Am J Physiol Cell Physiol 291: C726–C739, 2006.
First published May 17, 2006; doi:10.1152/ajpcell.00003.2006.

Bitter stimuli induce Ca^{2+} signaling and CCK release in enteroendocrine STC-1 cells: role of L-type voltage-sensitive Ca^{2+} channels

Monica C. Chen, S. Vincent Wu, Joseph R. Reeve, Jr., and Enrique Rozengurt

Division of Digestive Diseases, Department of Medicine, CURE: Digestive Diseases Research Center and Molecular Biology Institute, David Geffen School of Medicine, University of California at Los Angeles

Submitted 5 January 2006; accepted in final form 9 May 2006

Chen, Monica C., S. Vincent Wu, Joseph R. Reeve, Jr., and Enrique Rozengurt. Bitter stimuli induce Ca^{2+} signaling and CCK release in enteroendocrine STC-1 cells: role of L-type voltage-sensitive Ca^{2+} channels. *Am J Physiol Cell Physiol* 291: C726–C739, 2006. First published May 17, 2006; doi:10.1152/ajpcell.00003.2006.—We previously demonstrated the expression of bitter taste receptors of the type 2 family (T2R) and the α-subunits of the G protein gustducin ($G\alpha_{gust}$) in the rodent gastrointestinal (GI) tract and in GI endocrine cells. In this study, we characterized mechanisms of Ca^{2+} fluxes induced by two distinct T2R ligands: denatonium benzoate (DB) and phenylthiocarbamide (PTC), in mouse enteroendocrine cell line STC-1. Both DB and PTC induced a marked increase in intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) in a dose- and time-dependent manner. Chelating extracellular Ca^{2+} with EGTA blocked the increase in $[Ca^{2+}]_i$ induced by either DB or PTC but, in contrast, did not prevent the effect induced by bombesin. Thapsigargin blocked the transient increase in $[Ca^{2+}]_i$ induced by bombesin, but did not attenuate the $[Ca^{2+}]_i$ increase elicited by DB or PTC. These results indicate that Ca^{2+} influx mediates the increase in $[Ca^{2+}]_i$ induced by DB and PTC in STC-1 cells. Preincubation with the L-type voltage-sensitive Ca^{2+} channel (L-type VSCC) blockers nitrendipine or diltiazem for 30 min inhibited the increase in $[Ca^{2+}]_i$ elicited by DB or PTC. Furthermore, exposure to the L-type VSCCs opener BAY K 8644 potentiated the increase in $[Ca^{2+}]_i$ induced by DB and PTC. Stimulation with DB also induced a marked increase in the release of cholecystokinin from STC-1 cells, an effect also abrogated by prior exposure to EGTA or L-type VSCC blockers. Collectively, our results demonstrate that bitter tastants increase $[Ca^{2+}]_i$ and cholecystokinin release through Ca^{2+} influx mediated by the opening of L-type VSCCs in enteroendocrine STC-1 cells.

type 2 family taste receptors; gastrointestinal peptides; phospholipase C β_2; Ca^{2+} fluxes; enteroendocrine cells; cholecystokinin secretion

THE GASTROINTESTINAL (GI) tract responds to a wide array of signals originating in the lumen, including nutrient and nonnutrient chemicals, mechanical factors, and microorganisms (3, 13, 19, 60). Molecular sensing by GI cells plays a critical role in the control of multiple fundamental functions, including digestion, food intake, and metabolic regulation. Although these fundamental control systems have been known for a considerable time, the initial molecular recognition events that sense the chemical composition of the luminal contents have remained poorly understood.

The gustatory system has been selected during evolution to detect nonvolatile nutritive and beneficial (sweet) compounds as well as potentially harmful (bitter) substances (24, 34). In particular, bitter taste has evolved as a central warning signal against the ingestion of potentially toxic substances, including plant alkaloids and other environmental toxins (21, 65). Specialized neuroepithelial taste receptor cells, organized within taste buds in human and rodent lingual epithelium, expressed a family of bitter taste receptors (referred as T2Rs) (1, 6, 46). These putative taste receptors belong to the guanine nucleotide-binding regulatory protein (G protein)-coupled receptor (GPCR) superfamily (1), which are characterized by seven transmembrane α-helices (32). Extensive genetic and biochemical evidence indicate that specific G proteins, gustducin and transducin, mediate bitter and sweet gustatory signals in the taste buds of the lingual epithelium (47, 48, 62, 63, 73). More recently, phospholipase Cβ_2 (PLCβ_2) and TRPM5, a member (melastatin subtype 5) of the transient receptor potential (TRP) family (49), have been linked to bitter and sweet signal transduction (55, 56, 81). There is evidence for the activation of multiple second messenger pathways and ion channels in individual taste cells (1, 82). Clearly, taste signal transduction is complex and multifactorial and there is still much that is unknown about individual taste cell regulation.

Outside the tongue, expression of the α-subunit of gustducin ($G\alpha_{gust}$) has been also localized to gastric (28, 75) and pancreatic (27) cells, suggesting that a taste-sensing mechanism may also exist in the digestive system. Indeed, we demonstrated the expression of members of the bitter taste receptors of the T2R family in the mouse and rat GI tract and in mouse and rat enteroendocrine cells in culture (74, 75). More recently, these results have been confirmed (45) and extended to the expression of the sweet taste receptors of the T1R family (15). Collectively, these findings demonstrated the expression of taste signal transduction pathways in cells of the GI tract of mice and rats.

The intracellular signal transduction cascades initiated by bitter stimuli in GI endocrine cells have not been explored. As a first step to examine these processes, we used the mouse enteroendocrine STC-1 cell line as a model system (61). These cells have been used for studying the regulation of GI hormone release in response to bombesin/gastrin-releasing peptide (10, 54, 67), pituitary adenylate cyclase-activating polypeptide (7), leptin (22), fatty acids (8, 26, 66), orexin (36), amino acids (10, 38, 41, 43, 67), and peptidomimetic compounds (50, 53). STC-1 cells produce and release cholecystokinin (CCK), glucose-dependent insulinotropic polypeptide, secretin, and glucagon-like peptide-1 (GLP-1) (8, 9, 11, 26, 38) and have also served as a model for studies of enteroendocrine cell differentiation (59). In the present study, we used STC-1 cells as a

Address for reprint requests and other correspondence: E. Rozengurt, 900 Veteran Ave., Warren Hall, Rm. 11-115, Dept. of Medicine, David Geffen School of Medicine, Univ. of California at Los Angeles, Los Angeles, CA 90095-1786 (e-mail: erozengurt@mednet.ucla.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

model system to elucidate Ca^{2+} signaling and secretory responses initiated by bitter tastants in enteroendocrine GI cells.

Activation of bitter taste receptors promotes the synthesis of second messengers leading to the release of Ca^{2+} from intracellular stores and/or modulates the gating of ion channels that mediate Ca^{2+} entry into neuroepithelial taste cell (25, 44). To determine whether these pathways also operate in enteroendocrine cells, we examined the effect of the structurally unrelated bitter stimuli denatonium benzoate (DB) and phenylthiocarbamide (PTC) on intracellular Ca^{2+} ($[Ca^{2+}]_i$) in STC-1 cells. Our results demonstrate that these bitter tastants increase $[Ca^{2+}]_i$ through Ca^{2+} influx mediated by the opening of L-type voltage-sensitive Ca^{2+} channels (VSCCs) leading to CCK release in enteroendocrine STC-1 cells.

MATERIALS AND METHODS

Cell lines and DNA. The STC-1 cell line is derived from an intestinal endocrine tumor that developed in a double-transgenic mouse expressing the rat insulin promoter linked to the simian virus 40 large T antigen and the polyoma small T antigen (61). STC-1 cells (a gift from D. Hanahan, University of California at San Fransisco) were cultured in DMEM-F12 supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics (100 U/ml penicillin plus 50 mmol/l streptomycin and gentamycin) in a humidified atmosphere with 10% CO_2 and 90% room air at 37°C. STC-1 cDNA was synthesized from 50–100 ng of poly A^+-RNA (RNeasy, Qiagen; Valencia, CA) using a thermostable RT-PCR kit (Invitrogen; Carlsbad, CA).

RT-PCR using mRNA from STC-1 cells. Partial or full-length coding sequences of mT2Rs, PLCβ_2, TRPM4, TRPM5, $Ca_v1.2$, and $Ca_v1.3$ were amplified from cDNA using oligodeoxynucleotide primers designed for each specific mouse sequence, as listed in Table 1. PCR was performed in a total volume of 30 μl containing 100 ng of DNA, 300 nM of each primer in ExTaq buffer, and 2.5 units of ExTaq polymerase (TaKaRa; Madison, WI). An initial denaturation step of 94°C for 2 min was followed by 31 cycles of denaturation at 92°C for 40 s, annealing at 57°C for 40 s, and extension at 72°C for 2 min and finished with a final extension at 75°C for 5 min on a thermocycler (model PTC-200, MJ Research; San Francisco, CA). The housekeeping gene acetic ribosomal protein (ARP) was used as a control for cDNA quality and relative abundance. All PCR products were separated on 1% agarose gels and stained with ethidium bromide. Gel images were recorded from the UV illuminator and analyzed by imaging software (1D Image Analysis; Kodak, Rochester, NY). The predicted gene products were cloned into pCR II-TOPO vectors (Invitrogen), and their identities were confirmed by sequencing at least three positive clones.

Immunocytochemistry. Subconfluent STC-1 cells cultured in 35 mm were fixed for 20 min in 10% Formalin/PBS. Cells were then washed three times in PBS and permeabilized with 0.1% Triton X-100/PBS for 5 min. Cells were then blocked with PBS containing 6% normal sheep serum and 2% bovine serum albumin for 2 h and then incubated overnight at 4°C with rabbit polyclonal anti-Gα gusducin antibodies (1:500; sc-395; Santa Cruz; Santa Cruz, CA). Thereafter, the cells were washed three times in PBS, incubated for 1 h in Alexa Fluor 488-conjugated goat anti-rabbit secondary antibodies (1:1,000, Molecular Probes; Eugene, OR), and then washed again three times in PBS. Fluorescence images were observed with an epifluorescent microscope (Axioskop, Zeiss) and a Zeiss water-immersion objective (Achoplan 40/0.75w). Images were captured as uncompressed TIFF files with a SPOT digital camera driven by SPOT version 2.1 software (Diagnostic Instruments, Sterling Heights, MI). Images were processed using Adobe Photoshop CS.

Assay of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured by calcium fluorometry using fura-2 AM as previously described (56). Cells were grown on 9 × 22 mm glass coverslips in 35-mm dishes. The cells were washed twice with Hanks' balanced salt solution (HBSS; Gibco) supplemented with HEPES, pH 7.4, 1.26 mM $CaCl_2$, and 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$ and 0.1% BSA (referred as Ca^{2+} buffer) and were incubated at 37°C for 15 min in 1 ml of the same buffer with 1.0 μM fura-2 AM. The cultures were then washed three times with Ca^{2+} buffer and the coverslips inserted into a quartz cuvette containing 2 ml of Ca^{2+} buffer. The cuvette with the coverslip was placed into a Hitachi F-2000 fluorospectrophotometer. The incubation medium was continuously stirred at 37°C. The excitation wavelengths were set at 340 and 380 nm, and the emission wavelength was set at 510 nm. The

Table 1. *Oligonucleotide primers used in the study*

Gene	Forward	Reverse	Size
T2R			
mT2R108	AGATGCTCTGGGAACTGTATG	GAAATTCTGCTACTTGTAGAAACAG	894
	GGCACCAAACGAGGAAAGATG	TCAGGACCAAAGAGGCTACTAACG	565
mT2R138	ATGCTGAGTCTGACTCCTGTCT	TCAGAGTGTCCTGGGAGGAAC	996
mT2R135	ATGCTGAGTCTGACTCCTGTCT	TCAGAGTGTCCTGGGAGGAAC	966
mT2R137	ATGAGATTTATGAACAGAACAA	TTATGAAGCAGAGGGTCCCTT	1,002
mT2R144	ATGGCAATAATTACCACAAATTC	CTACCTTTTAAGGTAAAGATGAA	960
G Proteins			
$G\alpha_{gust}$	GATGGGAAGTGGAATTAGTTC	GCTCAGAAGAGCCCACAGTC	1,067
Gαt-2	ATGGGGAGTGGCATCAGTGCT	TTAAAAGAGCCCACAGTCCTT	1,065
Gβ3	GTGCACCATGGGGGAGATGGAGCA	TCAGTTCCAGATTTTGAGGAAGCT	1,030
Gγ13	TCCAAAACCTCAGGCTGGCTAC	AGAGAGTGTGGGTCAGGCTCATAG	265
Channels			
TRPM4	ATCGCCATGTTCAGCTACAC	TCAGCTTTGGAAAGTGG	537
TRPM5	TGTGACATGGTGGCCATCTTCCTGT	ATTTCAGGTGTCAGAGGGTGGCAA	1,056
$Ca_v1.2$	ATGGTCAATGAAAACACGAGG	TCTGTCCAGCCCTCCATGGT	1,100
$Ca_v1.3$	GAGGCAAACTATGCAAGAGG	TCTGTCCAGCCCTCCATGGT	937
Enzymes			
PLC-β_2	CATGTTTGGAGAGACCTTGCTCACA	TGCAGAGAACTCGAAGGAGATAAAC	422
Controls			
ARP	GTTGAACATCTCCCCCTTCTC	ATGTCCTCATCGGATTCCTCC	402

T2R, type 2 family taste receptors; TRPM, transient receptor potential member; PLC, phospholipase C; Ca_v, voltage-gated Ca^{2+} channel; $G\alpha_{gust}$, α-subunit of G protein gustducin; ARP, acetic ribosomal protein.

maximum fluorescence was determined by injecting 100 μl of 5 mM digitonin into the cuvette, and the minimum fluorescence was measured after injection of 100 μl of 0.5 M EGTA, pH 8.0. A K_d of 224 nM was used for the Ca^{2+} dissociation constant from fura-2 in the cells at 37°C. $[Ca^{2+}]_i$ was determined automatically by the cation measurement software of the F-2000 fluorospectrophotometer.

