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RESEARCH ARTICLE | *Hormones, Reproduction and Development*

Cholecystokinin, gastrin, cholecystokinin/gastrin receptors, and bitter taste receptor TAS2R14: trophoblast expression and signaling

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Taher S, Borja Y, Cabanela L, Costers VJ, Carson-Marino M, Bailes JC, Dhar B, Beckworth MT, Rabaglino MB, Post Uiterweer ED, Conrad KP. Cholecystokinin, gastrin, cholecystokinin/gastrin receptors, and bitter taste receptor TAS2R14: trophoblast expression and signaling. *Am J Physiol Regul Integr Comp Physiol* 316: R628–R639, 2019. First published March 20, 2019; doi:10.1152/ajpregu.00153.2018.—We investigated expression of cholecystokinin (CCK) in humans and mice, and the bitter taste receptor TAS2R14 in the human placenta. Because CCK and gastrin activate the CCKBR receptor, we also explored placental gastrin expression. Finally, we investigated calcium signaling by CCK and TAS2R14. By RT-PCR, we found *CCK/Cck* and *GAST/Gast* mRNA expression in both normal human and mouse placentas, as well as in human trophoblast cell lines (TCL). Although both *Cckar* and *–br* mRNA were expressed in the mouse placenta, only *CCKBR* mRNA was detected in the human placenta and TCL. mRNA expression for *TAS2R14* was also observed in the human placenta and TCL. Using immunohistochemistry, CCK protein was localized to the syncytiotrophoblast (ST) and extravillous trophoblast (EVT) in the human term placenta, and to trophoblast glycogen cells in mouse and human placentas. Gastrin and TAS2R14 proteins were also observed in ST and EVT of the human placenta. Both sulfated and nonsulfated CCK elicited a comparable rise in intracellular calcium in TCL, consistent with *CCKBR* expression. Three TAS2R14 agonists, flufenamic acid, chlorhexidine, and diphenhydramine, also evoked rises in intracellular calcium in TCL. These results establish CCK, gastrin, and their receptor(s) in both human and mouse placentas, and TAS2R14 in the human placenta. Both CCK and TAS2R14 agonists increased intracellular calcium in human TCL. Although the roles of these ligands and receptors, and their potential cross talk in normal and pathological placentas, are currently unknown, this study opens new avenues for placental research.

extravillous trophoblast; intracellular calcium; placenta; syncytiotrophoblast; trophoblast glycogen cell

INTRODUCTION

In a microarray analysis of first-trimester chorionic villous samples (CVS; GSE12767), we observed expression of cholecystokinin (CCK), which was one of the most highly upregulated genes in CVS from women who later developed preeclampsia compared with women who experienced a normal pregnancy (9, 28). Interestingly, microarray studies available in the public domain of delivered placentas (or cytotrophoblast isolated thereof) from preeclamptic women also demonstrated increased *CCK* expression relative to normal pregnancy [GSE40182 (52) and (32)]. In fact, as portrayed in Fig. 1 (previously unpublished), by using bioinformatics approaches similar to those we earlier described (28), *CCK* was found to be one of the few differentially expressed genes in common between first-trimester CVS and delivered placentas from women afflicted with preeclampsia. Therefore, we reasoned that this virtually unique confluence could be more than coincidental, and placental CCK may be of particular biological significance and worthy of further consideration. CCK mRNA was previously identified in placentas of women and mice during genome-wide gene expression profiling and showed increased expression in cells of unidentified origin in the metrial region of placentas from diabetic mice by in situ hybridization (37, 39). Otherwise, to the best of our knowledge, placental CCK has received little, if any, investigative attention.

Bitter taste receptor (TAS2R) stimuli were recently linked to CCK secretion in enteroendocrine cells of the gastrointestinal tract [(6, 13, 14, 49); and DISCUSSION]. We unexpectedly observed expression of *TAS2R14* in our CVS microarray (GSE12767 but not of other *TAS2R* genes in the microarray: *TAS2R1, TAS2R3–5, TAS2R7–10, TAS2R13, TAS2R16, TAS2R19, TAS2R38–41, TAS2R45* or *TAS2R50*), although TAS2R38 was recently reported in normal delivered placentas (48). Because of the potential linkage between CCK and bitter taste receptors, we also set out to investigate the bitter taste receptor TAS2R14 in the human placenta.

Admittedly, the work reported herein was mainly discovery based rather than being hypothesis driven, because it arose serendipitously in large measure from the tantalizing data found in microarray analyses (vide supra). Nevertheless, the specific objectives of this work were a logical extension of the observations derived from the microarray analyses, and they were carefully conceived and designed. Specifically, we inves-

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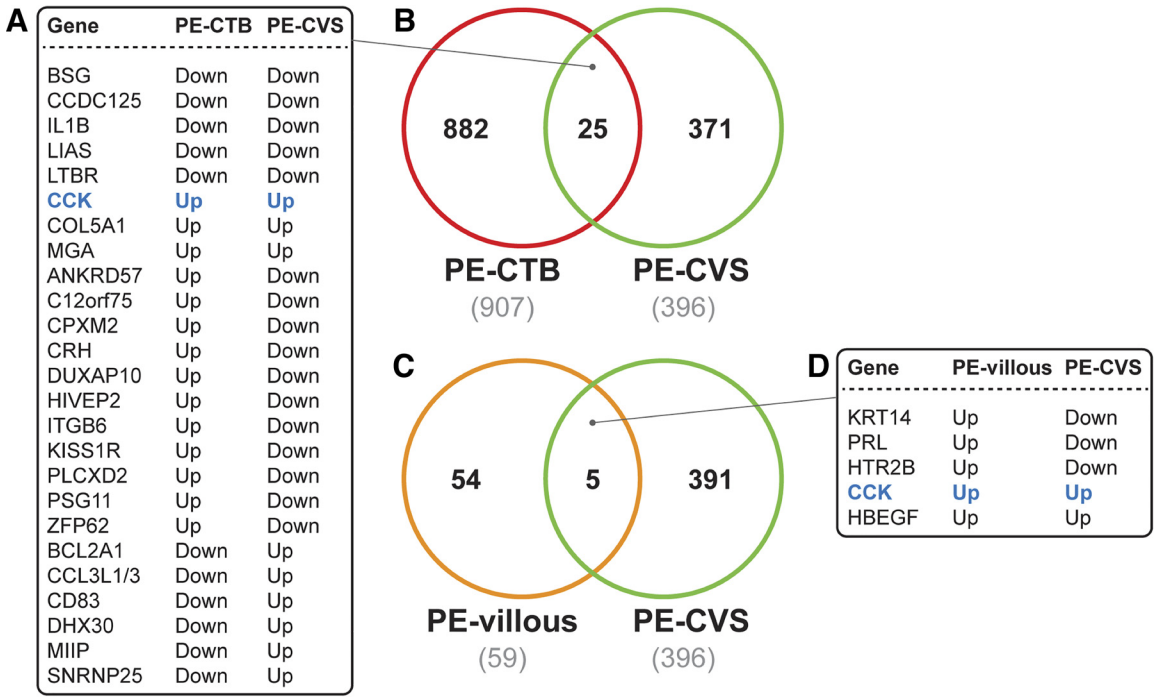

