; and rats were from Charles River Breeding Labora-
tories. The following materials were gifts. Monitor peptide was from Gary Green (University of Texas, San Antonio); MK-329 was from Merck Sharp & Dohme; and CCK-8 was from Squibb.

**Cell Preparation.** Male Sprague-Dawley rats, weighing 250–300 g, were sacrificed, and the proximal 20 cm of small intestine beginning 3 cm distal to the pylorus was removed. The intestine was everted and washed for 1 min with saline at room temperature to remove mucus. The tissue was then incubated for 5 min at room temperature under agitation in 15 ml of calcium-free modified Krebs–Henseleit bicarbonate buffer (KHB) containing 2.5 mM EDTA (23). This buffer was enriched with mixed amino acids and was equilibrated to pH 7.4 in an atmosphere of 95% O<sub>2</sub>/5% CO<sub>2</sub>. Tissue was then placed in 10 ml of KHB with 1.2 mM CaCl<sub>2</sub> containing 1 mg of collagenase and agitated by gentle inversion for 10 min. After this incubation, the suspension was centrifuged for 3 min at 100 x g. The supernate was discarded and the pellet (containing cells) was resuspended in 2 ml of Hepes buffer containing 10 mM Hepes, 1.2 mM CaCl<sub>2</sub>, 103 mM NaCl, 1 mM Na<sub>2</sub>HPO<sub>4</sub>, 4.7 mM KCl, 0.56 mM MgCl<sub>2</sub>, 11.1 mM glucose, 0.1% bovine serum albumin, and mixed amino acids (adjusted to pH 7.4). At this point, single cells were identified microscopically. For perfusion studies, these cells were added to a vial containing 2.5 ml of Sephadex G-50 medium resin that had been preswollen in saline overnight at 4°C. The cells and resin were mixed for 1 min.

**Perfusion of Intestinal Mucosal Cells.** A perfusion appara-
utus was modified as described (24, 25). Cells were loaded into a perfusion chamber consisting of a 3-ml disposable plastic syringe whose plunger was perforated with a 19-gauge needle connected to polyethylene tubing. Two to 2.5 ml of Sephadex resin/cell mixture was layered over a double layer of 5-μm nylon filters. Perfusion solutions were made of Hepes buffer (pH 7.4) supplemented as described above and bubbled with 100% O<sub>2</sub>. Solutions were maintained in a 37°C water bath and pumped through silicone-coated polyethylene tubing (0.02 inch, inner diameter; 0.037 inch, outer diameter; 1 inch = 2.54 cm) by a peristaltic pump through the perfusion column at a rate of 1 ml/min. The tip of the syringe was fitted with a 19-gauge needle, which, in turn, was attached to polyethylene tubing that emptied into a fraction collector.

Once loaded into the column, cells were equilibrated for 1 h with Hepes buffer to obtain a steady baseline level of CCK release. The perfusion medium could be changed instantly by means of switching flasks. In this way, perfusion medium containing different reagents were sequentially perfused through the chamber containing the mucosal cells. Column fractions were passed through octadecylsilylsilica (C<sub>18</sub> Sep-
Pak) cartridges prewashed with 5 ml of methanol and 20 ml of deionized water, to concentrate CCK (11). Cartridges were then washed with 20 ml of deionized water and stored at −20°C or used immediately for assay.

All periods of stimulation with secretagogues were 15 min long followed by a period of at least 20 min during which the column was perfused with Hepes buffer. Fractions for assay were collected at 5-min intervals. The dead space of the system was 4.6 ml; therefore, the effect of a test substance lagged approximately one fraction behind its initiation. All solutions to be perfused were maintained at 37°C in a water bath and were oxygenated with 100% O<sub>2</sub>.

For perfusion of cells after cell sorting, ~10<sup>5</sup> cells were incubated in DMEM supplemented with 10% (wt/vol) bovine serum albumin and layered over 1 ml of Sephadex G-50 resin. Cells were incubated at 37°C in 95% O<sub>2</sub>/5% CO<sub>2</sub> for 18 h. The cells and Sephadex mixture were then pipetted into a 3-ml column and perfused as described above.

**Flow Cytometric Analysis of Intracellular Ca<sup>2+</sup> and Cell Sorting.** Indo-1 AM was used to measure [Ca<sup>2+</sup>]i, in intestinal mucosal cells before and after exposure to monitor peptide. The AM group of Indo-1 AM allows this compound to enter cells (21, 22). Intracellular esterases then cleave the AM group, rendering the dye impermeable and, thus, trapped within cells. Binding of Ca<sup>2+</sup> to Indo-1 alters its fluorescence spectrum. [Ca<sup>2+</sup>]i can be measured by comparing the ratio of Indo-1 emission at 405 nm and 485 nm when excited at 350 nm. This ratio increases as Ca<sup>2+</sup> enters cells and complexes with Indo-1 (26, 27).