For the Na^+ replacement studies, STC-1 cells grown on coverslips were loaded with fura-2 and washed, as described above. Coverslips were washed once with Na^+-free Ca^{2+} buffer (same as HBSS, except that NaCl, $NaHCO_3$, and Na_2HPO_4 were replaced by equal molarities of sucrose and ChCl and the pH was adjusted to 7.4 with KOH) and immediately inserted into the cuvette containing 2 ml of Na^+-free buffer for the fluorometry studies.

Secretion of CCK from STC-1 cells. To determine the secretion of CCK from STC-1 cells, suspensions of these cells were plated at 1×10^5 cells/cm^2 in 6-well plates (Costar 3516) and assays were performed on cultures that reached at least 80% confluency. Before treatment with agonists, tastants, and/or inhibitors, culture medium was removed and dishes were rinsed with HBSS adjusted to pH 7.4 and supplemented with 20 mM HEPES. Test agents dissolved in HBSS were then added immediately to the culture plate to give their final concentrations in a volume of 1 ml/well. Cells were then incubated at 37°C for various times (15–60 min). The medium was collected and centrifuged at 4°C for 5 min at 1,000 *g* to remove cell debris and the supernatants were stored at −20°C. CCK was measured by RIA using rabbit antiserum R016 raised against sulfated CCK-10 conjugated to keyhole limpet hemocyanin (a gift from Dr. T. E. Solomon). The detection limit and the ID_{50} were 5 and 50 pM, respectively (72).

Materials. Tissue culture medium, fetal bovine serum and HBSS were purchased from Gibco (Grand Island, NY). Bitter tastant DB was from ICN Biomedicals (Aurora, OH) and PTC was from Sigma (St. Louis, MO). Channel blockers nitrendipine and glybenclamide were from Sigma, diltiazem was from Biomol Research Laboratories (Plymouth Meeting, PA). BAY K 8644, a voltage-sensitive Ca^{2+} channel activator, was from Biomol Research Laboratories. U-73122, an inhibitor of $PLC\beta_2$ was from Calbiochem (BMD Biosciences, La Jolla, CA).

RESULTS

Expression of signal transducing proteins implicated in taste perception in enteroendocrine STC-1 cells. Our previous studies (74, 75) indicated that enteroendocrine STC-1 cells express transcripts encoding for several receptors of the T2R family and α-subunits of gustducin and transducin (74, 75). Here, we extended these results showing that STC-1 cells express mT2R108, mT2R137, mT2R138, mT2R144, and mT2R135 (Fig. 1*A*). Furthermore, the results presented in Fig. 1*A* also show expression of $G\alpha_{gust}$, Gβ3, Gγ13, PLCβ2, in STC-1 cells, all of which have been associated with taste signaling (30, 81). We verified that ~95% of the STC-1 cells used in the upcoming experiments were immunostained with antibodies directed against $G\alpha_{gust}$ (Fig. 1*B*), indicating that most cells in the population express the α-subunit of this G protein.

DB and PTC elicit rapid increase in $[Ca^{2+}]_i$ in STC-1 cells. Next, we characterized the effect of bitter stimuli on Ca^{2+} signaling in STC-1 cells. In agreement with our previous results (75), stimulation of STC-1 cells with DB, an agonist of mT2R108, induced a rapid increase in $[Ca^{2+}]_i$ in a concentration-dependent manner. The baseline level of $[Ca^{2+}]_i$ was 233.7 ± 10.2 nM (means ± SE; from total of 40 samples, duplicates of 20 different cell preparations, therefore, $n = 20$). Typical traces of $[Ca^{2+}]_i$ in response to DB in these cells are presented in Fig. 2, *A–E*. For example, addition of 5 mM DB to STC-1 cells induced an increase in $[Ca^{2+}]_i$ that reached peak values of 791 ± 24 nM (means ± SE; $n = 20$) at 35 s and subsequently declined toward a plateau phase of 393 ± 31 nM. DB stimulated peak increase in $[Ca^{2+}]_i$ in a concentration-dependent fashion (Fig. 2*F*). These results demonstrate that the bitter tastant DB induces a striking increase in $[Ca^{2+}]_i$ and the increased $[Ca^{2+}]_i$ sustained for minutes in STC-1 cells with exception of high concentrations of DB (10 mM).

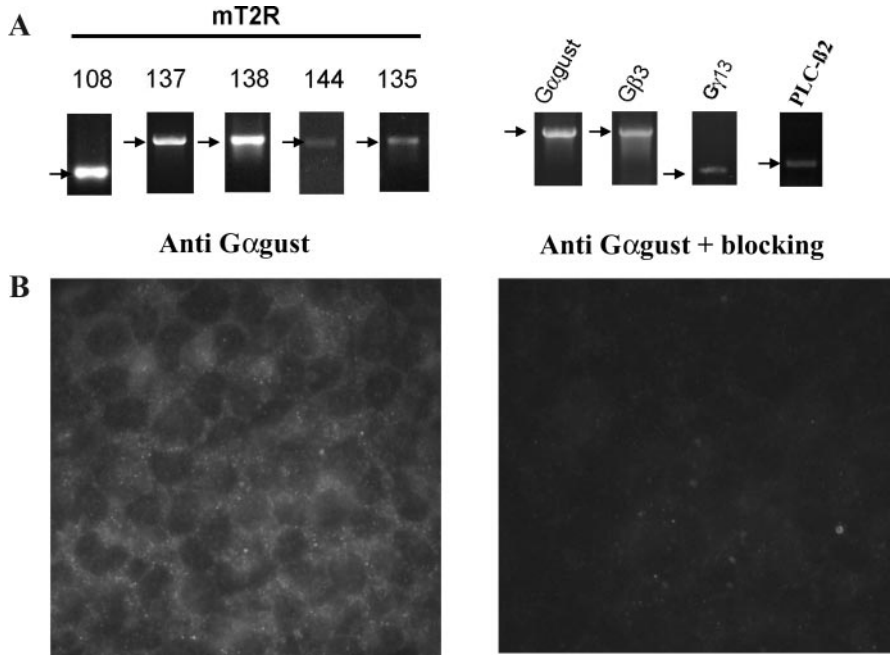

Fig. 1. *A*: expression of bitter taste receptors and signal transducing proteins in STC-1 cells. RT-PCR was performed using mouse-specific primers listed in Table 1 to detect the expression of functional murine type 2 family taste receptors (mT2Rs; i.e., mT2R108, mT2R137, mT2R138, mT2R144, and mT2R135) and the signal transducers α-subunit of G protein gustducin ($G\alpha_{gust}$), $G\beta_3$, Gγ13, and phospholipase C β_2 ($PLC\beta_2$). Arrows indicate the corresponding PCR products, all of which were confirmed by sequencing. *B*: immunostaining of $G\alpha_{gust}$ in the absence and presence of $G\alpha_{gust}$ blocking peptide in STC-1 cells.

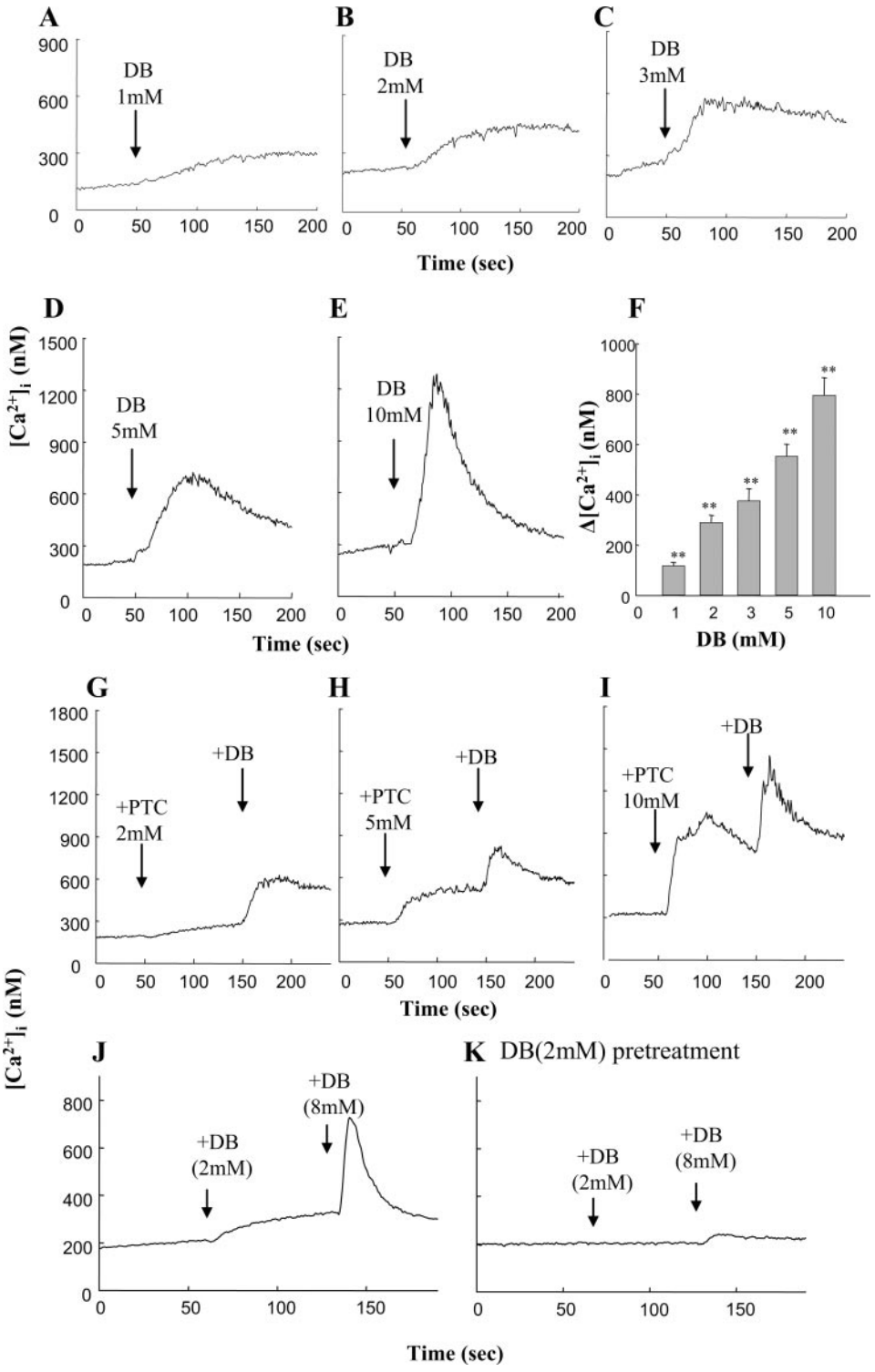

Fig. 2. The bitter stimuli denatonium benzoate (DB) and phenythiocarbamide (PTC) rapidly increase intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) in a dose-dependent manner in STC-1 cells. Cells grown on coverslips were washed and loaded with fura-2 AM. The cells on the coverslip were then washed and inserted into a quartz cuvette and placed into a Hitachi F-2000 fluorospectrophotometer, as described in MATERIALS AND METHODS. *A–E*: dose-dependent increases in $[Ca^{2+}]_i$ concentrations induced by DB in STC-1 cells. Real-time measurement of $[Ca^{2+}]_i$ after stimulation with increasing concentrations of DB (1 to 10 mM) was performed and the representative tracing of duplicate samples of 4 independent experiments was illustrated. *F*: peak increases in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) from baseline were measured in cells after treatment with DB at the indicated concentrations. Values are means ± SE of at least duplicate samples of four independent experiments ($n = 4$) and the statistical significance of the difference to controls ($^{**}P < 0.01$) is indicated. *G–I*: dose-dependent increases in $[Ca^{2+}]_i$ induced by PTC in STC-1 cells. Varying concentrations of PTC (2, 5, and 10 mM) were added at the time indicated by the arrow. After 75 s, the PTC-treated cells received a subsequent addition of DB (2 mM). $[Ca^{2+}]_i$ was measured throughout the incubation period. PTC was dissolved in DMSO and the maximal final concentration of DMSO was 0.5% for the Ca^{2+} studies. *J* and *K*: STC-1 cells were pretreated with or without DB (2 mM) for 15 min during fura-2 loading. After being washed, cells were transferred to cuvette and were challenged with sequential addition of DB (2 and 8 mM) with 60 to 75 s apart. Traces show duplicate samples of 3 separate experiments.