Fig. 1. Overlap of differentially expressed genes (DEG) between preeclamptic (PE) and normal pregnancies in chorionic villous samples (CVS) and delivered placentas. *A* and *B*: PE-CTB, DEG between cytotrophoblasts isolated from villous placentas delivered by women with severe PE (PE-CTB) relative to gestational age-matched preterm (noninfected) controls (52; GSE40182); PE-CVS, DEG between CVS from women who developed severe PE relative to normal pregnancy outcome (28; GSE12767). Twenty-five DEG overlapped between the CVS and PE-CTB, but only 8 DEG changed in the same direction in the CVS and PE-CTB; CCK was upregulated in both. *C* and *D*: PE-villous, DEG between villous tissue from placentas delivered by women with PE compared with normal pregnancy controls (data obtained from Table II in Ref. 32); PE-CVS. Five DEG overlapped between the CVS and villous tissue from delivered placentas with only 2 changing in the same direction, one of which was CCK that was upregulated.

tigated *1*) *CCK*/CCK and *TAS2R14*/TAS2R14 mRNA and protein expression in early and late gestational human placentas, and in human trophoblast cell lines; *2*) *Cck*/CCK mRNA and protein expression in mouse placentas; *3*) CCK mRNA receptor expression (*CCKAR*/*Cckar* and *–BR*/*br*) in early and late gestational human placentas, trophoblast cell lines, and in mouse placentas; and *4*) CCK and TAS2R14 calcium signaling in trophoblast cell lines. Because gastrin (GAST) utilizes CCKBR as its receptor, we also investigated *GAST*/GAST mRNA and protein expression in human placental tissues and trophoblast cell lines, as well as *Gast* mRNA in mouse placenta. Although a complete understanding of the physiological and potential pathophysiological roles of CCK, gastrin, and TAS2R14, as well as their potential interactions in the placenta require further investigation, herein we establish CCK, GAST, and CCK receptor expression by human and mouse trophoblasts, TAS2R14 expression by human trophoblasts, and further demonstrate that CCK and TASR14 receptor ligands are both coupled to calcium signaling, CCK mostly, if not exclusively, through CCKBR.

MATERIALS AND METHODS

Human placentas. Human placentas were obtained from uncomplicated pregnancies after delivery under Institutional Review Board (IRB) approval from the University of Florida (IRB 201602330). Tissue cryosections of first- and second-trimester placentas were provided by the National Institutes of Health Placental Bank at the University of California San Francisco (NICHD/NIH HD055764) under IRB approval from the University of Florida (IRB 201600936).

Human trophoblast cell lines. Trophoblast cell lines were propagated in T75 flasks at 37°C under standard tissue culture conditions (5% CO_2-balance room air). The HTR-8/SVneo trophoblast cell line derived from first-trimester human villous trophoblasts was generously provided by Dr. Charles Graham, Queens University (12). We previously corroborated the trophoblast origin of these cells in our laboratory (26). HTR-8/SVneo cells were cultured in T75 flasks containing complete media: RPMI 1640 with glucose (2.0 g/l), L-glutamine (0.3 g/l), and sodium bicarbonate (2.0 g/l). JAR, JEG, and BeWo choriocarcinoma cells are trophoblast derived and they were purchased from the American Type Culture Collection. JAR cells were cultured in the same media as described for the HTR-8/SVneo cells. JEG cells were cultured in EMEM containing L-glutamine (0.3 g/l) and sodium pyruvate (0.1 g/l). BeWo cells were propagated in F12 Nutrient Mixture Kaighn's Modification with L-glutamine (0.3 g/l). All media were supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml; Sigma, St. Louis, MO, or Corning, Mediatech, Manassas, VA), and 10% FBS (GE Healthcare Life Sciences, Hyclone Laboratories).

Mouse placentas. Placentas were harvested from pregnant C57BL/6J mice of 13.5, 14.5, 15.5, and 17.5 gestational days under Institutional Animal Care and Use Committee approval from University of Florida (IACUC 201508950).

RT-PCR. Total RNA was extracted from placental tissues and trophoblast cell lines using TRI Reagent (Ambion/ThermoFisher Scientific) according to the manufacturer's instructions and then dissolved in RNA secure solution (Ambion/ThermoFisher Scientific). RNA was treated with DNase (Ambion/Thermo Fisher Scientific) to remove any contaminating genomic DNA. The yield and purity of the resulting RNA preparations were determined by NanoDrop spectrophotometer. Total RNA from mouse placenta (E17) was obtained

from Zyagen (San Diego, CA), and human brain cortex, stomach, and duodenum RNA were purchased from Clontech Laboratories (Mountain View, CA). First-strand cDNA was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) or M-MLV Reverse Transcriptase (Promega, Madison, WI) and Oligo (dT_{15}) primers (Promega) according to the manufacturer's instructions. PCR was performed with 1 μl of cDNA in a 19-μl reaction containing (final concentration) 1× Green Go Taq Flexi Buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM each of forward and reverse primers, and 0.5 units GoTaq DNA Polymerase per reaction (Promega). Intron or exon/exon-spanning primers were designed using OligoPerfect (Invitrogen/ThermoFisher Scientific) or Primer-Blast (NCBI-NIH), and synthesized by Integrated DNA Technologies (Coralville, IA) (see Supplemental Table S1; all supplemental material can be accessed on the journal website). Twenty to forty cycles were performed on a Mastercycler Gradient PCR machine (Eppendorf, Hauppauge, NY) at 94°C for 30 s, 50–60°C for 1 min, and 72°C for 1 min with an initial 3 min at 80°C and a final 10 min at 72°C. PCR products were then electrophoresed on 1–2% agarose gels containing ethidium bromide or GelRed (ThermoFisher Scientific) and visualized under UV light using a VersaDoc system (Bio-Rad, Hercules, CA). A 100-bp DNA ladder (ThermoFisher Scientific) was run on each gel. For negative controls, nuclease-free water was substituted for RT or nuclease-free water replaced the cDNA in the PCR reaction.

