Effect of five taste ligands on the release of CCK from an enteroendocrine cell line, STC-1

Mutsuki MIYATA, Mako KUROGI, Mai ODA, and Osamu SAITO
Department of Animal Bioscience, Faculty of Bio-Science, Nagahama Institute of Bio-Science and Technology, 1266 Tamura-cho, Nagahama-shi, Shiga 526-0829, Japan

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ABSTRACT
Here, we investigated which taste ligand induces the CCK (cholecystokinin) release from intestinal STC-1 cells. We first developed a new assay to measure the release of CCK. The expression vector for CCK type A receptor (CCKAR) was permanently introduced into HEK293T cells and a cell line was established (CCKAR/HEK). Then, STC-1 cells were treated with taste ligands and the incubated buffer of STC-1 cells containing released CCK was applied to CCKAR/HEK cells. Since CCKAR couples to Gq-signaling, the CCK-induced receptor activation can be monitored by the method of Ca\(^{2+}\)-imaging. Therefore, when CCK is released from STC-1 cells to culture medium with taste stimulation, Ca\(^{2+}\) activation of CCKAR/HEK should be observed. Among five different taste ligands (saccharin, Na-glutamate, NaCl, denatonium benzoate, HCl), only denatonium benzoate and HCl induced the release of CCK in STC-1 cells. Thus, we found that only specific taste ligands induce the CCK release, and that other three taste ligands cannot induce the release of CCK despite of their ability to elevate the intracellular Ca\(^{2+}\) level in STC-1 cells.

In animals, the gastrointestinal tracts have many important functions, including secretion, digestion, motility and absorption. These functions are initiated and regulated by molecular sensing of luminal contents such as nutrient and non-nutrient chemicals. The enteroendocrine cells are thought to function as a specialized transducer receiving luminal factors. STC-1 cells have been known as an established cell line of enteroendocrine cells of mouse small intestine (16). Later, it was reported that STC-1 cells express many T2R receptors responding to bitter taste substances (19). We also characterized how STC-1 cells respond to bitter tastants (13). Then, we further investigated the cellular responses of intestinal STC-1 cells to compounds of five basic tastants using a calcium-imaging technique. In addition to bitter tastants, compounds of four other basic tastants also stimulated STC-1 cells. Therefore, it was suggested that the gastrointestinal system can sense all five of the basic taste stimuli, and that it might contain a taste receptor signaling system similar to the oral taste system (17). The expression of T1R taste receptors in mouse enteroendocrine cells has also been reported by Dyer et al. (6) and Margolskee et al. (12). Further, we recently found that the STC-1 cells can respond to the astringent compound of green tea, EGCG. When EGCG was applied to STC-1 cells, a significant increase in the intracellular Ca\(^{2+}\) concentration occurred. Mouse transient receptor potential ankyrin 1 (TRPA1) was identified as a sensor molecule for EGCG in STC-1 cells (11). Thus, this intestinal STC-1 cell can detect many taste stimulation.

STC-1 cells produce and release cholecystokinin (CCK), glucose-dependent insulinotropic polypeptide (GIP), secretin, glucagon-like peptide-1 (GLP-1), and serotonin (1–4, 9, 14). It has been reported
that the release of CCK from STC-1 cells is stimulated by dibutyryl cAMP, forskolin, KCl, phenylalanine, sodium oleate, bitter tastants, and allyl isothiocyanate (AITC) (1–3, 8, 15). It has been considered that the increase of the intracellular Ca²⁺ level and the resultant Ca²⁺-induced signaling are required to release CCK in STC-1 cells. Do all of taste stimulations that elevate Ca²⁺ level activate the release of CCK in these intestinal cells? CCK is gastrointestinal hormone which stimulates pancreatic exocrine secretion and gallbladder contraction, inhibits gastric emptying and acid secretion, and suppresses food intake. Enteroeocrine cells might have to discriminate between nutritive compounds and potentially harmful substances and show different responses.

To investigate which taste stimulation induces the CCK release from intestinal STC-1 cells, we developed a new assay to measure the release of CCK. Mouse CCK type A receptor (CCKAR) cDNA (1313 bp) was isolated using total RNA from mouse stomach and cloned into pcDNA3.1/Hygro(-)-3 × HA (generously provided by Dr. M. Itoh) to produce a HA-tagged protein. The expression vector for HA-tagged CCKAR was permanently introduced into HEK293T cells and a cell line was established (CCKAR/HEK). Almost all cells of CCKAR/HEK were positively stained with anti-HA antibody (Fig. 1A). It is known that CCKAR couples to Gq-signaling (18). When CCK was applied to CCKAR/HEK cells, the increase of intracellular Ca²⁺ level was easily detected with the Ca²⁺-imaging technique. The Ca²⁺-imaging analysis was performed as previously described (17). As shown in Fig. 1B, this cellular response was apparently dose-dependent and the Ca²⁺ elevation was observed from 0.5 nM CCK. The detection limit was 0.25–0.5 nM (Fig. 1B). Treatment of control HEK cells with CCK (0.1–100 nM) did not induce the increase in intracellular Ca²⁺ (data not shown). This procedure of Ca²⁺-imaging using CCKAR/HEK cells was utilized to detect the CCK release from intestinal STC-1 cells.