As shown in Fig. 2, *G–I*, STC-1 cells also displayed an increase in $[Ca^{2+}]_i$ in response to the addition of increasing concentrations of PTC (2–10 mM), the ligand of mT2R138 (4, 35). PTC has been used at similar (in mM) concentrations in taste studies in mouse (52, 68) and rats (12). In agreement with the notion that PTC and DB act through different receptors, the addition of PTC did not prevent the increase in $[Ca^{2+}]_i$ elicited by a subsequent addition of 2 mM DB. The initial activation by PTC further enhanced the $[Ca^{2+}]_i$ effect elicited by 2 mM DB (compare the rapid rise to the peak level in Fig. 2, *G–I*, to the slow rise stimulated by DB without PTC priming in Fig. 2*B*). In contrast to the results obtained with STC-1 cells, addition of DB (1–10 mM) or PTC (0.1–10 mM) did not induce any detectable change in $[Ca^{2+}]_i$ in a variety of cell lines that do

not express T2Rs and G proteins implicated in bitter taste reception, including mouse Swiss 3T3 fibroblasts, Rat-1 fibroblasts, rat intestinal epithelial cells (IEC-18, IEC-6) or human colon (T84), pancreatic (BxPC3 and Panc-1), and kidney (HEK-293) cells (Refs. 74 and 75, and results not shown). These findings are consistent with the notion that the effects of bitter compounds on $[Ca^{2+}]_i$ in STC-1 cells are mediated by specific signal transducers that are expressed in these GI cells.

To determine whether addition of DB to STC-1 cells attenuates the increase in $[Ca^{2+}]_i$ induced by a subsequent addition of DB (i.e., homologous desensitization), cells were exposed to 2 mM DB for 15 min and after being washed and loaded into the cuvette, cells were then challenged with two different doses of DB sequentially. As shown in Fig. 2, *J* and *K*, pretreatment of DB strikingly reduced the ability of a subsequent additions of DB at 2 and 8 mM to induce an increase in $[Ca^{2+}]_i$ in STC-1 cells. Our data suggest that like many other biological responses initiated by agonist binding to receptors and channels, DB exhibit the phenomenon of receptor-mediated adaptation or desensitization.

DB and PTC elicit rapid increase in $[Ca^{2+}]_i$ through Ca^{2+} influx. To determine the contribution of Ca^{2+} influx from the extracellular medium to the increase in $[Ca^{2+}]_i$ induced by DB or PTC in STC-1 cells, we prevented Ca^{2+} influx by chelating extracellular Ca^{2+} with EGTA and then sequentially challenged the cells with DB, PTC, and bombesin. As shown in Fig. 3, *A* and *B*, addition of EGTA blocked the increase in $[Ca^{2+}]_i$ induced by either DB or PTC but, in contrast, did not prevent the peak increase in $[Ca^{2+}]_i$ induced by bombesin, which is caused by mobilization of Ca^{2+} from intracellular stores (Fig. 3, *C* and *D*).

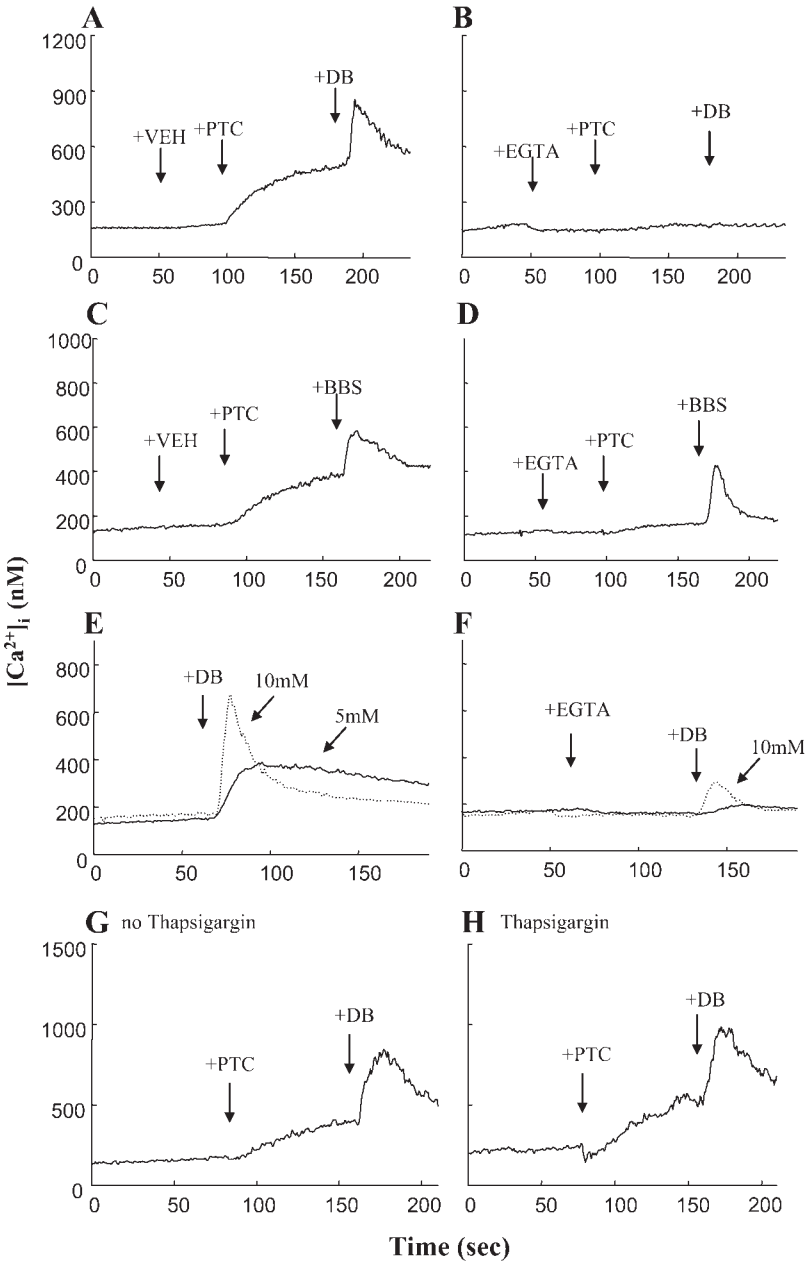

Fig. 3. DB and PTC increase $[Ca^{2+}]_i$ through Ca^{2+} influx in STC-1 cells. *A–D*: cells treated with either vehicle (VEH, water) or 1.25 mM EGTA for 50 s before stimulation with 5 mM PTC. After 80 s, either 2 mM DB (*A* and *B*) or 2.5 nM bombesin (BBS; *C* and *D*) was added. EGTA effect on higher concentrations of DB (5 and 10 mM in *E* and *F*) was examined. *G* and *H*: cells were preincubated with or without thapsigargin (50 nM) for 30 min during the fura-2 loading. Thapsigargin was added back to the cuvette after fura-2 was washed off, then DB (2 mM) or PTC (3 mM) was added at the indicated time points. Each tracing was a representative of duplicate samples of 3 separate experiments.

EGTA (1.25 mM) completely blocked $[Ca^{2+}]_i$ increases elicited by 2 and 5 mM DB, but attenuated the peak increases of $[Ca^{2+}]_i$ stimulated by 10 mM DB to 25.2% ± 6.3% (means ± SE; $n = 3$) (Fig. 3, *E* and *F*). These results indicate that Ca^{2+} influx is responsible for a major component of the increase in $[Ca^{2+}]_i$ induced by DB or PTC in STC-1 cells.

We explored further this conclusion using thapsigargin, an agent that selectively inhibits endoplasmic reticulum Ca^{2+} ATPase and thereby depletes intracellular Ca^{2+} stores (71). As shown in Fig. 3, *G* and *H*, treatment with 50 nM thapsigargin for 30 min did not prevent the increase in $[Ca^{2+}]_i$ induced by either PTC or DB in STC-1 cells but completely prevented the increase in $[Ca^{2+}]_i$ induced by bombesin (results not shown). Collectively, these results indicate that DB and PTC elicit an increase in $[Ca^{2+}]_i$ by stimulating Ca^{2+} influx into the cells.

Inhibitors of L-type VSCCs prevent the increase in $[Ca^{2+}]_i$ induced by DB. Subsequent experiments were designed to identify the Ca^{2+} permeability pathways activated by bitter stimuli in STC-1 cells. L-type VSCCs mediate influx of extracellular Ca^{2+} into neuronal and neuroendocrine cells in response to membrane depolarization (5, 14). STC-1 cells have been shown to express functional L-type VSCCs that are opened by addition of KCl (42) but very little is known about the role of these channels in mediating responses initiated by bitter stimuli. Using RT-PCR, we verified that STC-1 cells express the pore-forming α_1 subunit isoforms $Ca_v1.2$ and $Ca_v1.3$ of L-type VSCCs, as shown in Fig. 4*A*. Accordingly, the addition of depolarizing concentrations of KCl (5–25 mM) to STC-1 cells induced a robust early spike in $[Ca^{2+}]_i$, followed by a sustained plateau phase (Fig. 4, *B–D*). The increase in $[Ca^{2+}]_i$ induced by KCl was abrogated by chelation of extracellular Ca^{2+} by EGTA (Fig. 4, *E* and *F*) but it was unaffected by prior exposure to thapsigargin (Fig. 4, *G* and *H*).

The common pharmacological feature of all isoforms of L-type VSCCs is their sensitivity to dihydropyridines. Consequently, we examined the effects of these L-type VSCCs blockers on $[Ca^{2+}]_i$ increases elicited by KCl, bombesin, PTC, and DB in STC-1 cells. Our results show that pretreatment with nitrendipine (1 μM) inhibited the increase in $[Ca^{2+}]_i$ elicited by KCl, DB and PTC (Fig. 5, *A–D*). We also demonstrated that another blocker of L-type VSCCs, diltiazem (0.1 mM), abrogated the increase in $[Ca^{2+}]_i$ induced by DB, PTC, or KCl in STC-1 cells (Fig. 5, *E* and *F*). Both nitrendipine and diltiazem blocked the increase in $[Ca^{2+}]_i$ elicited by higher concentration of DB (5 mM, data not shown). In contrast, neither nitrendipidine nor diltiazem blocked bombesin-elicited $[Ca^{2+}]_i$ increase in these cells. These results indicate that L-type VSCCs play a major role in mediating the increase in $[Ca^{2+}]_i$ induced by bitter tastants in STC-1 cells.