Immunohistochemistry. Human villous and decidual basal plate tissues from normal delivered placentas, and whole mouse placentas including decidua were fixed in fresh 4% phosphate-buffered formalin for 18–24 h at 4°C followed by decanting and then three rinses in 10 ml ice-cold PBS for 10 min each on ice using a magnetic stir bar. The tissues were stored in 70% ethanol at 4°C until further processing. After being embedded in paraffin, 4-μm-thick sections were cut from the tissue blocks and transferred to SuperFrost Plus slides (ThermoFisher Scientific). After drying, the slides were deparaffinized in xylene and ethanol solutions. The sections were next dried for >5 min before permeabilization with 0.3% Triton X-100 and quenching of endogenous peroxidase with 1% hydrogen peroxide in methanol. After blocking of the sections with 1.5% normal horse serum, the sections were incubated with the primary or non-immune isotype antibody overnight at 4°C in a humidified chamber. In some instances, the primary antibody was preabsorbed with blocking peptide as an additional negative control. Supplemental Table S2 contains the list of primary and nonimmune isotype antibodies used in each figure or supplemental figure. The Supplemental Methods, including Supplemental Figs. S1 and S2, provide further details about the primary antibodies.

On the following day, the sections were incubated with a secondary anti-goat or anti-mouse/anti-rabbit biotinylated antibody for 30 min at room temperature in a humidified chamber. Immunodetection was subsequently performed using a Vectastain ABC Elite kit and DAB Peroxidase Substrate Kit (both from Vector Laboratories, Burlingame, CA). After dehydration of the sections in ethanol and xylene solutions, they were sealed with coverslips using Cytoseal XYL (ThermoFisher Scientific). The immunostained sections were visualized using an Olympus BX41 System Microscope (Olympus, Tokyo, Japan), photographed with an Olympus DP71 Microscope Digital Camera, and saved with DP Controller and Manager.

The early placental human placental cryosections were warmed to room temperature and fixed for 20 min with 4% phosphate-buffered formalin followed by three rinses in PBS. Thereafter, the procedures were identical to those described above.

Intracellular calcium. After reaching confluence in the T75 flasks, the trophoblast cell lines were passaged into 35-mm glass bottom petri dishes (MatTek, Ashland, MA). When the cells were 75–90% confluent, they were rinsed once with DPBS containing Ca^{2+}/Mg^{2+}, and then incubated in 0.1% Pluronic F-127 containing 5.0 μg/ml (4.6 μM) of Fluo 4-AM (ThermoFisher Scientific) in physiological saline solution (PSS: 140 mM NaCl, 5.0 mM KCl, 1.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, and 0.1% wt/vol BSA) for 25 min at 37°C. The cells were then rinsed three times with PSS and the dishes placed on a heated stage of the fluorescent microscope. Baseline fluorescence (480-nm excitation, 535-nm emission) was measured for 60 s followed by the addition of sulfated CCK (sCCK) or nonsulfated cholecystokinin (CCK). Alternatively, three agonists of TAS2R14 were introduced, either chlorohexidine (CHLX), flufenamic acid (FFA), or diphenhydramine (15, 16, 24, 33). The calcium ionophore A23187 was used as a positive control. Fluorescent intensity was subsequently measured for an additional 4 min. The fluorescent signal was captured with a Nikon Eclipse Ti-E Inverted Fluorescent Microscope (Nikon Instruments, Melville, NY) using a Andor Zyla sCMOS Monochrome Camera (Andor Technology, Belfast, UK). The signals were subsequently analyzed using Nikon NIS-Elements software. The fluorescent excitation source was a Lumencor Spectra X Light Engine (Lumencor, Beaverton, OR).

RESULTS

RT-PCR: mRNA expression. Because our objective was to detect mRNA expression, we used standard RT-PCR. Human placental villous and decidual basal plate tissues, as well as two trophoblast cell lines expressed *CCK* mRNA as determined by two different primer pair sets (Fig. 2*A*, *top* and *middle*). Mouse placenta (*E.17*) expressed *Cck* mRNA, predominantly *variant 1* as assessed by two different primer pair sets (Fig. 2*B*, *left* and *right*). *Variant 2* was faintly detected with one of the primer pair sets (Fig. 2*B*, *right*). Either *CCKBR variant 1*, *variant 2*, or both mRNAs (but not *CCKAR* mRNA; data not shown) were weakly expressed by human placental villous and decidual basal plate tissues, as well as by trophoblast cell lines as measured by partial nested primer RT-PCR (Fig. 2*C*). In contrast, both *Cckbr* and *–ar* mRNA were detected in mouse placenta (*E.17*) using either one primer pair set or nested primer pairs (Fig. 2*D*, *left* and *right*, respectively). Figure 3*A* (*top* and *middle*) shows *TAS2R14* and *GAST* mRNA expression, respectively, in human placental villous and decidual basal plate tissues, as well as two trophoblast cell lines. Finally, *gast* mRNA was also detected in the *E.17* mouse placenta (Fig. 3*B*).

Immunohistochemistry: protein expression. We identified CCK expression using two different CCK antibodies, one directed against the COOH-, and another against the NH_2-terminus. Importantly, these CCK antibodies were generated against human peptide sequences spanning amino acids 50–100 ("COOH"-terminus) and 20–70 ("NH_2"-terminus) each with little or no overlap with preprogastrin: 7/50 and 8/50 amino acids, respectively. These antibodies did not discriminate among the numerous active CCKs generated by proteolysis of the 115-amino acid prepropeptide; however, such discrimination was not an objective of this work and will be the subject of future investigation. In contrast, there is marked homology of human and mouse CCK such that 44/50 and 35/50 amino acids were overlapping for recognition of mouse CCK by the COOH- and NH_2-terminus antibodies, respectively.

With the use of the CCK antibody directed against the COOH-terminus, CCK (Fig. 4*A*, *a*, *c*, *e*, and *g*) was colocalized with cytokeratin (Fig. 4*Ah*), demonstrating expression by syncytiotrophoblasts (ST) and extravillous trophoblasts (EVT) in normal human term placenta ($n = 5$ placentas). CCK staining was inhibited by preabsorption with the COOH-, but not NH_2-terminus-blocking peptide, the latter serving as another

