Protein hydrolysate-induced cholecystokinin secretion from enteroendocrine cells is indirectly mediated by the intestinal oligopeptide transporter PepT1

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Liou AP, Chavez DI, Espero E, Hao S, Wank SA, Raybould HE. Protein hydrolysate-induced cholecystokinin secretion from enteroendocrine cells is indirectly mediated by the intestinal oligopeptide transporter PepT1. Am J Physiol Gastrointest Liver Physiol 300: G895–G902, 2011. First published February 10, 2011; doi:10.1152/ajpgi.00521.2010.—Dietary protein is a major stimulant for cholecystokinin (CCK) secretion by the intestinal I cell, however, the mechanism by which protein is detected is unknown. Indirect functional evidence suggests that PepT1 may play a role in CCK-mediated changes in gastric motor function. However, it is unclear whether this oligopeptide transporter directly or indirectly activates the I cell. Using both the CCK-expressing enteroendocrine STC-1 cell and acutely isolated native I cells from CCK-enhanced green fluorescent protein (eGFP) mice, we aimed to determine whether PepT1 directly activates the enteroendocrine cell to elicit CCK secretion in response to oligopeptides. Both STC-1 cells and isolated CCK-eGFP cells expressed PepT1 transcripts. STC-1 cells were activated, as measured by ERK1/2 phosphorylation, by both peptone and the PepT1 substrate Cefaclor; however, the PepT1 inhibitor 4-aminomethyl benzoic acid (AMBA) had no effect on STC-1 cell activity. The PepT1-transportable substrate glycyl-sarcosine dose-dependently decreased gastric motility in anesthetized rats but had no affect on activation of STC-1 cells or on CCK secretion by CCK-eGFP cells. CCK secretion was significantly increased in response to peptone but not to Cefaclor, cephalaxin, or Phe-Ala in CCK-eGFP cells. Taken together, the data suggest that PepT1 does not directly mediate CCK secretion in response to PepT1 specific substrates. PepT1, instead, may have an indirect role in protein sensing in the intestine.

aromatic amino acids; enteroendocrine; peptide transporter

CHOLECYSTOKININ (CCK), a gastrointestinal peptide secreted by enteroendocrine “I” cells in the proximal small intestine, mediates gastrointestinal feedback and satiety in response to luminal nutrients. CCK is released in response to dietary protein, particularly protein hydrolysates, and stimulates gall-bladder contraction (18), pancreatic enzyme secretion (14, 21), and activation of the CCK1 receptor-mediated vagal afferent pathway to inhibit gastric motility and emptying (7, 9, 32, 43) and reduce food intake (1, 34). Although neural pathways and functional responses to protein detection by the gut wall are well established, it is unclear precisely how protein hydrolysates are detected by the intestinal I cell to stimulate CCK secretion.

Recently, there has been increasing evidence that luminal nutrients can be directly detected by enteroendocrine cells. G-protein-coupled receptors have been implicated in fatty acid sensing (15, 22, 36), glucose sensing (19, 30, 33, 38, 42), and protein/amino acid sensing (3–4). Interestingly, intestinal transporters may also provide a nutrient-sensing role in the enteroendocrine cell. The sodium-coupled glucose cotransporter SGLT1 (26, 30, 33) and its related protein SGLT3 (10) are increasingly being acknowledged for their role in luminal glucose sensing by enteroendocrine cells. However, the precise mechanism for protein detection by gut enteroendocrine cells remains unclear.

Given that glucose sensing can occur via an intestinal transporter protein, we were interested in determining whether a similar proton–coupled oligopeptide transporter, PepT1 (8, 25), could function as a protein-sensing mechanism for intestinal I cells. PepT1 is primarily expressed in terminally differentiated small intestinal absorptive epithelial cells (29) and functions as the major conduit for protein absorption in the intestine. Along with a broad array of dipeptides and tripeptides, PepT1 also transports a variety of peptidomimetic drugs such as β-lactam antibiotics, antihypertensive agents (angiotensin-converting enzyme inhibitors), and statins (35). Cefaclor, a β-lactam antibiotic, delays gastric emptying through CCK1 receptors in rats (2). Additionally, vagal afferent activation in response to both protein hydrolysates and Cefaclor requires functional PepT1-transporter activity (6), suggesting that PepT1 may also serve as a protein sensor mediating CCK secretion from the I cell in response to dietary protein.