Because PKCs have been implicated in both stimulation as well as inhibition of L-type VSCCs in different cell types (51, 77), we determined whether PKCs play any role in the activation of these channels elicited by DB. Activation of PKC by addition of tumor promoting agents of the phorbol ester family (e.g., PDB, 100 nM for 10 min) to STC-1 cells prior to DB (3 mM), profoundly inhibited the ability of DB to increase $[Ca^{2+}]_i$ in these cells (Fig. 5*G*). In contrast, an identical treatment with PDB did not prevent the increase in $[Ca^{2+}]_i$ induced by KCl, suggesting that PKC inhibits upstream of L-type VSCCs (data not shown). Exposure to the PKC selective inhibitors bisindolylmaleimide I (also known as GF 109203X or GFI) or Ro 31-8220 (each at 3.5 μM) for 30 min, enhanced the rate and peak of the increase in $[Ca^{2+}]_i$ induced by DB in STC-1 cells (Fig. 5*H*). These results support the hypothesis that PKCs do not directly activate L-type VSCCs elicited by DB, but they mediate negative feedback on DB-induced Ca^{2+} signaling in STC-1 cells.

To further substantiate that L-type VSCCs are involved in mediating the increase in $[Ca^{2+}]_i$ elicited by DB or PTC in STC-1 cells, we also determined whether the L-type VSCCs opener BAY K 8644 potentiates the increase in $[Ca^{2+}]_i$ induced by these bitter tastants. Addition of BAY K 8644 to STC-1 cells stimulated an increase in $[Ca^{2+}]_i$ in a concentration-dependent manner (Fig. 6, *A–D*). A detectable effect was seen at 0.3 μM and a robust elevation in $[Ca^{2+}]_i$ was obtained at either 3 or 10 μM. Interestingly, prior exposure to low concentrations of BAY K 8644 potentiated the ability of DB to elicit an increase in $[Ca^{2+}]_i$. As shown in Fig. 6, *E–G*, addition of BAY K 8644 at 0.3–1 μM strikingly enhanced the increase in $[Ca^{2+}]_i$ induced by a subsequent addition of 1 mM DB, a concentration that induced a modest and slower increase in $[Ca^{2+}]_i$, as shown previously in Fig. 2*A*. Collectively, the results presented in Figs. 4–6 demonstrate that the bitter compounds DB and PTC induce a striking increase in $[Ca^{2+}]_i$ through the opening of L-type VSCCs that mediate Ca^{2+} influx into STC-1 cells. In addition, the small increase in $[Ca^{2+}]_i$ originally elicited by either very low concentrations of BAY K 8644 or PTC primed subsequent challenge by low concentration of DB (1 mM) to a robust elevation through a mechanism of Ca^{2+} entry into STC-1 cells, possibly the opening of L-type VSCCs.

Expression of TRPM4 and TRPM5 and requirement of Na^+ for the increase in $[Ca^{2+}]_i$ stimulated by DB or PTC in STC-1 cells. Subsequently, we determined the role of signaling elements that could mediate the activation of L-type VSCC in response to bitter stimuli. TRPM5, a member of the transient receptor potential (TRP) family (49), has been linked to bitter and sweet signal transduction (55, 56, 81). TRPM5 and the related TRPM4 have been identified as Ca^{2+}-activated, monovalent cation channels (29) that induce membrane depolarization (39, 57). As shown in Fig. 7*A*, *inset*, RT-PCR of mRNA from STC-1 cells revealed the expression of TRPM4 and TRPM5 in these cells. It is therefore possible that tastant-induced activation of these channels produces Na^+ influx leading to membrane depolarization and thereby, to amplification of L-type VSCCs activation in STC-1 cells. In agreement with this possibility, we found that the increase in $[Ca^{2+}]_i$ induced by addition of DB or PTC was attenuated when STC-1 cells were stimulated with these compounds in a Na^+-free medium (Fig. 7, *A–D*). In contrast, the increase in $[Ca^{2+}]_i$ elicited by addition of 25 mM KCl was not affected (Fig. 7, *E–F*). These results indicate that L-type VSCCs activation that mediates Ca^{2+} influx into STC-1 cells in response to bitter tastants requires Na^+ in the extracellular medium.

U-73122 prevented the increase in $[Ca^{2+}]_i$ induced by DB or bombesin, whereas glybenclamide did not affect basal or stimulated $[Ca^{2+}]_i$ in response to DB, KCl, or BBS. Phospholipase $C\beta_2$ ($PLC\beta_2$) has been linked to bitter and sweet signal transduction in the lingual epithelium (81) and more recently in STC-1 cells (45). Consequently, we deter-

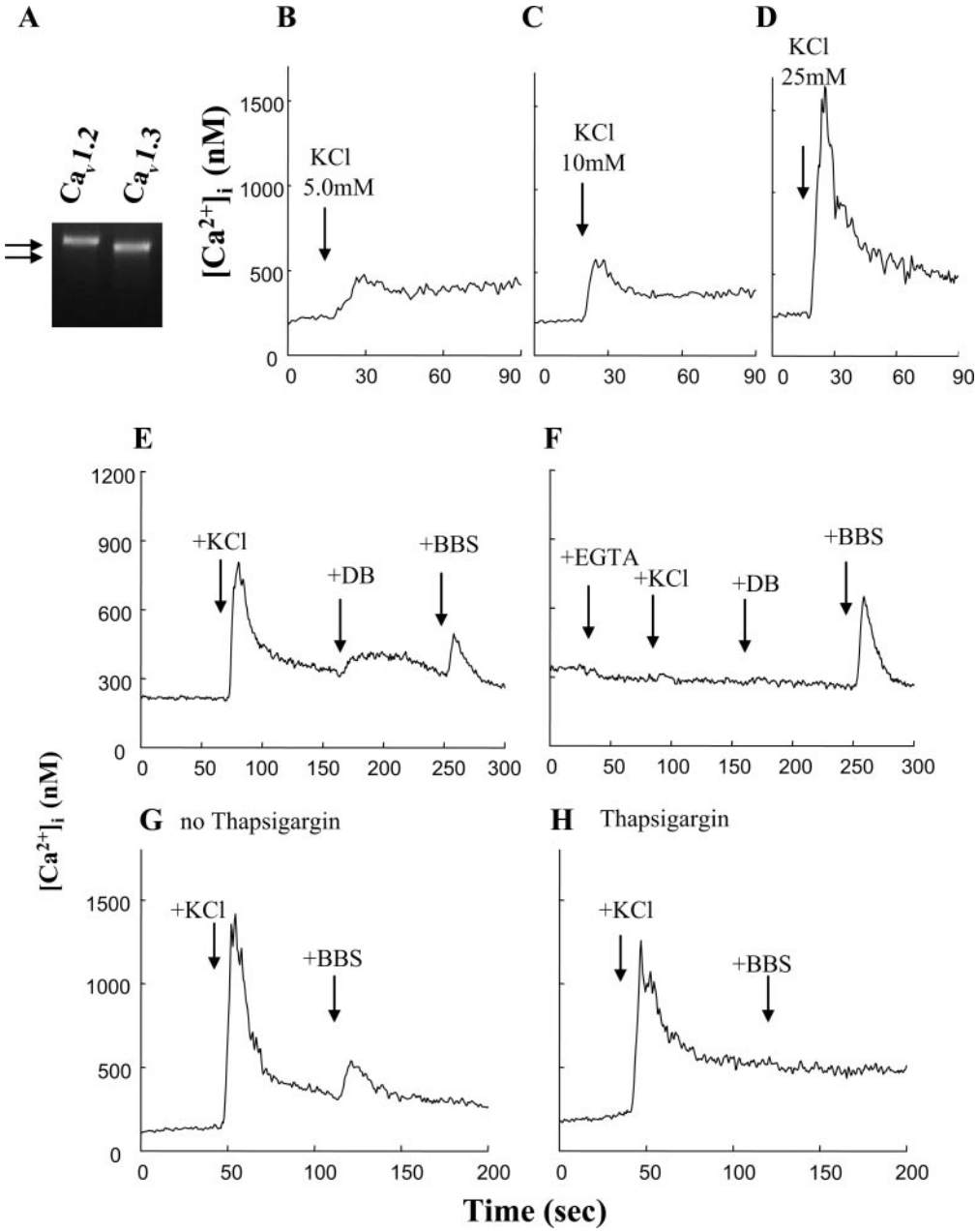

Fig. 4. Bitter stimuli increase $[Ca^{2+}]_i$ through Ca^{2+} influx mediated by the opening of L-type voltage-sensitive Ca^{2+} channels (VSCCs) in STC-1 cells. *A*: expression of the pore-forming α1 subunit isoforms $Ca_v1.2$ and $Ca_v1.3$ of L-type VSCCs in STC-1 cells. RT-PCR was carried out as described in MATERIALS AND METHODS using the primers described in Table 1. *B–D*: cells on the coverslips were exposed to increasing concentrations of KCl (5.0 to 25 mM) added to the cuvette, as indicated by the arrows. *E* and *F*: vehicle or EGTA (1.25 mM) was added to the cuvette for 50 s before the cells were sequentially stimulated with 25 mM KCl, 2 mM DB, and 2.5 nM BBS, as indicated. *G* and *H*: cells were treated with or without 50 nM thapsigargin for 30 min during fura-2 loading. After washing off the fura-2, cells were then inserted into the cuvette where the thapsigargin was also added back to the solution. KCl (25 mM) and 2.5 nM BBS were added, as indicated. Each trace was a representative of duplicate samples of 3 separate experiments.

mined whether the PLC inhibitor U-73122 prevents $[Ca^{2+}]_i$ signaling in response to DB. Preincubation of STC-1 cells with U-73122 for 30 min at 5 μM profoundly inhibited, and at 10 μM fully blocked, DB and bombesin-induced increase in $[Ca^{2+}]_i$ (Fig. 8, *A–C*), suggesting a role of the PLC pathway in bitter tastant signal transduction in enteroendocrine cells. Although these results are in agreement with a previous report (45), they should be interpreted with caution because U-73122 has also been reported to block L-type VSCC activity (70). Therefore, it is likely that the inhibitory effects of U-73122 on DB-induced $[Ca^{2+}]_i$ signaling in STC-1 cells are due to inhibition of both PLC and L-type VSCCs.

It has been reported that DB stimulated insulin secretion in clonal HIT-T15 β-cells and rat pancreatic islets through the closure of the K^+ channel Kir6.2, causing depolarization of the membrane of these cells and thereby triggering L-type VSCC activation (69). To test whether the Ca^{2+} response elicited by