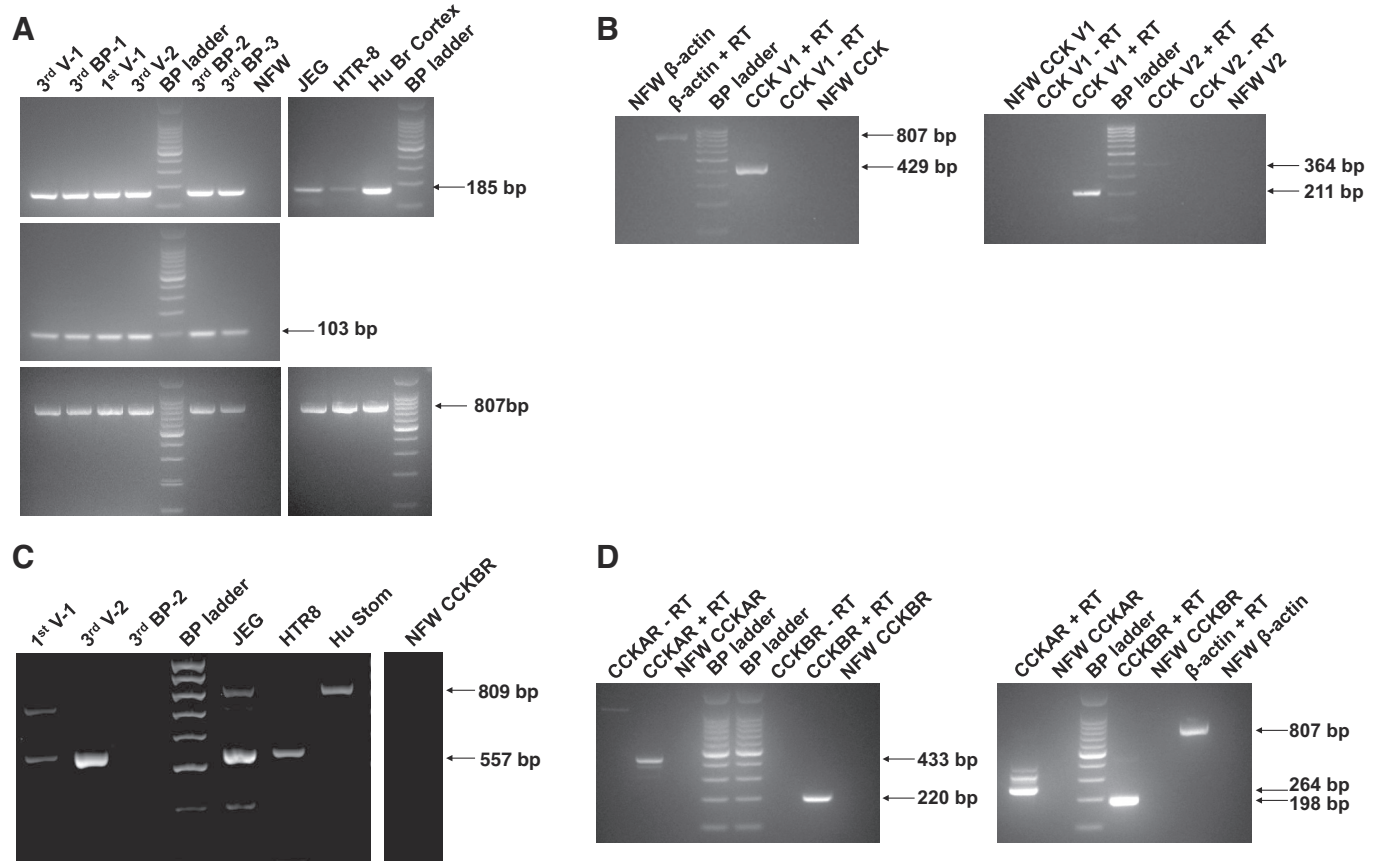

Fig. 2. *A*: expression of cholecystokinin (*CCK*) mRNA by human placenta and trophoblast cell lines using RT-PCR. *Top*: one primer pair yielded an amplicon with a predicted size of 185 bp. *Middle*: a second primer set yielded an amplicon with a predicted size of 103 bp. *Bottom*: actin β (*ACTB*, 807 bp). See Supplemental Table S1A for details of human primer sets. 3rd, third trimester; 1st, first trimester; V, villous; BP, basal plate; −1, −2, −3, 3 different placentas; JEG, JEG choriocarcinoma cells; HTR-8, HTR-8/SVneo immortalized 1st-trimester extravillous trophoblast cells; Hu Br Cortex, human brain cortex (positive control); NFW, nuclease-free water substituted for cDNA in the PCR (negative control); BP ladder, base pair ladder (bright band of 500 bp). *B*: expression of *Cck* mRNA by mouse placenta (*E.17*) using RT-PCR. *Left*: one primer pair yielded an amplicon with a predicted size of 429 bp consistent with *Cck variant 1* (V1). However, using the same primer pair, an amplicon with a predicted size 529 bp for *Cck variant 2* (V2) was absent. *Actb* (807 bp). *Right*: using a second primer set unique for *Cck* V1 yielded an amplicon with a predicted size of 211 bp, and a faint band of 364 bp was obtained using another primer set specific for *Cck* V2. See Supplemental Table S1B for details of mouse primer sets. + and – RT, with and without reverse transcriptase. *C*: expression of *CCKBR* receptor mRNA by human placenta and trophoblast cell lines using RT-PCR. Partial nested primer PCR revealed amplicons of predicted size of 557 and 809 bp for *CCKBR* V2 and *CCKBR* V1, respectively. *D*: expression of *Cckar* and *-br* receptor mRNA by mouse placenta (*E.17*) using RT-PCR. *Left*: outer primer pairs for *Cckar* and *–br* yielded amplicons of predicted size, i.e., 433 and 220 bp, respectively. *Right*: amplicons generated using the outer primer pairs were subsequently amplified with inner primer pairs producing amplicons of predicted size for *Cckar* and *–br*, respectively, 264 and 198 bp. There were 2 higher, unidentified bands generated by the *Cckar* nested primer reaction. *Actb* (807 bp).

control (Fig. 4*A*, *d* and *f*, respectively). CCK expression in ST and EVT was corroborated using the CCK antibody directed against the NH_2-terminus (Fig. 4*B*). CCK was also expressed by EVT and ST in early gestational placentas ($n = 10$ placentas; Fig. 4*C* and 6*C*, and Supplemental Fig. S3, respectively). In normal human term placenta, there was colocalization of CCK (Fig. 5*A*, *a* and *e*) and cytokeratin (Fig. 5*A*, *b* and *f*) with periodic acid-Schiff-positive, glycogen-expressing trophoblast cells (Fig. 5*A*, *c* and *g*), although many trophoblast cells expressing glycogen did not express CCK and vice versa. Similar coincidences of CCK, cytokeratin, and periodic acid-Schiff staining were observed in trophoblast glycogen cells located in the mouse junctional zone and decidua (Fig. 5*B*, *a*, *b*, and *c*, respectively). Finally, neither CCK-AR nor –BR was detectable by immunohistochemistry in human or mouse placenta (data not shown).

In addition to CCK, GAST was also expressed in ST and EVT of normal human term placenta ($n = 4$ placentas; Fig. 6*A*). Importantly, the antibody for human GAST, which was directed against amino acids 1–13, showed minimal overlap with CCK: 4/13 amino acids. The bitter taste receptor TAS2R14 was detected by immunohistochemistry in EVT of normal human term placenta identified by cytokeratin staining ($n = 5$ placentas) using two different antibodies (Fig. 6*Ba*, rabbit polyclonal anti-TAS2R14 antibody; Fig. 6*Bf*, goat polyclonal anti-TAS2R14 antibody). Many, but not all EVT expressing TAS2R14 also coexpressed CCK (Fig. 6*Bd*). TAS2R14 was also detected in ST (see Supplemental Fig. S3*C*) and EVT of early gestation human placentas and colocalized with CCK at least in some EVT (Fig. 6*C*; $n = 2$ placentas). However, because of the limited number of early-gestation placental sections available for investigation of TAS2R14 expression, additional studies are needed.