Whether PepT1 acts indirectly or directly on the intestinal I cell is unknown; however, evidence in the murine CCK-secreting pancreatic tumor cell line STC-1 suggests that a direct mechanism exists. Protein hydrolysates and cephalosporin drugs activate cAMP and MAP kinase (ERK1/2) pathways and elevate intracellular calcium concentrations, resulting in an upregulation of CCK gene transcription and CCK secretion, respectively (5, 12, 28). PepT1 may be responsible for membrane depolarization, increased intracellular calcium flux, and hormone secretion in response to direct dipeptide stimulation in the STC-1 cell (23).

Here, we tested the hypothesis that PepT1 directly mediates CCK secretion in response to protein hydrolysates through examination of the role of this oligopeptide transporter in both the activation of the STC-1 cell line and in hormone secretion from acutely isolated intestinal I cells in response to protein hydrolysates, dipeptides, and cephalosporin antibiotics.

MATERIALS AND METHODS

Materials

Unless otherwise stated, all chemicals and drugs were obtained from Sigma Chemicals (St. Louis, MO). BD BactoTryptone was used for bacterial growth and CLONTECH eGFP reporter mice were used for in vivo studies. N-Ethylmaleimide (NEM) and 4-aminomethyl benzoic acid (AMBA) were obtained from Sigma Chemicals (St. Louis, MO). Anti-ERK1/2, anti-p-ERK1/2, and anti-CCK antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA). Anti-NHE1 and anti-NHE3 antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-CCK1 and anti-CCK2 antibodies were purchased from Enzo Life Sciences (Farmington, CT). Anti-ATP synthase antibodies were purchased from BD Biosciences (San Jose, CA). All other chemicals and drugs were obtained from Sigma Chemicals (St. Louis, MO) unless otherwise stated.
purchased from BD Biosciences (San Jose, CA). 4-Aminomethylbenzoic acid (4-AMBA) is a competitive nontranslocated inhibitor of PepT1 with a Michaelis constant of ~3 mM (24). 4-Aminophenylalanine (4-APAA) is an inactive analog that is translocated by PepT1 (37). Cefaclor (1 mM) and glycyrl-sarcosine (Gly-Sar, 10 mM) are known PepT1 substrates (37). β-Ala-Lys-Ne-AMCA labeled peptide, a known PepT1-transportable substrate used to measure peptide uptake into the cell cytosol (13), was synthesized by J. R. Reeve [University of California Los Angeles (UCLA), School of Medicine].

Animals

Transgenic mice with cholecystokinin promoter-driven enhanced green fluorescent protein (CCK-eGFP) were obtained from the Mutant Mouse Regional Resource Center (University of California, Davis), bred and maintained on regular chow, and used under protocols approved by the National Institutes of Health Institutional Animal Care and Use Committee guidelines. Sprague Dawley male rats weighing 200 –300 g were maintained and used in compliance with protocols approved by the University of California, Davis Institutional Animal Use and Care Committee.

Cell Culture Conditions and Maintenance

STC-1 cells, Caco-2 cells, and BON cells were donated by Doug Hanahan (University of California, San Francisco), Susan Kelleher (Department of Nutrition, University of California, Davis), and C. M. Townsend Jr. (University of Texas, Galveston, TX), respectively, and maintained in a humidified incubator (37°C, 5% CO2). Following incubation, cover slips were washed two times with Earl’s balanced salt solution (EBSS), followed by a 15-min equilibration with a pH-adjusted EBSS (pH 6.0). After preincubation, buffer was removed, and a 500-μl volume of either 0, 5, or 50 μM AMCA-labeled peptide was added and incubated for 20 min at 37°C, 5% CO2. Following incubation, cover slips were washed two times with ice-cold EBSS and fixed for 20 min in fresh cold 4% paraformaldehyde in PBS (PFA-PBS) before mounting on slides. Images were taken with the Provis microscope. Cells that were easily defined were counted across three to five different slides. Clumps of cells that could not be singly differentiated were excluded from counts. Images were analyzed using Scion Image Analysis software (version beta 4.02) by encircling an area of interest drawn around individual whole cells and their nuclei. Total and illuminated (marked) pixels were counted within each area of interest to calculate the cytoplasmic pixel count = [(total pixels – marked pixels)/total pixels]. Data were expressed as the percent marked pixels relative to total pixels. Data were analyzed using a one-way ANOVA with post hoc test. Significance was accepted at P < 0.05.