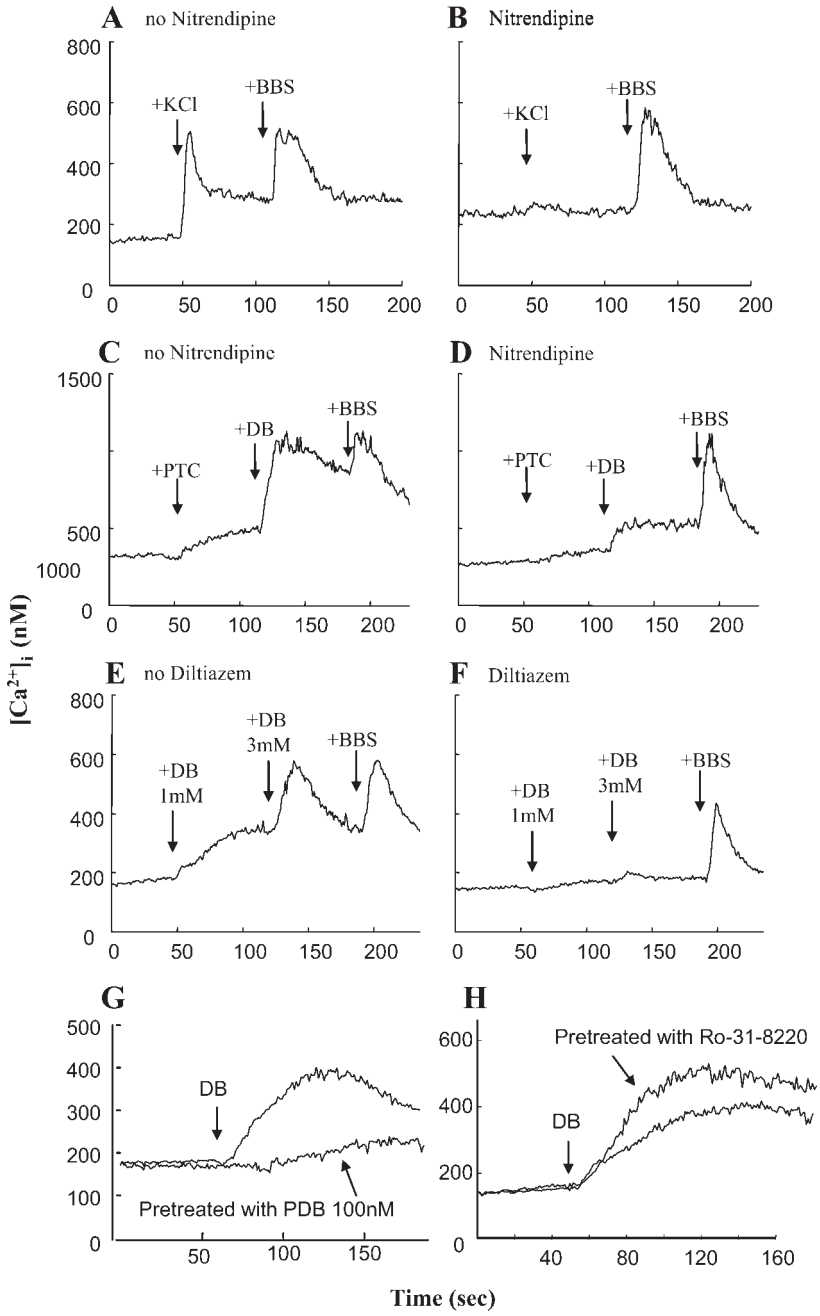

Fig. 5. Inhibitors of L-type VSCCs prevent the increase in $[Ca^{2+}]_i$ induced by DB or PTC. *A–D*: cells were pretreated with or without 1 μM nitrendipine for 30 min and 25 mM KCl, 3 mM PTC, 2 mM DB, and 2.5 nM BBS were added as indicated. Nitrendipine was dissolved in DMSO and final concentration for DMSO was 0.1%. *E* and *F*: STC-1 cells were pretreated without (*E*) or with (*F*) the L-type VSCC blocker diltiazem (0.1 mM) for 30 min. The same concentration of diltiazem was readded to the cells in *F*. DB (1 mM or 3 mM), and 2.5 nM bombesin (BBS) were added sequentially, as indicated. *G* and *H*: STC-1 cells were pretreated with or without either PDB (100 nM) for 10 min or PKC specific inhibitor Ro-31–8220 (3.5 μM) for 30 min during fura-2 loading. After fura-2 was washed off, cells were then inserted into the cuvette and treated with 3 mM DB as indicated. Each tracing was a representative of duplicate samples of 3 separate experiments.

DB in STC-1 cells also involves regulation of KATP channel activity, we examined whether glybenclamide, a well-established blocker of this channel in pancreatic β-cells, can mimic the increase in $[Ca^{2+}]_i$ elicited by DB in STC-1 cells or can potentiate Ca^{2+} signaling in response to KCl, bombesin, or DB in these cells. Our results show that in contrast to the results obtained with HIT-T15 β-cells, addition of glybenclamide (50 μM) did not elicit any detectable increase in $[Ca^{2+}]_i$ in STC-1 cells or enhance the increase in $[Ca^{2+}]_i$ elicited by a subsequent addition of DB (low and high concentrations), bombesin, or KCl in these cells (Fig. 8, *D–F*). Furthermore, we also verified that pretreatment of STC-1 cells with glybenclamide (1 to 50 μM) for 1 h had no effect on the subsequent increase in $[Ca^{2+}]_i$ induced by DB (data not shown). In contrast, a concentration of glybenclamide of 1 μM was sufficient to mimic DB effects in HIT-T15 β-cells (69).

CCK secretion from STC-1 cells in response to bitter tastants. STC-1 cells are known to produce and release CCK in response to multiple stimuli. We verified that ~95% of the STC-1 cells used in our experiments were immunostained with antibodies directed against CCK (data not shown), indicating that virtually all the cells in the population produce this gastrointestinal hormone.

To determine the effect of DB on the secretion of CCK from STC-1 cells, cultures of these cells were washed with HBSS adjusted to pH 7.4 and incubated in this solution at 37°C for

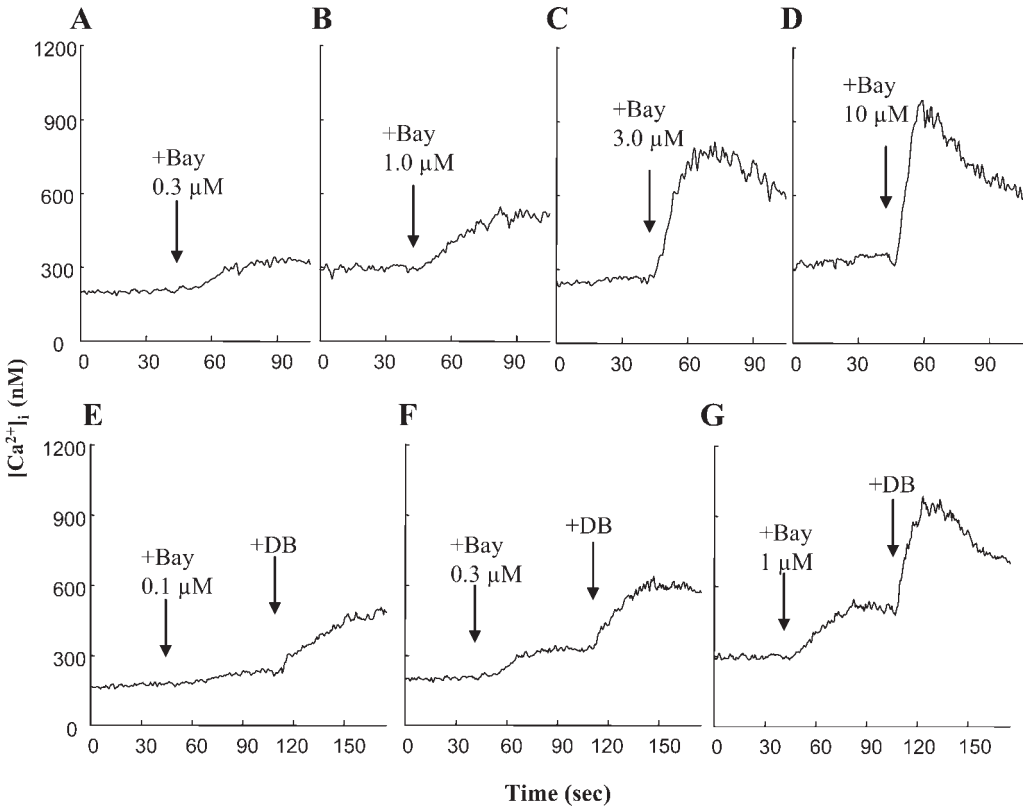

Fig. 6. The L-type VSCCs opener BAY K 8644 increases $[Ca^{2+}]_i$ and potentiates the increase in $[Ca^{2+}]_i$ induced by DB. *A–D*: addition of increasing concentrations of BAY K 8644 (0.3 μM to 10 μM) to fura-2 loaded STC-1 cells elicited a dose-dependent increase in $[Ca^{2+}]_i$. *E–G*: low concentrations of BAY K 8644 (0.1 to 1.0 μM) were added to the cuvette for 65 s and followed by 1 mM DB, as indicated. BAY K 8644 was dissolved in absolute ethanol and the final concentration for ethanol was 0.5%. Each trace represents duplicate samples of 4 separate experiments.

various times (15–60 min) in the absence or in the presence of DB at 5 mM. As shown in Fig. 9*A*, DB induced a marked increase in CCK release from STC-1 cells, compared with cells treated with the solvent control. In other experiments, we demonstrated that 5 mM DB stimulated CCK release to a degree comparable to that produced by 100 nM bombesin, a potent stimulus of CCK release in STC-1 cells (data not shown). In addition, DB increased CCK output into the medium in a dose-dependent manner in STC-1 cells (Fig. 9*B*).

Secretory cells typically release hormones and neurotransmitters by regulated, Ca^{2+}-dependent exocytosis of vesicles or granules (33). Consequently, we determined whether the increase in $[Ca^{2+}]_i$ plays a major role in promoting CCK release from STC-1 cells stimulated by DB. As shown in Fig. 9*C*, chelation of extracellular Ca^{2+} with EGTA to prevent Ca^{2+} influx, completely prevented the increase in CCK release induced by DB in STC-1 cells. Because our results demonstrated that DB increases $[Ca^{2+}]_i$ through the opening of L-type VSCCs, we determined the role of these channels in mediating DB-stimulated secretion of CCK from STC-1 cells. Exposure of STC-1 cells to nitrendipine (1 μM), a treatment that inhibited the increase in $[Ca^{2+}]_i$ elicited by DB (see Fig. 5), also prevented CCK release induced by DB in STC-1 cells (Fig. 9*C*). These results indicate that the bitter tastant DB induces CCK release from enteroendocrine STC-1 cells via an increase in $[Ca^{2+}]_i$ mediated by the opening of L-type VSCCs.

DISCUSSION

Molecular sensing of the luminal contents of the GI tract not only regulates motility, release of GI hormones, and pancreatobiliary secretion, but it is also responsible for the detection of ingested drugs and toxins thereby initiating responses critical for survival. The enteroendocrine cells have been thought to play a critical role in the integration and coordination of these physiological responses but the initial molecular recognition events that sense the chemical composition of the luminal contents have remained elusive.

In view of the importance of chemical sensing in the regulation of food intake, digestion, and poison rejection, we started to determine whether bitter taste receptors are expressed in the GI tract. Our previous studies identified transcripts encoding members of the T2R family of bitter taste receptors in the gastric mucosa as well as in the lining of the intestine of mice and rats and in cultured enteroendocrine cells (74, 75). In the present study, we extended our previous findings demonstrating the expression of additional T2R receptors and effectors implicated in intracellular taste signal transduction, namely $G\alpha_{gust}$, $PLC\beta_2$, and TRPM5 in STC-1 cells.

The complex pathways that mediate taste signaling in taste cells of the lingual epithelium are becoming increasingly understood (24, 44, 56) but it is not known whether these pathways operate in GI endocrine cells. In recent years, STC-1 cells have emerged as a valuable model system for studying the