Fluo 4 calcium-sensitive dye: calcium signaling. As a point of reference, 1.0 μM A23187, a calcium ionophore, increased intracellular calcium in JEG cells on average by fourfold, with

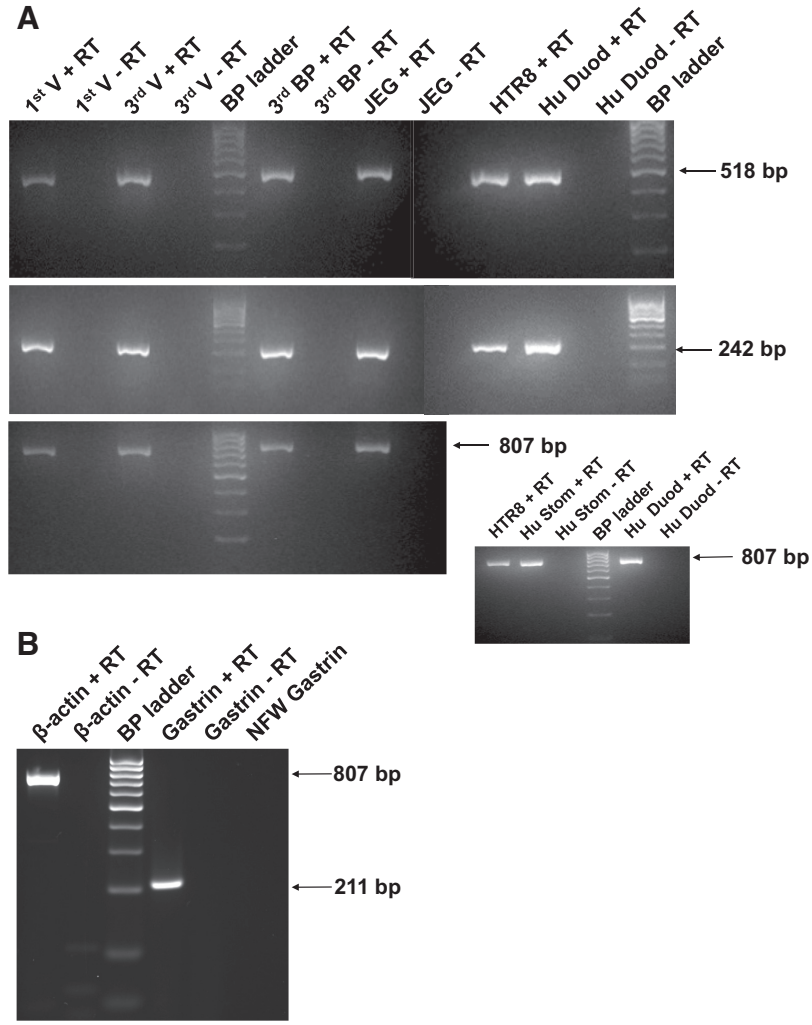

Fig. 3. *A*: expression of human *TAS2R14*, gastrin (*GAST*), and actin β (*ACTB*) mRNA by human placenta and trophoblast cell lines using RT-PCR. *Top*: RT-PCR for *TAS2R14* generated an amplicon of predicted size, 518 bp. *Middle*: RT-PCR for *GAST* resulted in an amplicon with the predicted size of 242 bp. *Bottom*: *ACTB* (807 bp). See Supplemental Table S1A for details of human primer sets. 3rd, third trimester; 1st, first trimester; V, villous; BP, basal plate; JEG, JEG choriocarcinoma cells; HTR-8, HTR-8/SVneo immortalized 1st-trimester extravillous trophoblast cells; Hu Duod, human duodenum (positive control); + and – RT, with and without reverse transcriptase; BP ladder, base pair ladder (bright band of 500 bp). *B*: expression of *Gast* mRNA by mouse placenta (*E.17*) using RT-PCR. RT-PCR generated an amplicon of predicted size, 211 bp. *Actb* (807 bp). See Supplemental Table S1B for details of mouse primer sets. NFW, nuclease free water substituted for cDNA in the PCR reaction (negative control).

100% of cells responding. Both nonsulfated and sulfated CCK stimulated rises in intracellular calcium in JEG cells (Fig. 7, *A* and *B*, respectively). The equimolar doses of sulfated and nonsulfated CCK (10 nM) elicited a comparable increase, suggesting a predominant action through CCKBR. A selective CCKBR antagonist (YM-022) was more effective in blocking the calcium response than a selective CCKAR antagonist (devazepide) (7); see Supplemental Fig. S4). Sulfated CCK was also tested in two other trophoblast cells lines, JAR and BeWo, but the fold-increase in intracellular calcium and the percentage of responding cells were less than for JEG cells (Fig. 7, *C* and *D*). Interestingly, neither 10 nM ($n = 9$ dishes) or 1.0 μM ($n = 6$ dishes) sulfated CCK increased intracellular calcium in the HTR-8/SVneo trophoblast cell line (data not shown).

The TAS2R14 agonist FFA (10, 30, and 100 μM) increased intracellular calcium in a more or less dose-responsive fashion in JEG cells both in regard to the maximum calcium response and percentage of responding cells (Fig. 8*A*, *a–c*). FFA also increased intracellular calcium in JAR, BeWo, and HTR-8/SVneo trophoblast cell lines (Fig. 8*A*, *d–g*). The calcium response to FFA was replicated by another TAS2R14 agonist, CHLX (10 μM) (Fig. 8*B*). However, the pattern of increase was gradual and not immediate for the JEG and HTR-8/SVneo cells. Lower CHLX concentrations of 1.0 and 3.0 μM failed to increase intracellular calcium in any of the four cell lines ($n = 3$ dishes each; data not shown). Yet diphenhydramine, another TAS2R14 agonist, also increased intracellular calcium in JAR cells. A concentration of 0.25 mM did not raise intracellular calcium (data not shown), being below the threshold dose in JAR cells of 0.5 mM with the maximum stimulus being 1.0 mM (see Supplemental Fig. S5) The DMSO vehicle for FFA and CHLX, when diluted to the same degree as 30 and 100 μM FFA, did not affect intracellular calcium ($n = 2$ dishes each; data not shown).