Activation of the ERK1/2 Phosphorylation Pathway

Response to peptone, cefaclor, and Gly-Sar. STC-1 cells were exposed to increasing concentrations (0–10%) of milk or soy peptone (n = 6), a single concentration of 1 mM Cefaclor (n = 7), or 10 mM Gly-Sar (n = 6) for 2 min. PepT1 inhibition studies. To determine whether PepT1 is required for neuroendocrine cell activation in response to peptone, STC-1 cells (n = 3–5) were preincubated for 5 min in either EBSS or varying concentrations (50 μM, 0.5 mM, or 5 mM) of 4-AMBA or 4-APAA, followed by a 2-min treatment of either 1% soy peptone, 5% milk peptone, or 1 mM Cefaclor.

Protein Extraction, SDS-PAGE, and Western Blot

Protein was extracted from STC-1 cells by scraping into Tris-EDTA suspension buffer [15 mM Tris-Cl, 1.5 mM EDTA in double-distilled H2O (ddH2O), pH 8.0], supplemented with 1% protease inhibitor cocktail (Sigma) and 1% Triton X-100. Cells were homogenized by running samples through a 25-gauge needle two to six times, sonicated, and centrifuged (10,000 g, 15 min, 4°C). Protein was quantified with a DC Protein Assay (Bio-Rad, Hercules, CA) and underwent SDS-PAGE and transfer onto a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated overnight with anti-p44/p42 phosphorylated MAP kinase [phosphorylated ERK1/2 (pERK1/2), 1:1,000; Cell Signalling, Danvers, MA] in 5% BSA-TBST, followed by appropriate horseradish peroxidase-linked secondary antibodies (1 h, room temperature). The desired protein bands of 44 and 42 kDa were semiquantitatively measured by densitometric analysis.
To obtain total ERK₁/₂ protein concentrations, the PVDF membranes were stripped (7.6 g Tris base, 20 g SDS, 7 ml 2-mercaptoethanol in 1-liter final volume of ddH₂O, pH 6.8), reblocked with 5% milk-TBST, and incubated with anti-p44/p42 MAP kinase (ERK₁/₂) primary antibody (1:1,000; Cell Signaling) using the same protocol as the phosphorylated antibody above to obtain total ERK₁/₂. Phosphorylation activity was expressed as a ratio of pERK₁/₂ to total ERK₁/₂ and normalized to the EBSS control, such that the result was a fold change from no treatment, arbitrarily set to one.

**Pept1 Immunohistochemistry**

For frozen tissue sections, anesthetized rats were intracardially perfused with fresh cold 4% PFA-PBS. The duodenum was collected and fixed in 4% PFA-PBS for an additional 2 h and stored in 25% sucrose solution with 0.01% sodium azide until cryosectioned. After being blocked in 5% donkey serum, slides were incubated overnight in goat anti-Pept1 (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-CK/gastrin (1:1,000; UCLA CURE no. 9303) primary antibodies, followed by incubation with donkey anti-goat IgG AlexaFluor 488 secondary antibody and donkey anti-mouse IgG AlexaFluor 568 (1:500; Invitrogen). Slides were cover slipped and examined under confocal microscopy.