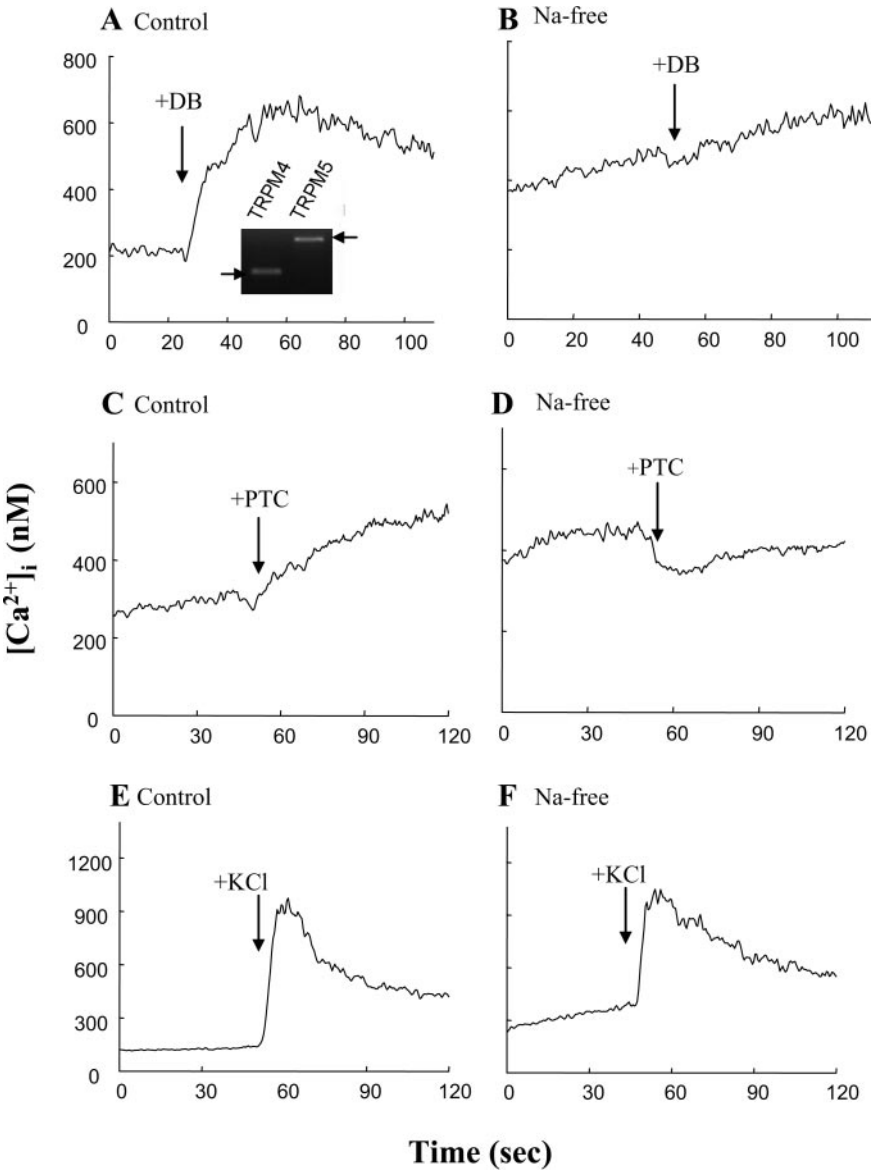

Fig. 7. Expression of TRPM4 and TRPM5 in STC-1 cells and requirement of Na^+ for the increase in $[Ca^{2+}]_i$ stimulated by DB or PTC in STC-1 cells. *A*, *inset*: RT-PCR of mRNA from STC-1 cells revealed the expression of TRPM4 and TRPM5. Arrows indicate the confirmed PCR products corresponding to the predicted sequences of TRPM4 and TRPM5. *A–F*: STC-1 cells were grown on coverslips, fura-2 loaded, and washed as described in Fig. 2. Coverslips were quickly washed once in Na^+-free media and placed into the cuvette with 2 ml of a solution with (control; *A*, *C*, and *E*) or without Na^+ (Na^+ free; *B*, *D*, and *F*), as described in MATERIALS AND METHODS. The cultures were challenged with 2 mM DB, 3 mM PTC or 25 mM KCl, as indicated. Each trace represents duplicate samples of 3 separate experiments.

regulation of GI peptide synthesis and release from intestinal endocrine cells. STC-1 cells synthesize and store CCK and release this hormonal peptide in response to bombesin (10, 54, 67), pituitary adenylate cyclase-activating polypeptide (7), leptin (22), fatty acids (8, 66), orexin (36), aromatic amino acids (10), and peptidomimetic compounds (50, 53). STC-1 cells have been also used as a model for studies of enteroendocrine cell differentiation (59), GI peptide processing (79, 80) and regulation of CCK, glucose-dependent insulinotropic polypeptide, and proglucagon gene expression (2, 20, 31, 37, 58, 78). In this study, we demonstrate that the bitter tastants DB and PTC induced a marked increase in $[Ca^{2+}]_i$ in dose- and time-dependent fashion in STC-1 cells. In contrast, these tastants did not induce any detectable change in $[Ca^{2+}]_i$ in multiple cell lines that do not express T2Rs and G proteins implicated in bitter taste reception or GI peptides, including mouse Swiss 3T3 fibroblasts, rat intestinal epithelial IEC-18 and IEC-6 cells and human colonic (T84), pancreatic (BxPC3), or kidney (HEK-293) cells (74, 75). These findings are consistent with the notion that the effects on $[Ca^{2+}]_i$ signaling elicited by bitter stimuli in STC-1 cells are mediated by specific transducers that are expressed in these GI cells. We produced several lines of evidence indicating that either DB or PTC increases Ca^{2+} influx into SCT-1 cells. For example, chelating extracellular Ca^{2+} with EGTA blocked the increase in $[Ca^{2+}]_i$ induced by DB and PTC but in contrast, did not prevent the effect induced by the Ca^{2+}-mobilizing neuropeptide bombesin. Reciprocally, thapsigargin, a compound that depletes the intracellular stores of Ca^{2+}, blocked the transient increase in $[Ca^{2+}]_i$ induced by bombesin, but did not attenuate the $[Ca^{2+}]_i$ increase elicited by DB or PTC. These results indicate that Ca^{2+} influx mediates a substantial component of the increase in $[Ca^{2+}]_i$ induced by DB or PTC in STC-1 cells.

L-type VSCCs mediate influx of extracellular Ca^{2+} into neuronal and neuroendocrine cells in response to membrane depolarization (5, 14, 40). Accordingly, enteroendocrine

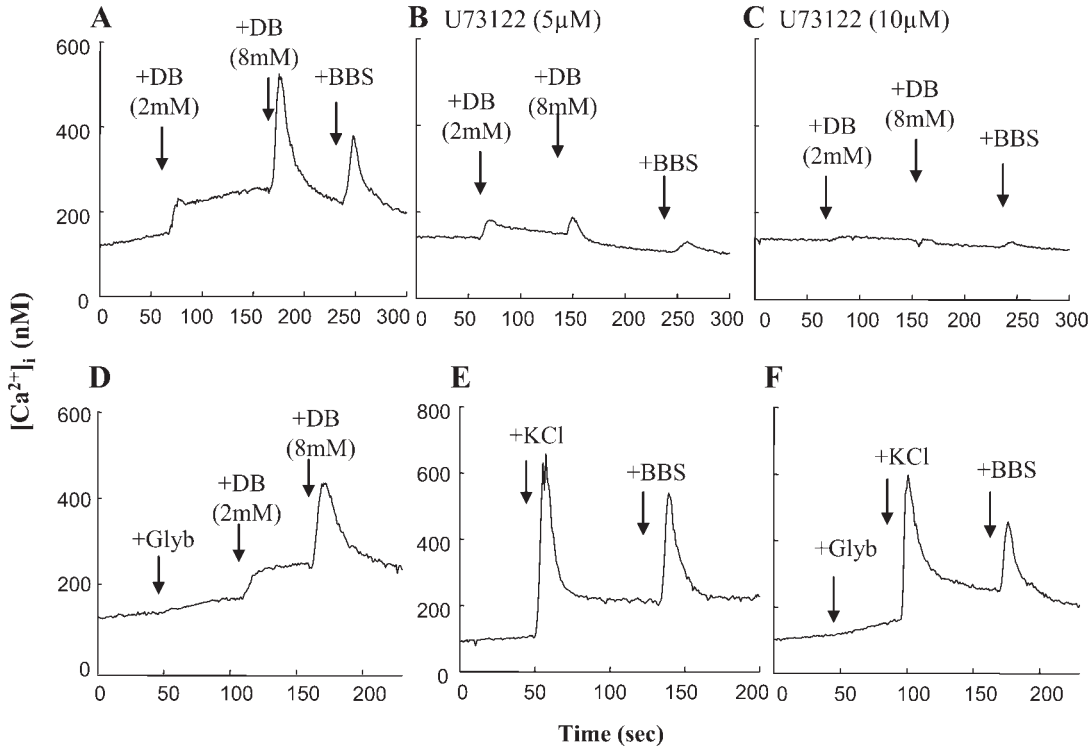

Fig. 8. U-73122 prevented the increase in $[Ca^{2+}]_i$ induced by DB or BBS, whereas glybenclamide did not affect basal or stimulated $[Ca^{2+}]_i$ in response to DB, KCl, or BBS. *A–C*: cells were pretreated with or without 5 or 10 μM of the PLC inhibitor U-73122 for 30 min, and subsequently, 2 and 8 mM DB and 2.5 nM BBS were added, as indicated. U-73122 was dissolved in DMSO and final concentration for DMSO was 0.1%. *D–F*: glybenclamide (50 μM) was added to cells for 60 s, followed by addition of DB, KCl (20 mM), or BBS (2.5 nM). Glybenclamide was dissolved in DMSO and the final concentration for DMSO was 0.5%. Each tracing was a representative of duplicate samples of three separate experiments ($n = 3$).

STC-1 cells have been shown to express functional L-type VSCCs that are opened by addition of KCl (42). Using RT-PCR, we demonstrated that STC-1 cells express the pore-forming α1 subunit isoforms $Ca_v1.2$ and $Ca_v1.3$ of L-type VSCCs and verified that cell depolarization by KCl or addition of the L-type VSCC opener BAY K 8644 mimicked the increase in $[Ca^{2+}]_i$ induced by DB or PTC. Consequently, we determined whether the opening of L-type VSCCs could mediate the increase in $[Ca^{2+}]_i$ elicited by bitter stimuli in STC-1 cells. A salient feature of the results presented here is that treatment with the L-type VSCC blockers nitrendipine or diltiazem profoundly inhibited the increase in $[Ca^{2+}]_i$ elicited by tastants and KCl, indicating that the opening of these channels plays a major role in mediating the increase in $[Ca^{2+}]_i$ in response to DB or PTC in STC-1 cells. In line with this conclusion, exposure to BAY K 8644 potentiated the increase in $[Ca^{2+}]_i$ elicited by DB. Thus our results demonstrate for the first time that bitter tastants increase $[Ca^{2+}]_i$ through Ca^{2+} influx mediated by the opening of L-type VSCCs in enteroendocrine STC-1 cells.

Having demonstrated robust increases in $[Ca^{2+}]_i$ in STC-1 cells challenged with bitter tastants, we assessed whether these compounds also stimulate the release of CCK from these cells. Our results show that DB is a potent stimulant of CCK release from enteroendocrine STC-1 cells and that treatment with either EGTA or nitrendipine prevented this effect. We conclude that tastant-elicited CCK release is also mediated by an increase in $[Ca^{2+}]_i$ produced by the opening of L-type VSCCs.

We found that DB-induced Ca^{2+} influx required the presence of Na^+ in the medium while KCl, which directly depolarizes the cells by reducing the outward gradient of K^+, was still effective in stimulating Ca^{2+} influx even in the absence of extracellular Na^+. These results imply that bitter stimuli stimulate Ca^{2+} influx into STC-1 cells through PLC- and Na^+-dependent steps, possibly mediated by TRPM5 and TRPM4. Recent studies, using genetically modified mice, demonstrated that TRPM5 plays a critical role in the transduction of bitter stimuli in taste cells. Interestingly, this channel was expressed strongly not only in taste cells of the lingual epithelium but also in gastrointestinal cells. Taking together, these experiments suggest that DB and PTC induce a complex signaling cascade in enteroendocrine STC-1 cells leading to CCK release that appears to involve T2Rs, PLC, Na^+, and Ca^{2+} influx and a PKC feedback loop, as presented schematically in Fig. 10. In agreement with this model, a previous study (69) indicated that DB does not exert any direct effect on L-type VSCC activity. It is interesting that the signaling cascade proposed in Fig. 10 can be distinguished from the bombesin-induced Ca^{2+} mobilization from thapsigargin-sensitive internal stores in the same STC-1 cells. There is increasing evidence indicating important differences between local and global changes in $[Ca^{2+}]_i$ response to different pools of Ins(1,4,5)-trisphosphate in a variety of cell types (17, 18, 76). It is therefore plausible that local changes in $[Ca^{2+}]_i$ in response to βγ-mediated PLCβ2 stimulation by bitter stimuli activate TRPM4 and TRPM5 channels that mediate Na^+-

dependent depolarization leading to the activation of L-type VSCCs, as proposed in Fig. 10, while bombesin receptor activation, which is known to elicit Gαq-mediated PLCβ$_1$ activation, leads to an increase in $[Ca^{2+}]_i$ that is not sufficient to activate TRPMs that mediate membrane depolarization and trigger L-type VSCC activation. Further experimental work is warranted to elucidate the differences between the signaling pathways triggered by taste receptors and hormonal neurotransmitters.