DISCUSSION

CCK: ligands, receptors, and cell signaling. Major findings were that *CCK* mRNA was expressed in third-trimester villous and decidual basal plate of placentas delivered following normal pregnancies, and in first-trimester villous. *CCK* mRNA was observed in choriocarcinoma and HTR-8/SVneo cell lines. CCK protein was detected in the ST and EVT of early-gestation and normal delivered placentas, the latter using two different antibodies, one directed against a COOH-terminus peptide and the other against a NH_2-terminus peptide (with neither peptide having significant homology with gastrin). *Cck*

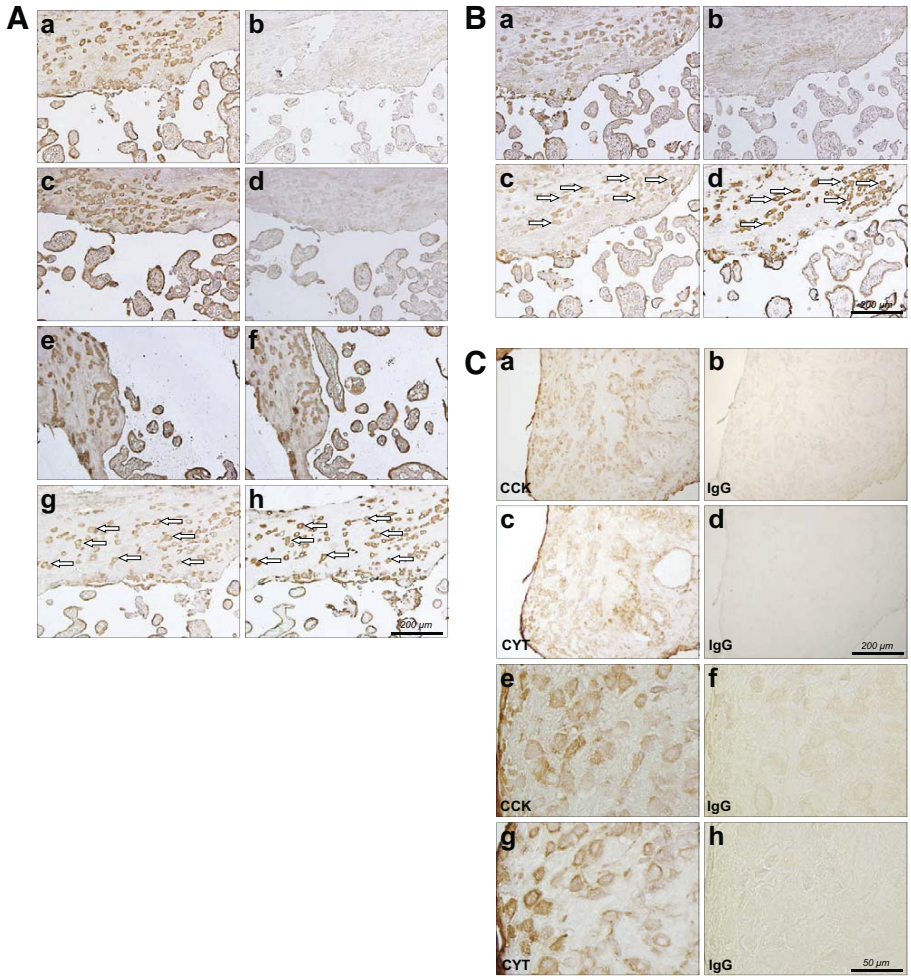

Fig. 4. *A*: localization of cholecystokinin (CCK) protein in syncytiotrophoblast and extravillous trophoblasts of normal delivered human placenta using an antibody directed against the COOH-terminus (×100 magnification). *a*, *c*, *e*, and *g*: Immunostaining with COOH-terminus CCK antibody. *b*: Goat IgG, negative control. *d*: COOH-terminus CCK antibody preabsorbed with CCK COOH-terminus blocking peptide, *f*: COOH-terminus CCK antibody preincubated with CCK NH_2-terminus blocking peptide. *h*: Cytokeratin 18 (CYT) antibody. Arrows indicate cells colocalizing with CCK and cytokeratin 18. *B*: localization of CCK protein in syncytiotrophoblast and extravillous trophoblasts of normal delivered human placenta using an antibody directed against the NH_2-terminus (×100 magnification). *a* and *c*: Immunostaining with NH_2-terminus CCK antibody. *b*: Goat IgG, negative control. *d*: Cytokeratin 18 antibody. Arrows indicate cells colocalizing with CCK and cytokeratin 18. *C*: localization of CCK protein in extravillous trophoblast of early-gestation human placenta (15.4 wk). *a–d*: ×100 Magnification. *e–h*: ×400 Magnification. *a* and *e*: Immunostaining with COOH-terminus CCK antibody. *b* and *f*: Goat IgG, negative control. *c* and *g*: Cytokeratin 18 antibody. *d* and *h*: Mouse IgG, negative control.

mRNA was readily detected in mouse placenta, and protein expression was also observed, predominantly in trophoblast glycogen cells located in the junctional zone and decidua. Finally, *CCKBR/br*, but not *–AR/ar* receptor mRNA, was detected at low levels in human placenta, and both receptor mRNAs were expressed in the mouse placenta. CCK receptors were not detected in the placenta of either species by immunohistochemistry, most likely because of low expression of these GPCRs and/or ineffective antibodies.

Another major observation was that sulfated and nonsulfated CCK stimulated increases in intracellular calcium in human trophoblast cell lines. Equimolar doses of sulfated and nonsulfated CCK elicited comparable increases in intracellular calcium and percentage of responding cells, suggesting a predominate action through CCKBR, because nonsulfated CCK has a much lower affinity than sulfated CCK for CCKAR, whereas their affinities for CCKBR are comparable (7). The concept that CCKBR is the major receptor mediating CCK calcium signaling was further supported by data showing that a CCKBR antagonist, YM-022, completely blocked the calcium response, whereas a CCKAR antagonist, devazepide, was only partly effective (7). Finally, *CCKBR*, but not *–AR* mRNA was detected in human placenta and trophoblast cell lines. A caveat is that at the higher concentrations of CCK, YM-022, and devazepide used in the antagonist protocol, this experiment by itself was not definitive. However, by taking into consideration the other evidence (vide supra), the primacy of the CCKBR receptor is likely.

Interestingly, CCK elicited calcium signaling in all three choriocarcinoma cell lines, but not in the HTR-8/SVneo cells. In future studies, primary cytotrophoblasts require interrogation. The nanomolar concentrations of CCK needed to consistently stimulate increases in intracellular calcium were high compared with circulating levels that are in the picomolar range (10, 29). Nevertheless, CCK concentration could be high in the immediate vicinity of placental ST and EVT, or the placental CCKBR might be a low-affinity receptor. Because CCK is expressed by trophoblasts, and trophoblasts respond to CCK by increasing intracellular calcium (at least in choriocarcinoma cell lines), these results suggest an autocrine role for CCK, which remains to be further elucidated. By analogy to other cell systems, CCK might stimulate acetylcholine release from trophoblasts, which is highly expressed in this cell type, or upregulate the ABCB1 transporter (4, 13, 27, 38). Trophoblast CCK could also play a vasodilatory role (4), and have other paracrine or even endocrine roles. For example, both directly (21, 25, 35, 36, 51) and indirectly through acetylcholine release (3, 30, 46), CCK might exert immunomodulatory actions at the maternal-fetal interface. Finally, because fasting plasma CCK increases during pregnancy (10) [although not all agree (29)], it is not inconceivable that the placenta might contribute to this rise.