From 1 to 5 x 10<sup>5</sup> intestinal mucosal cells were loaded with 5 μM Indo-1 AM for 45 min at 24°C in 1 μM Pluronic F-127 in Hepes buffer as recommended by the manufacturer and washed twice. Loading of these cells with Indo-1 AM was measured by the dual emission method using an EPICS 753 flow cytometer equipped with an argon ion laser operating in the ultraviolet at 40 mW. Forward and orthogonal light scattering were used to gate analysis to signals from intact cells. Fluorescence emission ratios for intracellular Indo-1 were calculated in real time for each cell (21, 22).

Once baseline fluorescence ratios were established, cells were stimulated with monitor peptide (10 nM). Cells that exhibited an increased fluorescence ratio, reflecting an increased [Ca<sup>2+</sup>], were sorted into tissue culture medium at rates of 500–1000 cells per sec.

**Immunohistochemical Staining.** Cells were prepared in suspension for immunohistochemical labeling by using previously described methods (28). From 1 to 50 x 10<sup>5</sup> cells were fixed using 2% (wt/vol) paraformaldehyde. Prior to staining, cells were incubated with 10% (vol/vol) goat serum for 1 h and then washed three times with phosphate-buffered saline (PBS). Cells were stained for CCK content using antibody AB 1972 (which is directed against the midportion of CCK-33) at 1:100 dilution (28). Goat anti-rabbit antiserum was used as secondary antibody at a 1:40 dilution in PBS. To confirm the specificity of antibody staining for CCK-containing cells, AB 1972 was preabsorbed with 10 nmol of CCK-33 (Peninsula Laboratories). This preabsorption eliminated specific cell staining. In addition, staining with antibodies OAL-656 (which is specific for the sulfated region of CCK) (29) and AB 1972 yielded similar results.

**CCK Assay.** CCK values were measured by bioassay as described (11, 30). In brief, Sep-Pak cartridges (containing CCK from the column fractions) were eluted with 1 ml of ethanol/1% trifluoroacetic acid, 4:1 (vol/vol), into incubation vials and dried under nitrogen. Extracts were then incubated with isolated rat pancreatic acini for 30 min at 37°C, and the amylase released into the incubation medium was assayed using prion yellow-coupled starch as substrate. Amylase release expressed as percent of total amylase content was compared with a dose–response curve for CCK-8. With this method, CCK concentrations in the original column effluent as low as 1 fmol per fraction could be detected.

The use of animals for these studies was approved by the Duke University Institutional Animal Care and Use Committee.

**RESULTS**

**Effect of Monitor Peptide on CCK Release.** Dispersed intestinal mucosal cells were mixed with Sephadex G-50 resin, introduced into the perfusion column, and perfused with Hepes buffer at 1 ml/min. Fractions were collected at 5-min intervals and measured for CCK activity. Monitor
peptide produced a prompt increase in CCK release that persisted for the duration of exposure (Fig. 1). The ability of monitor peptide to stimulate CCK release was dependent upon extracellular calcium ion concentration since monitor peptide had no effect on CCK release in calcium-free buffer.

Several controls were used to confirm that the CCK activity measured by bioassay was an accurate estimate of the amount of CCK bioactivity present in column fractions. (i) Perfusion buffer either with or without EGTA, after passage through Sep-Pak cartridges (identical to the manner in which column fractions were processed), did not modify basal or CCK-stimulated amylase release. (ii) Monitor peptide in concentrations of up to 1 μM, when incubated with pancreatic acini, had no effect on amylase release. (iii) The CCK receptor antagonist MK-329 (1 μM), when added to column fractions immediately before bioassay, completely blocked the bioactivity of column fractions.

Cell Sorting with Monitor Peptide. Dispersed mucosal cells were loaded with Indo-1 AM, washed, and resuspended in Heps buffer containing 1.2 mM CaCl₂. Basal fluorescence ratios ranged between 0 and 0.4 (n = 10) and remained stable for up to 1 h. A change in fluorescence ratio was observed with addition of monitor peptide. A concentration of 10 nM was sufficient to detect a change in fluorescence ratio in 15 of 17 cell preparations. The fluorescence signal increased within 30 sec of adding monitor peptide and was sustained for at least 30 min during which time cells were separated by selecting a gating ratio over that of basal (Fig. 2). The number of dispersed intestinal mucosal cells that responded to monitor peptide by demonstrating a change in fluorescence ratio (thus reflecting an increase in [Ca²⁺]) averaged 0.86 ± 0.16% (mean ± SEM; n = 15) of the total number of mucosal cells loaded with Indo-1. This compared with >95% of cells responding with a change in fluorescence ratio when stimulated with 1 μM ionomycin in the presence of 1.2 mM CaCl₂.

Immunohistochemical Staining of Sorted Cells. As a test of cell viability after sorting, >90% of cells excluded trypan blue. To determine the purity of CCK cells in this preparation, cells were analyzed by immunohistochemistry using a CCK-specific antibody to stain CCK-containing cells. In preparations of cells prior to sorting, 0.66% of cells (n = 455 cells) stained positively for CCK (Fig. 3 A and B). However, after sorting 92.9% of sorted cells (n = 225 cells) stained positively for CCK (Fig. 3C).