Stimulation with sweet, umami, or salty compound does not induce the CCK release from STC-1 cells. It was previously reported that STC-1 cells can respond to five basic tastants by using the Ca²⁺-imaging technique (17). First, STC-1 cells were treated with each of three taste ligands of saccharin, Glu-Na (Na-glutamate), and NaCl for 1 h and the incubated buffer (Hank’s balanced salt solution, HBSS) of STC-1 cells was applied to CCKAR/HEK cells. When CCK is released from STC-1 cells to culture medium with taste stimulation, Ca²⁺ activation of CCKAR/HEK should be observed. When monitoring Ca²⁺ level of STC-1 cells, it was evident that these cells are activated by three taste ligands of saccharin, Glu-Na, and NaCl. The CCK release, however, was not observed in each case, since none of the culture medium obtained after stimulation with each of three taste ligands could activate CCKAR/HEK cells (Fig. 2). Saccharin, Glu-Na, and NaCl had no direct effect on CCKAR/HEK cells (data not shown).

Stimulation with bitter or sour compound induces the CCK release from STC-1 cells. Next, we examined effect of other two taste ligands out of five basic tastes on the release of CCK from STC-1 cells. STC-1 cells were treated with denatonium benzoate (DB, bitter compound) or HCl for 1 h, and
then the incubated buffer of STC-1 cells was applied to CCKAR/HEK cells. By monitoring Ca²⁺ level in STC-1 cells, it was apparent that the treatment with either of DB or HCl activates these cells in a dose-dependent manner. Furthermore, in either case, the stimulation induced the CCK release from STC-1 cells. The CCK release was observed from 10 mM DB or 8 mM HCl. We measured the pH of HBSS containing 4–10 mM HCl. The results were as follows: HBSS with 4 mM HCl, pH 6.4; HBSS with 6 mM HCl, pH 3.2; HBSS with 8 mM HCl, pH 2.8; HBSS with 10 mM HCl, pH 2.6. The CCK release by sour compound seems to be induced, when the pH is below 3. We confirmed that DB and HCl had no direct effects on CCKAR/HEK cells (data not shown). For rough estimation of the content of CCK, the incubated buffer of STC-1 cells after stimulation with 30 mM DB (most effective ligand concentration) was diluted from one to twenty times (∗1, ∗5, ∗10, ∗15, ∗20) and the diluted buffer was applied to CCKAR/HEK cells. The buffer activated CCKAR/HEK even after fifteen times dilution (∗15), suggesting that the buffer of STC-1 cells after stimulation with 30 mM DB contains 3.75–7.5 nM CCK (Fig. 3A). In the case of the sour stimulation with 8 mM HCl (most effective ligand concentration), the buffer activated CCKAR/HEK even after forty times dilution (∗40). Therefore, it was considered that STC-1 cells release 10–20 nM CCK into culture medium by stimulation with 8 mM HCl (Fig. 3B). The treatment with sour compound quickly induces the CCK release from STC-1 cells, but bitter compound-induced release of CCK is relatively slow. Although each of five different ligands induces the elevation of intracellular Ca²⁺ levels of STC-1 cells,
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the incubated buffer of STC-1 cells was applied to CCKAR/HEK cells, respectively. The CCK release was already detected for the HCl stimulation for 5 min, but the DB treatment could not induce the release of CCK within 5 min. STC-1 cells were further treated with 30 mM DB for 10 min, 15 min, and 20 min. The significant CCK release by DB was only two reagents of DB and HCl induced the release of CCK. Do these two stimulations similarly activate the secretion process of CCK from vesicles present in STC-1 cells? We analyzed and compared the time to secrete CCK after stimulation. STC-1 cells were stimulated for 5 min at room temperature with 30 mM DB or 8 mM HCl, and then each of the incubated buffer of STC-1 cells was applied to CCKAR/HEK cells, respectively. The CCK release was already detected for the HCl stimulation for 5 min, but the DB treatment could not induce the release of CCK within 5 min. STC-1 cells were further treated with 30 mM DB for 10 min, 15 min, and 20 min. The significant CCK release by DB was