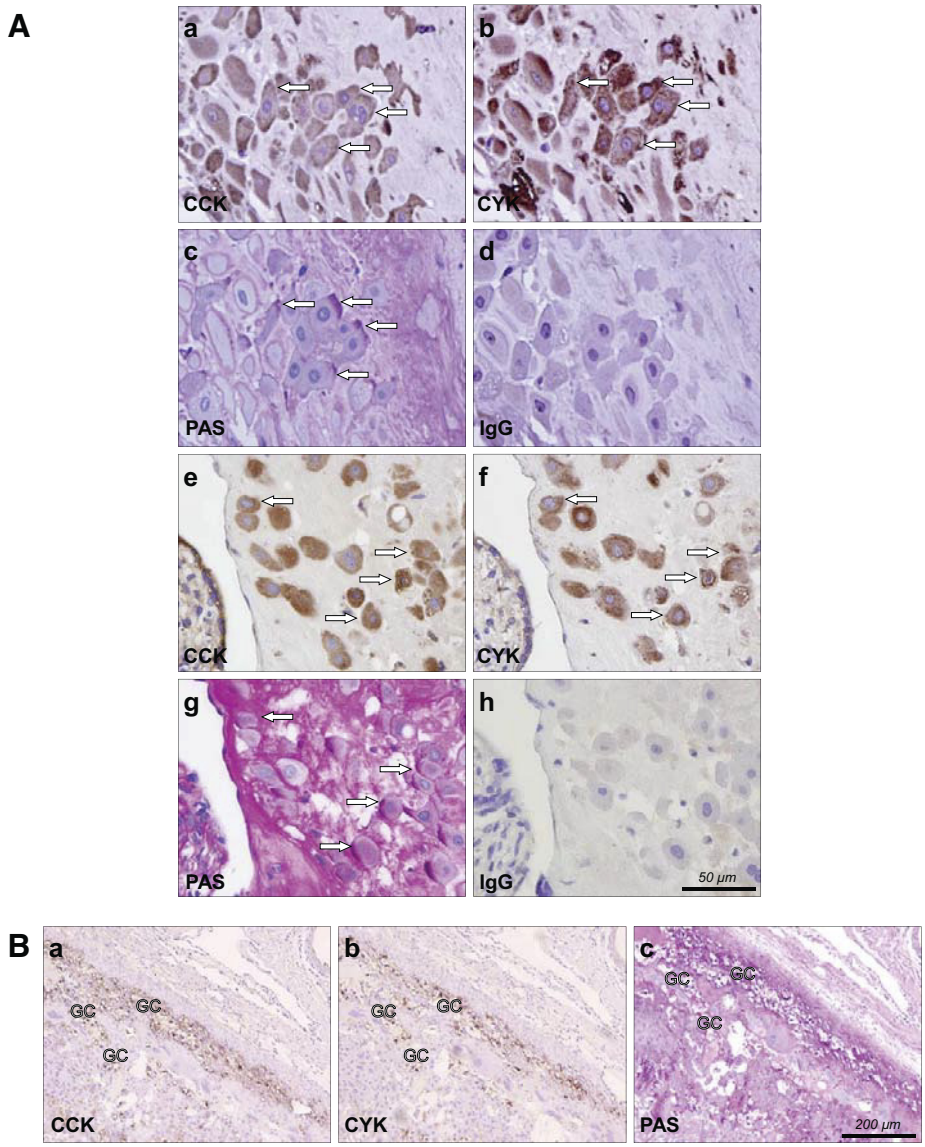

Fig. 5. *A*: localization of cholecystokinin (CCK) protein and periodic acid-Schiff (PAS) staining in trophoblast cells of normal delivered human placenta (×400 magnification). *a* and *e*: immunostaining with COOH-terminus CCK antibody. *b* and *f*: Cytokeratin 19 (CYK) antibody. *c* and *g*: PAS staining. *d* and *h*: Goat IgG, negative control. Arrows indicate cells colocalizing with CCK, cytokeratin 19, and PAS. *B*: colocalization of CCK protein and PAS staining in glycogen trophoblast cell of 16.5-day gestation mouse placenta (×100 magnification). *a*: Immunostaining with COOH-terminus CCK antibody. *b*: Cytokeratin 19 antibody. *c*: PAS staining. GC, glycogen cells.

The mechanism for CCK production and/or secretion by trophoblasts is unknown. We tried unsuccessfully to detect basal and stimulated sulfated CCK in conditioned media and lysates of choriocarcinoma cells and villous explants using an RIA specific for the sulfated form (ALPCO, Salem, NH; data not shown). We attempted to stimulate CCK production by raising intracellular calcium using A23187, BAY K 8644, methacholine, and TAS2R14 receptor agonists, as well as by augmenting intracellular cAMP with dibutyryl cAMP and human chorionic gonadotropin. We added protease inhibitors to the media to mitigate catabolism of CCK, if it was being secreted, and concentrated the conditioned media. However, these procedures did not result in detectable sulfated CCK either. However, as a positive control, we were able to measure sulfated CCK in the conditioned media of STC-1 cells, which possess many features of native mouse intestinal enteroendocrine cells (ATCC CRL-3254) (41, 42, 47). In contrast, we detected low levels of nonsulfated CCK in the conditioned media of JEG, HTR-8/SVneo, and STC-1 cells, but not villous explants by EIA (RayBiotech, Norcross, GA; data not shown). However, after further inquiry, we found that this assay did not distinguish nonsulfated CCK from gastrin, because the antibody was made against a homologous peptide in the C-terminus. Thus, whether nonsulfated CCK, gastrin, or both were detected cannot be distinguished. The reason for not readily detecting sulfated CCK in the conditioned media is also unclear, because the cells are clearly expressing CCK (vide supra). One possibility is that secretion rates are extremely low, and therefore undetectable by our RIA in the conditioned medium. Another potential explanation is that nonsulfated CCK may be the major form(s) produced by trophoblasts, consistent with the expression of CCKBR and not –AR (31). Yet another potential reason is that appropriate stimuli for CCK secretion were not used. For example, in the gut, CCK is released by various fatty acids through the ILDR1, CD-36, GPR120, and GPR40 receptors (5, 22, 41, 42), and amino acids through a CaSR receptor (47). Finally, although entirely speculative, it is possible that CCK is not secreted, but rather exerts an intracrine function within trophoblasts.