For immunostaining of partially digested duodenal mucosa of CCK-eGFP BAC transgenic mice, intestinal mucosa was incubated for 5 min in 2.5 mM EDTA-PBS, followed by collagenase (1 mg/ml; 10 min) digestion for 10 min. Cells were collected, filtered in a 40-μm cell strainer (BD Biosciences), and resuspended in a solution of 4% PFA-PBS, 0.1% saponin, and 1% BSA for 5 min. Cells were washed in PBS, blocked with 5% BSA-0.1% saponin in PBS (30 min), incubated with anti-Pept1 antibody (Abcam, Cambridge, MA) at a 1:50, 1:100, or 1:200 dilution overnight at 4°C, and subsequently incubated with an appropriate AlexaFluor 594 secondary antibody (1:500; Invitrogen). Slides were cover slipped and examined under epifluorescent microscopy.

**Measurement of Gastric Motility**

Gastric motility was assessed by manometry in urethane-anesthetized rats as previously described (31). Intragastric pressure (IGP) waves were measured in response to duodenal perfusion (0.05 mL/min for 10 min) of an increasing dose of Gly-Sar, a hydrolysis-resistant dipeptide and known Pept1-transportable substrate (11), followed by 8% meat peptone. A 10-min saline flush was given between each treatment. The phasic IGP, or amplitude of the contraction waves, was measured as the mean decrease in height over 2 min of perfusion treatment. The baseline, or tonic, IGP was taken as the average nadir of the trace over the same time period compared with a saline baseline. Differences between the Gly-Sar dose response were compared using a one-way ANOVA and post hoc Dunnett’s test against the saline control. Significance was accepted at P < 0.05.

**Data Analysis and Statistics**

All values are expressed as means ± SE, unless otherwise stated. Significance for Pept1 gene expression among the cell lines and between CCK-eGFP and non-eGFP cells was determined using a one-way ANOVA and a Student’s t-test of the ΔCT values, respectively. ERK₁/₂ signaling responses were normalized to no treatment (EBSS) and represented as a fold change from 1.0. Significance among the treatments was determined using a one-way ANOVA and post hoc Dunnett’s test.

For hormone secretion studies in CCK-eGFP cells, values were expressed as the percent change from baseline secretion, and significance was determined using a one-way ANOVA and appropriate post hoc test. Statistical analysis was performed using GraphPad Prism version 5. Significance was accepted at P < 0.05.

**RESULTS**

**STC-1 Cells Functionally Express Pept1**

To determine whether STC-1 cells have the potential to express functional Pept1, quantitative RT-PCR was performed. Pept1 mRNA expression was compared between the murine CCK-secreting STC-1 cell line (n = 3), the human serotonin-secreting BON cell line (n = 3), and the human intestinal epithelial Caco-2 cell line (n = 4) relative to GAPDH. STC-1 cells expressed less Pept1 transcript than the Caco-2 cells and at least 220-fold more Pept1 transcript level than the BON cell, which was rarely detectable (Fig. 1A).

To demonstrate functional evidence for Pept1 in the STC-1 cell line, the ability to transport AMCA-labeled peptide (13) was determined by analyzing fluorescent peptide uptake under epifluorescent microscopy and quantifying the percent of illuminated pixels from total pixels within the cell cytoplasm. A dose-dependent increase in cytoplasmic AMCA-labeled peptide was observed, starting from 14 ± 1.6% pixels (n = 21) at 0 μM AMCA, 19 ± 4.6% pixels (n = 9) at 5 μM AMCA, and 25 ± 3.8% pixels (n = 12) at 50 μM AMCA, with a significant pixel count compared with baseline (P < 0.05; Fig. 1, B–D).