CCK Secretion from Purified CCK Cells. The ability of enriched CCK cells to secrete hormone in response to secretagogue stimulation was tested by placing cells into short-term overnight culture with Sephadex G-50 beads. The cell and resin mixture was then introduced into a perfusion column for measuring dynamic hormone secretion. Enriched CCK cells were stimulated with four types of potential secretagogues (Fig. 4). Cells were continuously perfused with Heps buffer and then stimulated with 30 nM monitor peptide. After an intervening period with perfusion of buffer alone, cells were exposed to buffer to which 5 μM dibutyryl cAMP was added. This was followed by Heps in which 52 mM KCl was substituted for NaCl. Finally, the effects of the calcium ionophore A23187 (1 μM) on CCK release was tested. All four agents produced significant increases in CCK release. In between stimulations, CCK levels returned to basal levels, indicating that hormone release was not due to toxic effects on the CCK cells. Effects similar to those of A23187 were also achieved using 1 μM ionomycin. Control substances such as buffer alone, saline, and 1 μM bovine serum albumin had no effect on CCK release.

**DISCUSSION**

In the present study we used a specific CCK secretagogue to identify and purify a population of CCK cells from intestinal mucosa. These experiments tested the hypothesis that monitor peptide selectively stimulated CCK cells and, in so doing, increased [Ca²⁺]. This hypothesis suggests that, if a mixed population of intestinal mucosal cells was loaded with the dye Indo-1, then stimulation with monitor peptide would increase [Ca²⁺] only in CCK cells. The increase in [Ca²⁺] would then change the emission fluorescence within cells responding to monitor peptide. These changes could then be detected by a fluorescence-activated cell sorter and CCK cells could be separated from other cells in the mucosal preparation. Immunofluorescent antibody studies indicated that >90% of sorted cells stained positively for CCK. Therefore, it appears...
that monitor peptide signals an increase in $[\text{Ca}^{2+}]_i$, only in CCK cells. This selectivity of monitor peptide for CCK cells suggests that the major physiologic action of monitor peptide in the intestinal lumen is to stimulate CCK secretion.

The ability to highly enrich CCK cells from other cells of the enteric mucosa was dependent upon identifying a secretagogue specific for the CCK cell. Although monitor peptide is the first luminal factor selective for stimulating CCK release to be purified and sequenced, to our knowledge, other CCK releasing factors appear to exist (13, 14, 31). Furthermore, the relative physiologic importance of each type of releasing factor has yet to be determined. Characterization of specific releasing factors could be facilitated by the use of a pure population of responsive CCK cells.

Other approaches to studying CCK secretion in vitro have included enriching CCK cells by counterflow elutriation (20) and perfusion of isolated intestinal segments (18, 19). Barber et al. (20) performed the first studies on isolated canine CCK cells, which were enriched ~20-fold. Elevated extracellular potassium, dibutyryl cAMP, forskolin, and tryptophan were all shown to stimulate CCK release. The present data using a preparation of CCK cells enriched >100-fold support the findings that membrane depolarization with high concentrations of KCl stimulate CCK release. Furthermore, cAMP also appears to function as a second messenger pathway mediating CCK secretion since dibutyryl cAMP stimulated secretion from highly enriched CCK cells. Our studies with calcium ionophore have extended earlier observations by invoking intracellular calcium as an important intracellular regulator of CCK secretion. These findings raise the possibility that an increase in $[\text{Ca}^{2+}]_i$ occurs by activation of the inositol phospholipid cascade through a membrane-receptor-initiated event (32, 33). Additional studies on purified CCK cells should clarify the precise steps involved in CCK release.

Cuber et al. (18) have recently utilized an isolated vascu-
larly perfused duodenojejunum from rat to study CCK se-
cretion. Intraluminal administration of monitor peptide was found to stimulate CCK release (19). A theoretical advantage of the intestinal segment model is that cells maintain their orientation to the luminal surface and potential secretagogues can be administered either intravascularly or on the luminal surface. These studies indicated that monitor peptide was acting on the luminal surface of the CCK cell to stimulate release.
There are several advantages to studying a highly enriched population of hormone-secreting cells instead of a mixture of different cell types. The major advantage is that direct effects of secretagogues can be determined in a homogeneous population of CCK cells. In a mixed population of intestinal mucosal cells, it is extremely difficult to evaluate whether agents are acting directly on the CCK cell or indirectly on another cell type that in turn affects CCK secretion. In addition, an enriched population of CCK cells also provides a means for studying intracellular second messenger systems. By being able to measure enzyme and protein products that are activated or generated during secretagogue stimulation, this preparation of cells should be useful for studying stimulus-secretion coupling.

The ability of monitor peptide to stimulate CCK release from isolated CCK cells indicates that it is acting directly on the CCK cell. Because monitor peptide increases [Ca\(^{2+}\)], it is likely that monitor peptide binds to membrane receptors. In animals, monitor peptide is found in pancreatic juice and is secreted into the lumen of the small intestine. Therefore, for monitor peptide to regulate CCK release in vivo, it is likely to interact with specific receptors on the luminal surface of the CCK cell. Further studies using autoradiographic techniques will be required to definitively address this issue.

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