Fig. 3 Denatonium or HCl induces the CCK release from STC-1 cells. Ca^{2+}-imaging analysis of STC-1 and CCKAR/HEK cells was performed as Fig. 2. A, STC-1 cells were preloaded with Fluo8-AM, and stimulated with denatonium benzoate (DB). The $\Delta F/F$ is shown (left). To detect the CCK release, STC-1 cells were treated with DB for 1 h and the incubated buffer was applied to CCKAR/HEK preloaded with Fluo8-AM. The $\Delta F/F$ is shown (upper right). To estimate the content of CCK, the incubated buffer of STC-1 cells after stimulation with 30 mM DB was diluted from one to twenty times (×1, 5, 10, 15, 20) and the diluted buffer was applied to Fluo8-AM-preloaded CCKAR/HEK cells. The $\Delta F/F$ was determined (lower right). B, STC-1 cells preloaded with Fluo8-AM were activated with HCl. The $\Delta F/F$ was determined (left). To examine the CCK release, STC-1 cells were treated with HCl for 1 h and the incubated buffer was applied to Fluo8-AM-preloaded CCKAR/HEK cells. The $\Delta F/F$ is indicated (upper right). To estimate the CCK content, the incubated buffer of STC-1 cells after stimulation with 8 mM HCl was diluted (×1, 10, 20, 30, 40, 50) and the diluted buffer was applied to CCKAR/HEK cells preloaded with Fluo8-AM. The $\Delta F/F$ was determined (lower right). Arrows indicated the time points of application of ligands or the incubated buffer.
first detected after incubation of 15 min (Fig. 4). Thus, the treatment of STC-1 cells with HCl induces the quick release of CCK within 5 min, but the denatonium-dependent release of CCK requires more than 10 min.

Here, we investigated which taste stimulation induces the CCK release from intestinal STC-1 cells by a new assay system using CCKAR/HEK cells. STC-1 cells were treated with five taste ligands and the incubated buffer of STC-1 cells containing CCK was applied to CCKAR/HEK cells. We could analyze the CCK-release by examining whether the culture medium of STC-1 cells stimulated with a taste ligand may induce the increase in intracellular Ca$^{2+}$ of CCKAR/HEK cells or not. Among five different taste ligand (saccharin, Glu-Na, NaCl, DB, HCl), only DB and HCl induced the release of CCK in STC-1 cells. Thus, we found that only specific taste ligands induce the CCK release, and that other tastants cannot induce the release of CCK despite of their ability to elevate the intracellular Ca$^{2+}$ level.

Further, by roughly comparing the releasing levels of CCK among stimulation of DB and HCl, we found that DB was less effective on inducing the CCK-release from STC-1 cells. We also investigated the time course of tastant-induced secretion of CCK from STC-1 cells. The quick secretion within 5 min was observed for the treatment with HCl, but the DB-induced release was significantly slow and required more than 10 min after stimulation.

Quite recently, Daly et al. reported that glutamate stimulates STC-1 cells to secrete CCK (5). They also demonstrated that the expression of T1R1 and T1R3 in STC-1 cells. However, in a previous report, we could not detect the expression of T1R1 in STC-1 cells by RT-PCR analysis, and demonstrated that NMDA (N-methyl-D-aspartate) receptors are involved in glutamate-induced response of STC-1 cells (7). By culture or passage condition, a part of properties of STC-1 cells might change. From their report and our observation, it is suggested that Ca$^{2+}$-signaling induced through T1R1/3 causes the CCK release, but that Ca$^{2+}$-elevation induced by activation of NMDA receptors might not be able to trigger the release of CCK.

Why can only two ligands of DB and HCl induce the release of CCK in STC-1 cells? DB is reported to activate G protein-coupled system through T2R family in STC-1 cells (13, 19). It has been demonstrated that the Ca$^{2+}$ influx is required for the denatonium-dependent CCK release from STC-1 (3). The response of STC-1 cells to HCl, however, does not require the extracellular Ca$^{2+}$. Since thapsigargin, an agent that depletes endoplasmic reticulum Ca$^{2+}$, blocks the HCl-mediated response, the mobilization of Ca$^{2+}$ from intracellular stores plays crucial roles in the HCl-dependent CCK release from STC-1 (data not shown). Thus, two ligands activate distinct pathways involving Ca$^{2+}$, and it is not considered that some specific Ca$^{2+}$-signaling pathway may cause the CCK-release from STC-1 cells. Since sweet tastant is known to activate the secretion of GIP (10, 12) but does not induce the CCK-release (described here) in STC-1 cells, it seems that GIP-containing and CCK-containing vesicles may be independently distributed in the cytoplasm of STC-1 cells. Therefore, it is possible that stimulation with DB or HCl might cause some specific local and high level accumulation of Ca$^{2+}$ near CCK-containing vesicles, and trigger the fusion of plasma membrane and vesicles to release their content. As another possibility, it might be possible that STC-1 cells are mixed cells of different properties. For example, one cell population is able to release CCK with responding to
two ligands (DB or HCl), and the other population is able to release GIP with stimulation by sweet tastants. We could not deny this possibility completely. We, however, observed that about 50–60% cells of STC-1 respond to each of five taste ligands, and that a single STC-1 cell can be activated by multiple taste ligands (data not shown). Therefore, it is considered that the mechanism discriminating the signals induced by individual taste stimulation must be present.

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