**Phosphorylation Activity of the ERK₁/₂ Pathway in STC-1 Cells in Response to Peptone and Cefaclor**

To ensure that STC-1 cells behave similarly to other reported studies, the activation of STC-1 cells was measured in response to peptone and the cephalosporin antibiotic Cefaclor. Activation was measured as the ratio of pERK₁/₂ (pERK₁/₂/ERK₁/₂) in response to treatment, normalized to the pERK₁/₂/ERK₁/₂ ratio of EBSS alone (control). STC-1 cells demonstrated dose-responsive activation to both milk and soy peptone (Fig. 2, A and B). Based on the dose-response curves, the 1% soy peptone and 5% milk peptone dosages were used for further studies. Cefaclor significantly increased the pERK/ERK ratio by ~68 ± 14% compared with EBSS alone (n = 7; P = 0.001; Fig. 2C), similar to that observed by others (27).
PepT1 Antagonist 4-AMBA Does Not Significantly Attenuate ERK1/2 Phosphorylation in Response to Peptone and Cefaclor

To determine whether PepT1 was required for STC-1 cell activation in response to milk peptone, soy peptone, and Cefaclor, the effect of the commercially available PepT1 inhibitor 4-AMBA on ERK1/2 phosphorylation activation was tested and compared with its inactive analog 4-APAA. A dose response with 50 μM, 0.5 mM, and 5 mM 4-AMBA with all three substrates was performed, but the effect of 5 mM 4-AMBA against peptone-induced ERK1/2 activation was most consistent. Alone, 4-AMBA and 4-APAA had no endogenous effect on ERK1/2 activation. 4-AMBA or 4-APAA had no effect on pERK1/2 in response to milk peptone, soy peptone, and Cefaclor (Table 1 and Fig. 2D).

Gly-Sar Inhibits Gastric Motility But Does Not Directly Activate CCK-Secreting Enteroendocrine Cells

Duodenal perfusion of specific PepT1-translocatable synthetic dipeptide, Gly-Sar, caused a dose-dependent inhibition of gastric motility in anesthetized rats, primarily for phasic IGP, similar to that observed with 8% peptone (Fig. 3). To test whether Gly-Sar could directly activate enteroendocrine cell signaling pathways and hormone secretion, STC-1 cells underwent a 2-min exposure to Gly-Sar, which was ineffective at increasing pERK1/2 activity. Additionally, hormone
secretion from isolated CCK-eGFP cells \((n = 8)\) was not different from baseline in response to Gly-Sar (see Fig. 5). Together, these findings suggest that Gly-Sar may be inhibiting gastric motility via PepT1 indirectly and not by direct activation of CCK-secreting enteroendocrine cells.

**Endogenous PepT1 Expressed in Acutely Isolated Native Murine CCK-eGFP Cells Does Not Mediate CCK Secretion in Response to PepT1 Specific Ligands**

Isolated CCK-eGFP cells express PepT1 at a gene and protein level similar to that of the sorted non-eGFP cell population (Fig. 4, A and B), which supports immunohistochemical staining of PepT1 closely aligned to CCK-immunoreactive cells in rat frozen tissue sections (Fig. 4C). In the absence of primary antibodies, no nonspecific immunofluorescent staining was apparent (data not shown).

To evaluate hormone secretion activity in response to PepT1-transportable substrates, acutely isolated CCK-eGFP cells were exposed to various PepT1 substrates such as increasing concentrations of the dipeptide Phe-Ala, the hydrolysistransportable I/transportable substrate in transfected cells (40), did not significantly increase CCK secretion in acutely isolated murine I cells \((n = 3–4, \text{ data not shown})\). Neither were Cefaclor and cephalaxin effective at increasing CCK secretion compared with baseline (Fig. 5). The enzymatic casein digest tryptone evoked a more consistently robust increase in CCK secretion compared with baseline \((P < 0.05)\). A similar response was observed with a meat peptone source (data not shown).

**DISCUSSION**

In the present study, we investigated the role of the oligopeptide transporter PepT1 as a putative protein hydrolysates and cephalosporin antibiotics are known to activate STC-1 cells and elicit CCK secretion (27–28). We demonstrated similar activation in our cells by measuring ERK1/2 phosphorylation activity in response to the same substrates. However, although the PepT1 inhibitor 4-AMBA significantly reduced the effect of peptone

**Table 1. Activation of ERK1/2 in response to peptones or Cefaclor in the presence of absence of either the PepT1 competitive inhibitor 4-AMBA (5 mM) or its inactive analog 4-APAA (5 mM)**