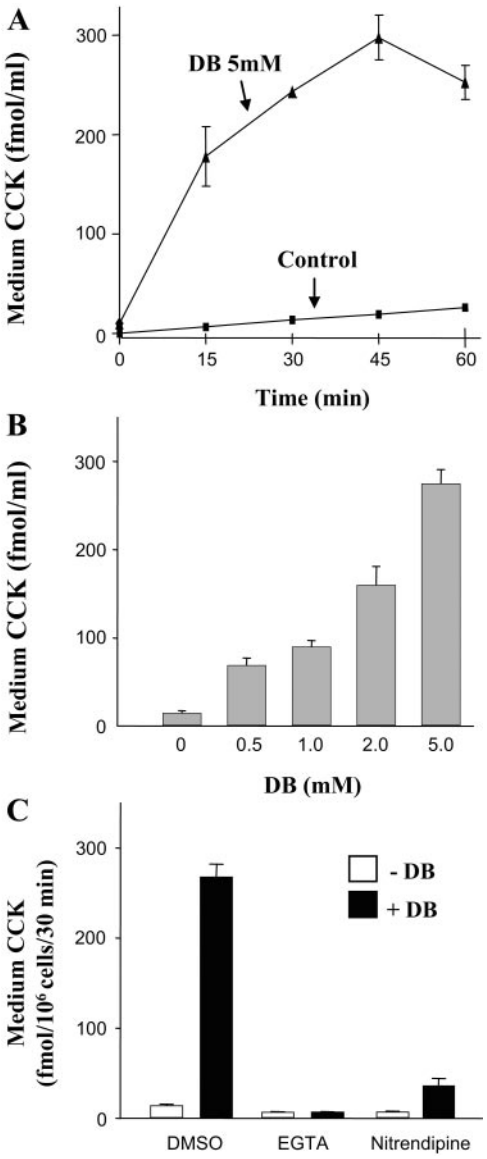

Fig. 9. CCK release from STC-1 cells in response to DB is mediated by L-type VSCCs. CCK secretion was measured as described in MATERIALS AND METHODS. *A*: time course of DB-induced CCK-8 release from STC-1 cells. Before stimulation with DB, medium was removed and cultures were rinsed with Hanks' balanced salt solution (HBSS) adjusted to pH 7.4 and supplemented with 20 mM HEPES. DB dissolved in HBSS was then added to give a final concentration of 5 mM. Cells were then incubated at 37°C for various times (15–60 min). The medium was collected and centrifuged at 4°C for 5 min at 1,000 *g* to remove cell debris and the supernatants were stored at −20°C. The CCK concentrations in the media were determined by RIA. *B*: dose response of DB on CCK-8 release in STC-1 cells. For the dose response, increasing concentrations of DB were added to the cultures for 45 min and the media was then collected for CCK measurements. *C*: exposure to EGTA or to the L-type VSCCs blocker nitrendipine prevents CCK-8 release from STC-1 cells. Cells were pretreated in the absence (DMSO) or in the presence of 1 μM nitrendipine for 30 min or 1.25 mM EGTA for 2 min. After 5 mM DB was added, cultures were incubated at 37°C for 30 min. Culture media were then collected for the measurement of CCK by RIA.

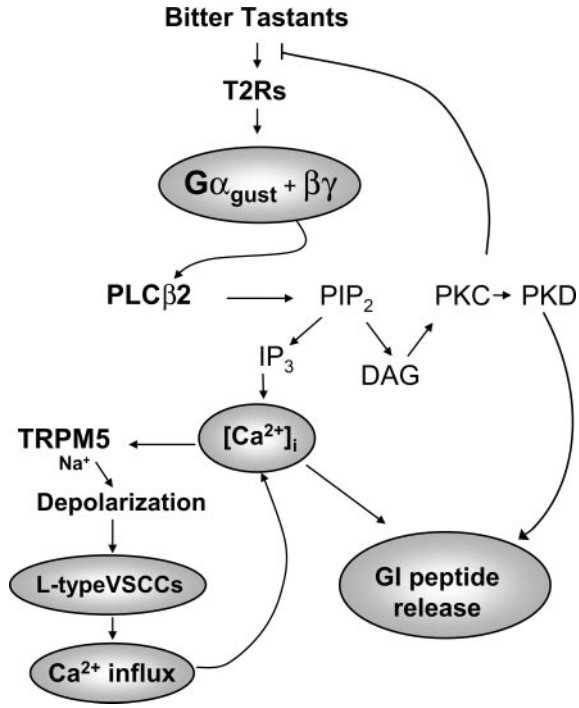

Fig. 10. Putative pathways triggered by bitter stimuli in GI enteroendocrine cells. The signaling cascade triggered by bitter stimuli in enteroendocrine STC-1 cells leading to CCK release, appears to involve T2Rs, PLC, Na^+, and Ca^{2+} influx and a negative PKC feedback loop. This model proposes a mechanism by which bitter stimuli elicit responses in GI enteroendocrine cells. IP_3, inositol trisphosphate; DAG, diacylglycerol; PKD, protein kinase D; PIP_2, phosphatidylinositol (4,5) bisphosphate.

Enteroendocrine cells respond to luminal factors by releasing signaling molecules, including hormones and paracrine regulators at the basolateral side. In view of the results presented in this study, it is plausible that luminal tastants trigger an increase in $[Ca^{2+}]_i$ in enteroendocrine cells leading to exocytosis and the release of signaling molecules, including GI peptides, that activate neural reflexes and/or act in a paracrine or endocrine manner to modulate the activity of adjacent or distant cells. Recently, we demonstrated the co-localization of the α-subunit of Gα$_{gust}$ with PYY and GLP-1, which are produced by open enteroendocrine L cells of the ileum and colon (N. Rozengurt, S. V. Wu, M. Chen, C. Huang, C. Sternini, and E. Rozengurt, unpublished observations). Interestingly, CCK, GLP-1, and PYY, like bitter stimuli, mediate an aversive food response in rodents (16, 23, 64) and the results presented here show that bitter stimuli trigger the release of CCK. Collectively, these findings raise the attractive possibility that the release of gastrointestinal peptides in response to bitter stimuli could play a role in protecting the organism against potentially toxic (bitter) substances.

ACKNOWLEDGMENTS

E. Rozengurt is the Ronald S. Hirshberg Professor of Pancreatic Cancer Research.

GRANTS

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-55003 and DK-56930 (to E. Rozengurt).

REFERENCES

1. **Adler E, Hoon MA, Mueller KL, Chandrashekar J, Ryba NJP, and Zuker CS.** A novel family of mammalian taste receptors. *Cell* 100: 693–702, 2000.
2. **Bernard C, Sutter A, Vinson C, Ratineau C, Chayvialle J, and Cordier-Bussat M.** Peptones stimulate intestinal cholecystokinin gene transcription via cyclic adenosine monophosphate response element-binding factors. *Endocrinology* 142: 721–729, 2001.
3. **Buchan AMJ.** Nutrient tasting and signaling mechanisms in the gut. III. Endocrine cell recognition of luminal nutrients. *Am J Physiol Gastrointest Liver Physiol* 277: G1103–G1107, 1999.
4. **Bufe B, Breslin PA, Kuhn C, Reed DR, Tharp CD, Slack JP, Kim UK, Drayna D, and Meyerhof W.** The molecular basis of individual differences in phenylthiocarbamide and propylthiouracil bitterness perception. *Curr Biol* 15: 322–327, 2005.
5. **Catterall WA, Striessnig J, Snutch TP, and Perez-Reyes E.** International Union of Pharmacology. XL. Compendium of voltage-gated ion channels: calcium channels. *Pharmacol Rev* 55: 579–581, 2003.
6. **Chandrashekar J, Mueller KL, Hoon MA, Adler E, Feng L, Guo W, Zuker CS, and Ryba NJP.** T2Rs function as bitter taste receptors. *Cell* 100: 703–711, 2000.
7. **Chang CH, Chey WY, Braggins L, Coy DH, and Chang TM.** Pituitary adenylate cyclase-activating polypeptide stimulates cholecystokinin secretion in STC-1 cells. *Am J Physiol Gastrointest Liver Physiol* 271: G516–G523, 1996.
8. **Chang CH, Chey WY, and Chang TM.** Cellular mechanism of sodium oleate-stimulated secretion of cholecystokinin and secretin. *Am J Physiol Gastrointest Liver Physiol* 279: G295–G303, 2000.
9. **Chang CH, Chey WY, Erway B, Coy DH, and Chang TM.** Modulation of secretin release by neuropeptides in secretin-producing cells. *Am J Physiol Gastrointest Liver Physiol* 275: G192–G202, 1998.
10. **Chang CH, Chey WY, Sun Q, Leiter A, and Chang TM.** Characterization of the release of cholecystokinin from a murine neuroendocrine tumor cell line, STC-1. *Biochim Biophys Acta* 1221: 339–347, 1994.
11. **Cheung AT, Dayanandan B, Lewis JT, Korbutt GS, Rajotte RV, Bryer-Ash M, Boylan MO, Wolfe MM, and Kieffer TJ.** Glucose-dependent insulin release from genetically engineered K cells. *Science* 290: 1959–1962, 2000.
12. **Dahl M, Erickson RP, and Simon SA.** Neural responses to bitter compounds in rats. *Brain Res* 756: 22–34, 1997.
13. **Dockray GJ.** Luminal sensing in the gut: an overview. *J Physiol Pharmacol* 54: 9–17, 2003.
14. **Dolphin GL.** The AC Brown Prize Lecture: voltage-dependent calcium channels and their modulation by neurotransmitters and G proteins. *Exp Physiol* 80: 1–36, 1995.
15. **Dyer J, Salmon KS, Zibrik L, and Shirazi-Beechey SP.** Expression of sweet taste receptors of the T1R family in the intestinal tract and enteroendocrine cells. *Biochem Soc Trans* 33: 302–305, 2005.
16. **Ervin GN, Birkemo LS, Johnson MF, Conger LK, Mosher JT, and Menius JA Jr.** The effects of anorectic and aversive agents on deprivation-induced feeding and taste aversion conditioning in rats. *J Pharmacol Exp Ther* 273: 1203–1210, 1995.
17. **Ferreri-Jacobia M, Mak DOD, and Foskett JK.** Translational mobility of the type 3 inositol 1,4,5-trisphosphate receptor Ca^{2+} release channel in endoplasmic reticulum membrane. *J Biol Chem* 280: 3824–3831, 2005.
18. **Finch EA and Augustine GJ.** Local calcium signalling by inositol-1,4,5-trisphosphate in Purkinje cell dendrites. *Nature* 396: 753–756, 1998.
19. **Furness JB, Kunze WAA, and Clerc N.** Nutrient tasting and signaling mechanisms in the Gut II. The intestine as a sensory organ: neural, endocrine, and immune responses. *Am J Physiol Gastrointest Liver Physiol* 277: G922–G928, 1999.
20. **Gevrey JC, Malapel M, Philippe J, Mithieux G, Chayvialle JA, Abello J, and Cordier-Bussat M.** Protein hydrolysates stimulate proglucagon gene transcription in intestinal endocrine cells via two elements related to cyclic AMP response element. *Diabetologia* 47: 926–936, 2004.
21. **Glendinning JI, Tarre M, and Asaoka K.** Contribution of different bitter-sensitive taste cells to feeding inhibition in a caterpillar (*Manduca sexta*). *Behav Neurosci* 113: 840–854, 1999.
22. **Guilmeau S, Buyse M, Tsocas A, Laigneau JP, and Bado A.** Duodenal leptin stimulates cholecystokinin secretion: evidence of a positive leptin-cholecystokinin feedback loop. *Diabetes* 52: 1664–1672, 2003.
23. **Halatchev IG and Cone RD.** Peripheral administration of PYY3–36 produces conditioned taste aversion in mice. *Cell Metabolism* 1: 159–168, 2005.
24. **Herness MS and Gilbertson TA.** Cellular mechanisms of taste transduction. *Annu Rev Physiol* 61: 873–900, 1999.
25. **Herness MS and Gilbertson TA.** Cellular mechanisms of taste transduction. *Annu Rev Physiol* 61: 873–900, 1999.
26. **Hirasawa A, Tsumaya K, Awaji T, Katsuma S, Adachi T, Yamada M, Sugimoto Y, Miyazaki S, and Tsujimoto G.** Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. *Nat Med* 11: 90–94, 2005.
27. **Hoefer D and Drenckhahn D.** Identification of the taste cell G-protein, α-gustducin, in brush cells of the rat pancreatic duct system. *Histochem Cell Biol* 110: 303–309, 1998.
28. **Hoefer D, Pueschel B, and Drenckhahn D.** Taste receptor-like cells in the rat gut identified by expression of α-gustducin. *Proc Natl Acad Sci USA* 93: 6631–6634, 1996.
29. **Hofmann T, Chubanov V, Gudermann T, and Montell C.** TRPM5 is a voltage-modulated and Ca^{2+}-activated monovalent selective cation channel. *Curr Biol* 13: 1153–1158, 2003.
30. **Huang L, Shanker YG, Dubauskaite J, Zheng JZ, Yan W, Rosenzweig S, Spielman AI, Max M, and Margolskee RF.** Gγ13 colocalizes with gustducin in taste receptor cells and mediates IP_3 responses to bitter denatonium. *Nat Neurosci* 2: 1055–1062, 1999.
31. **Jepeal LI, Fujitani Y, Boylan MO, Wilson CN, Wright CV, and Wolfe MM.** Cell-specific expression of glucose-dependent-insulinotropic polypeptide is regulated by the transcription factor PDX-1. *Endocrinology* 146: 383–391, 2005.
32. **Ji TH, Grossmann M, and Ji I.** G protein-coupled receptors. I. Diversity of receptor-ligand interactions. *J Biol Chem* 273: 17299–17302, 1998.
33. **Kasai H.** Comparative biology of Ca^{2+}-dependent exocytosis: implications of kinetic diversity for secretory function. *Trends Neurosci* 22: 88–93, 1999.
34. **Katz DB, Nicolelis MAL, and Simon SA.** Nutrient tasting and signaling mechanisms in the gut. IV. There is more to taste than meets the tongue. *Am J Physiol Gastrointest Liver Physiol* 278: G6–G9, 2000.
35. **Kim UK, Jorgenson E, Coon H, Leppert M, Risch N, and Drayna D.** Positional cloning of the human quantitative trait locus underlying taste sensitivity to phenylthiocarbamide. *Science* 299: 1221–1225, 2003.
36. **Larsson KP, Akerman KE, Magga J, Uotila S, Kukkonen JP, Nasman J, and Herzig KH.** The STC-1 cells express functional orexin-A receptors coupled to CCK release. *Biochem Biophys Res Commun* 309: 209–216, 2003.
37. **Lay JM, Bane G, Brunkan CS, Davis J, Lopez-Diaz L, and Samuelson LC.** Enteroendocrine cell expression of a cholecystokinin gene construct in transgenic mice and cultured cells. *Am J Physiol Gastrointest Liver Physiol* 288: G354–G361, 2005.
38. **Liddle RA.** Cholecystokine cells. *Annu Rev Physiol* 59: 221–242, 1997.
39. **Liu D and Liman ER.** Intracellular Ca^{2+} and the phospholipid PIP2 regulate the taste transduction ion channel TRPM5. *Proc Natl Acad Sci USA* 100: 15160–15165, 2003.
40. **Lomax RB, Gallego S, Novalbos J, Garcia AG, and Warhurst G.** L-type calcium channels in enterochromaffin cells from guinea pig and human duodenal crypts: an in situ study. *Gastroenterology* 117: 1363–1369, 1999.
41. **Lomax RB, McLaughlin JT, Dockray GJ, Thompson DG, and Warhurst G.** Fatty acids stimulate cholecystokinin secretion from STC-1 enteroendocrine cells by Ca^{2+} influx through L-type Ca^{2+} channels. *J Physiology (Cambridge)* 511P: 25P-26P, 1998.
42. **Mangel AW, Scott L, and Liddle RA.** Depolarization-stimulated cholecystokinin secretion is mediated by L-type calcium channels in STC-1 cells. *Am J Physiol Gastrointest Liver Physiol* 270: G287–G290, 1996.
43. **Mangel AW, Scott L, Prpic V, and Liddle RA.** Regulation of cholecystokinin secretion in STC-1 cells by nitric oxide. *Am J Physiol Gastrointest Liver Physiol* 271: G650–G654, 1996.