<table>
<thead>
<tr>
<th>Inhibitor or Inactive Analog</th>
<th>None</th>
<th>4-AMBA</th>
<th>4-APAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBSS</td>
<td>1.0 ± 0.00</td>
<td>1.0 ± 0.06</td>
<td>1.1 ± 0.10</td>
</tr>
<tr>
<td>Soy peptone (1%)</td>
<td>1.6 ± 0.18*</td>
<td>2.5 ± 0.44**</td>
<td></td>
</tr>
<tr>
<td>Milk peptone (5%)</td>
<td>2.1 ± 0.58*</td>
<td>2.7 ± 0.87</td>
<td></td>
</tr>
<tr>
<td>Cefaclor (1 mM)</td>
<td>1.2 ± 0.12*</td>
<td>1.3 ± 0.42</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE and are represented as a mean fold change over baseline \((1.0, \text{ solid line})\); \(n = 5\) separate passages performed in triplicate. PepT1, proton-coupled oligopeptide transporter; 4-AMBA, 4-aminomethyl benzoic acid; 4-APAA, 4-aminophenylacetic acid. *\(P < 0.05\), **\(P < 0.01\), and ***\(P < 0.001\) compared with Earl’s balanced salt solution (EBSS).

**Fig. 3. The effect of PepT1-transportable peptide glycyl-sarcosine (Gly-Sar) on gastric motility and STC-1 cell activity. Duodenal perfusion with Gly-Sar dose dependently inhibits intraluminal gastric pressure changes in the anesthetized rat. 8% peptone served as a positive control. Values are means ± SE; \(n = 5–11\) experiments per group. *\(P < 0.05\) compared with saline.**
on vagal afferent activation and gastric motility (6), it did not
significantly reduce ERK1/2 phosphorylation in the STC-1 cell
in response to peptone and Cefaclor. Furthermore, the PepT1
specific substrates had no effect on activation of STC-1 cells or
eliciting CCK secretion from isolated CCK-eGFP cells, even
though PepT1 specific dipeptide Gly-Sar had a significant
effect on inhibiting gastric motility. Taken together, the data
suggest that PepT1 is not directly involved in mediating
enteroendocrine cell activation in response to peptone,
which contradicts what has been reported by others (23).
There remains the possibility, however, that PepT1 plays an
indirect role in activating CCK1 receptor-mediated vagal
afferent signaling and its downstream effects on gastric
motor function.

Given that protein hydrolysates consistently activate and
elicit hormone secretion from STC-1 cells and native CCK-
eGFP cells, it is apparent that another protein sensor is
expressed by these cells. The G protein-coupled receptor
GPR93, which also utilizes the ERK1/2 pathways, has been
implicated in peptone sensing by the STC-1 cell (3).
Whether GPR93 or another, still unidentified protein sensor
is functionally expressed by the enteroendocrine I cell
remains to be elucidated.

Protein detection by the intestinal I cell likely incorporates
both indirect and direct detection mechanisms. Although
PepT1 is expressed by CCK-secreting enteroendocrine cells,
the evidence does not support its role in directly mediating
protein hydrolysate-induced hormone secretion. Given its in-
fluence on gastric motility, perhaps the transport of peptides
via PepT1 by the enterocyte may initiate secretion of signaling
factors that stimulate the I cell to secrete CCK. This signaling
factor may be diazepam-binding inhibitor (DBI), a CCK-
releasing peptide originally isolated in the rat intestinal mucosa
that has been demonstrated to elicit pancreatic secretion and
elevate plasma CCK levels in the rat (20) and elicit CCK
secretion by the STC-1 cell (44). It would be interesting to test
whether a connection exists between PepT1-transporter activity
and DBI secretion by the intestinal mucosa in CCK secretion.

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(National Institute of Diabetes and Digestive and Kidney Diseases) for tech-
nical assistance.
10. Interest in protein, amino acids, and health. However, she was only involved in DISCLOSURES Foundation, and the Dairy Marketing Initiative.

DISCLOSURES

Dr. S. Hao is currently working for Ajinomoto, a company that has an interest in protein, amino acids, and health. However, she was only involved in this project when she was employed by UC Davis.

REFERENCES