44. **Margolskee RF.** Molecular mechanisms of bitter and sweet taste transduction. *J Biol Chem* 277: 1–4, 2002.
45. **Masuho I, Tateyama M, and Saitoh O.** Characterization of bitter taste responses of intestinal STC-1 cells. *Chem Senses* 30: 281–290, 2005.
46. **Matsunami H, Montmayeur JP, and Buck LB.** A family of candidate taste receptors in human and mouse. *Nature* 404: 601–604, 2000.
47. **Ming D, Ninomiya Y, and Margolskee RF.** Blocking taste receptor activation of gustducin inhibits gustatory responses to bitter compounds. *Proc Natl Acad Sci USA* 96: 9903–9908, 1999.
48. **Ming D, Ruiz-Avila L, and Margolskee RF.** Characterization and solubilization of bitter-responsive receptors that couple to gustducin. *Proc Natl Acad Sci USA* 95: 8933–8938, 1998.
49. **Montell C, Birnbaumer L, and Flockerzi V.** The TRP channels, a remarkably functional family. *Cell* 108: 595–598, 2002.
50. **Murai A, Noble PM, Deavall DG, and Dockray GJ.** Control of c-fos expression in STC-1 cells by peptidomimetic stimuli. *Eur J Pharmacol* 394: 27–34, 2000.
51. **Nakamura J, Suda T, Ogawa Y, Takeo T, Suga S, and Wakui M.** Protein kinase C-dependent and -independent inhibition of Ca^{2+} influx by phorbol ester in rat pancreatic β-cells. *Cell Signal* 13: 199–205, 2001.
52. **Nelson TM, Munger SD, and Boughter JD Jr.** Taste sensitivities to PROP and PTC vary independently in mice. *Chem Senses* 28: 695–704, 2003.
53. **Nemoz-Gaillard E, Bernard C, Abello J, Cordier-Bussat M, Chayvialle JA, and Cuber JC.** Regulation of cholecystokinin secretion by peptones and peptidomimetic antibiotics in STC-1 cells. *Endocrinology* 139: 932–938, 1998.
54. **Nemoz-Gaillard E, Cordier-Bussat M, Filloux C, Cuber JC, Van Obberghen E, Chayvialle JA, and Abello J.** Bombesin stimulates cholecystokinin secretion through mitogen-activated protein-kinase-dependent and -independent mechanisms in the enteroendocrine STC-1 cell line. *Biochem J* 331: 129–135, 1998.
55. **Perez CA, Huang L, Rong M, Kozak JA, Preuss AK, Zhang H, Max M, and Margolskee RF.** A transient receptor potential channel expressed in taste receptor cells. *Nat Neurosci* 5: 1169–1176, 2002.
56. **Perez CA, Margolskee RF, Kinnamon SC, and Ogura T.** Making sense with TRP channels: store-operated calcium entry and the ion channel Trpm5 in taste receptor cells. *Cell Calcium* 33: 541–549, 2003.
57. **Prawitt D, Monteilh-Zoller MK, Brixel L, Spangenberg C, Zabel B, Fleig A, and Penner R.** TRPM5 is a transient Ca^{2+}-activated cation channel responding to rapid changes in $[Ca^{2+}]_i$. *Proc Natl Acad Sci USA* 100: 15166–15171, 2003.
58. **Ratineau C, Dreau S, Blanc M, Bernard C, Cordier-Bussat M, Abello J, Chayvialle J, and Roche C.** CCK expression in enteroendocrine cell is regulated by soluble factor(s) from underlying fibroblasts. *Mol Cell Endocrinol* 175: 5–13, 2001.
59. **Ratineau C, Plateroti M, Dumortier J, Blanc M, Kedinger M, Chayvialle JA, and Roche C.** Intestinal-type fibroblasts selectively influence proliferation rate and peptide synthesis in the murine enteroendocrine cell line STC-1. *Differentiation* 62: 139–147, 1997.
60. **Raybould HE.** Does your gut taste? Sensory transduction in the gastrointestinal tract. *News Physiol Sci* 13: 275–280, 1998.
61. **Rindi G, Grant SGN, Yiangou Y, Ghatei MA, Bloom SR, Bautch VL, Solcia E, and Polak JM.** Development of neuroendocrine tumors in the gastrointestinal tract of transgenic mice: Heterogeneity of hormone expression. *Am J Pathol* 136: 1349–1364, 1990.
62. **Ruiz-Avila L, McLaughlin SK, Wildman D, McKinnon PJ, Robichon A, Spickofsky N, and Margolskee RF.** Coupling of bitter receptor to phosphodiesterase through transducin in taste receptor cells. *Nature* 376: 80–85, 1995.
63. **Ruiz-Avila L, Wong GT, Damak S, and Margolskee RF.** Dominant loss of responsiveness to sweet and bitter compounds caused by a single mutation in α-gustducin. *Proc Natl Acad Sci USA* 98: 8868–8873, 2001.
64. **Schirra J and Goke B.** The physiological role of GLP-1 in human: incretin, ileal brake or more? *Regul Pept* 128: 109–115, 2005.
65. **Scott TR and Verhagen JV.** Taste as a factor in the management of nutrition. *Nutrition* 16: 874–885, 2000.
66. **Sidhu SS, Thompson DG, Warhurst G, Case RM, and Benson RS.** Fatty acid-induced cholecystokinin secretion and changes in intracellular Ca^{2+} in two enteroendocrine cell lines, STC-1 and GLUTag. *J Physiol* 528: 165–176, 2000.
67. **Snow ND, Prpic V, Mangel AW, Sharara AI, McVey DC, Hurst LJ, Vigna SR, and Liddle RA.** Regulation of cholecystokinin secretion by bombesin in STC-1 cells. *Am J Physiol Gastrointest Liver Physiol* 267: G859–G865, 1994.
68. **St. John SJ, Pour L, and Boughter JD, Jr.** Phenylthiocarbamide produces conditioned taste aversions in mice. *Chem Senses* 30: 377–382, 2005.
69. **Straub SG, Mulvaney-Musa J, Yajima H, Weiland GA, and Sharp GW.** Stimulation of insulin secretion by denatonium, one of the most bitter-tasting substances known. *Diabetes* 52: 356–364, 2003.
70. **Taylor CW and Broad LM.** Pharmacological analysis of intracellular Ca^{2+} signalling: problems and pitfalls. *Trends Pharmacol Sci* 19: 370–375, 1998.
71. **Thastrup O, Cullen PJ, Drøbak BK, Hanley MR, and Dawson AP.** Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. *Proc Natl Acad Sci USA* 87: 2466–2470, 1990.
72. **Turkelson CM, Dale WE, Reidelberger R, and Solomon TE.** Development of cholecystokinin radioimmunoassay using synthetic CCK-10 as immunogen. *Regul Pept* 15: 205–217, 1986.
73. **Wong GT, Gannon KS, and Margolskee RF.** Transduction of bitter and sweet taste by gustducin. *Nature* 381: 796–800, 1996.
74. **Wu SV, Chen MC, and Rozengurt E.** Genomic organization, expression, and function of bitter taste receptors (T2R) in mouse and rat. *Physiol Genomics* 22: 139–149, 2005.
75. **Wu SV, Rozengurt N, Yang M, Young SH, Sinnett-Smith J, and Rozengurt E.** Expression of bitter taste receptors of the T2R family in the gastrointestinal tract and enteroendocrine STC-1 cells. *Proc Natl Acad Sci USA* V99: 2392–2397, 2002.
76. **Wu X, Zhang T, Bossuyt J, Li X, McKinsey TA, Dedman JR, Olson EN, Chen J, Brown JH, and Bers DM.** Local $InsP_3$-dependent perinuclear Ca^{2+} signaling in cardiac myocyte excitation-transcription coupling 10.1172/JCI27374. *J Clin Invest* 116: 675–682, 2006.
77. **Yang L, Liu G, Zakharov SI, Morrow JP, Rybin VO, Steinberg SF, and Marx SO.** Ser1928 is a common site for Cav1.2 phosphorylation by protein kinase C isoforms. *J Biol Chem* 280: 207–214, 2005.
78. **Yi F, Brubaker PL, and Jin T.** TCF-4 mediates cell type-specific regulation of proglucagon gene expression by β-catenin and glycogen synthase kinase-3β. *J Biol Chem* 280: 1457–1464, 2005.
79. **Yoon J and Beinfeld MC.** Prohormone convertase 1 is necessary for the formation of cholecystokinin 8 in Rin5F and STC-1 cells. *J Biol Chem* 272: 9450–9456, 1997.
80. **Yoon J and Beinfeld MC.** Prohormone convertase 2 is necessary for the formation of cholecystokinin-22, but not cholecystokinin-8, in RIN5F and STC-1 cells. *Endocrinology* 138: 3620–3623, 1997.
81. **Zhang Y, Hoon MA, Chandrashekar J, Mueller KL, Cook B, Wu D, Zuker CS, and Ryba NJ.** Coding of sweet, bitter, and umami tastes: different receptor cells sharing similar signaling pathways. *Cell* 112: 293–301, 2003.
82. **Zhao FL, Lu SG, and Herness S.** Dual actions of caffeine on voltage-dependent currents and intracellular calcium in taste receptor cells. *Am J Physiol Regul Integr Comp Physiol* 283: R115–R129, 2002.