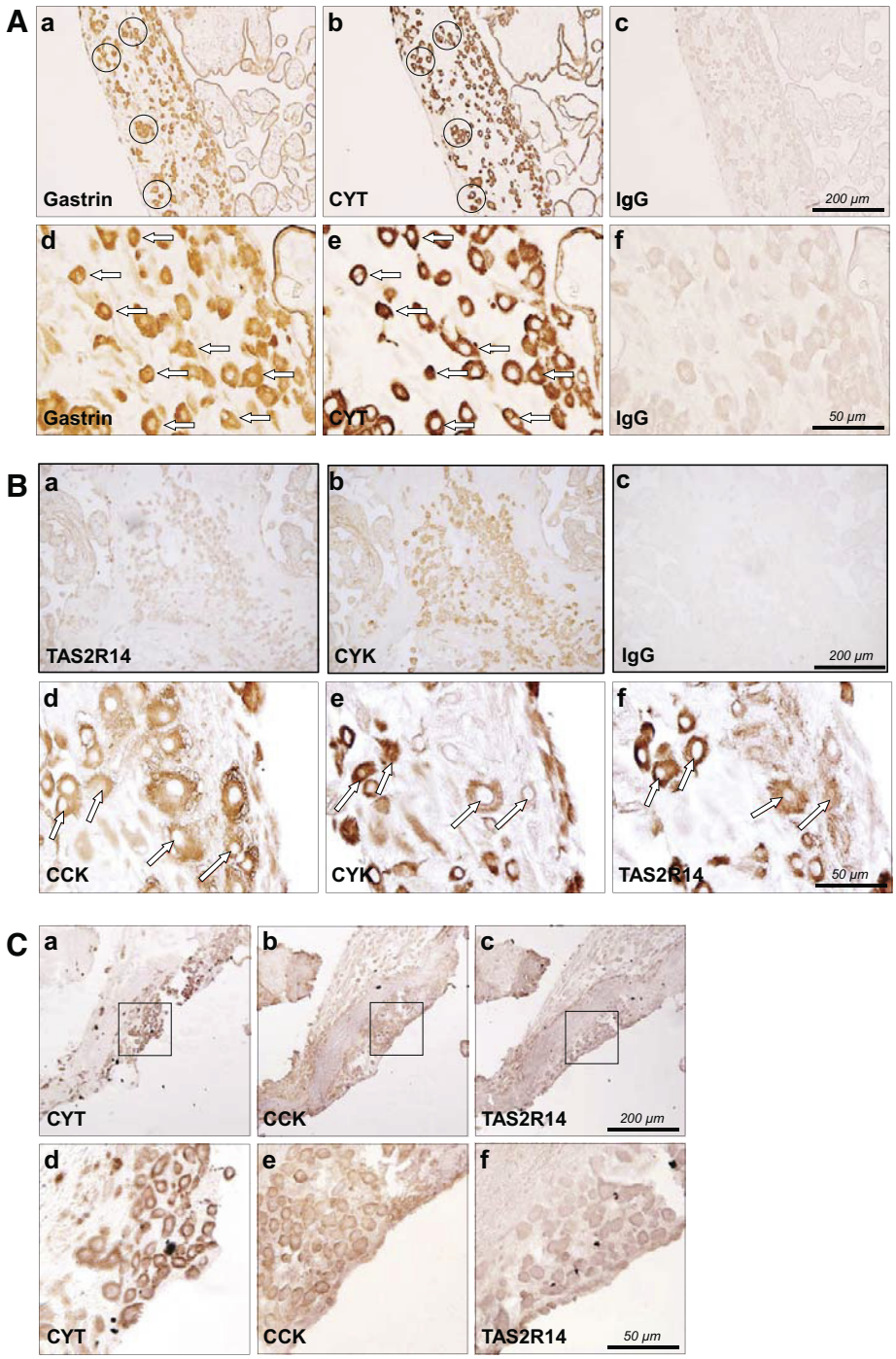

Fig. 6. *A*: localization of gastrin protein in syncytiotrophoblast and extravillous trophoblast of normal delivered human placenta (*a–c*: ×100 magnification; *d–f*: ×400 magnification). *a* and *d*: Immunostaining with gastrin antibody. *b* and *e*: Cytokeratin 18 (CYT) antibody. *c* and *f*: Mouse IgG, negative control. Circles and arrows indicate cells colocalizing with gastrin and cytokeratin 18. *B*, *top*: localization of TAS2R14 protein in extravillous trophoblast of normal delivered human placenta (×100 magnification). *a*: Immunostaining with rabbit polyclonal TAS2R14 antibody. *b*: Cytokeratin 19 (CYK) antibody. *c*: Rabbit IgG, negative control. *Bottom*: colocalization of TAS2R14 and CCK protein in extravillous trophoblast of normal delivered human placenta (×400 magnification). *d*: Immunostaining with COOH-terminus CCK antibody. *e*: Cytokeratin 19 antibody. *f*: Goat polyclonal TAS2R14 antibody. Arrows indicate cells colocalizing with CCK, TAS2R14, and cytokeratin 19. *C*: localization of TAS2R14, CCK, and cytokeratin in extravillous trophoblast of early gestation human placenta. *Top*: *a–c*: ×100 magnification. *Bottom*: *d–f*: ×400 magnification. *a* and *d*: Immunostaining with cytokeratin 18 antibody. *b* and *e*: COOH-terminus CCK. *c* and *f*: Goat polyclonal TAS2R14 antibody.

Gastrin. In the literature, whether gastrin mRNA was expressed in human or mouse placenta is contentious (18, 40, 50, 52). On the other hand, gastrin protein was detected using radioimmunoassay in a homogenate prepared from delivered placenta(s) (1). Because we identified *CCKBR/br* mRNA in both human and mouse placenta, we expanded our initial investigation to include gastrin, which interacts with CCKBR. Not only did we find abundant *GAST* mRNA in first-trimester villous, third-trimester villous, and decidual basal plate and trophoblast cell lines, we also identified mRNA expression in mouse placenta. Protein expression was observed in both ST and EVT of healthy, delivered human placenta. Because the gastrin antibody we used was specific for humans, we did not explore gastrin protein expression in mouse placenta.

TAS2R14. While conducting our work on placental TAS2R14, a report emerged showing TAS2R38 expression by immunohistochemistry specifically in the chorion layer of human fetal membranes, but not in ST or decidua basalis as we report herein for TAS2R14. TAS2R38 was also expressed by JEG cells, which responded to the TAS2R38 agonist phenylthiocarbamide by increasing intracellular calcium (48). In our work, *TAS2R14* mRNA was expressed in first-trimester villous and decidua basalis, third-trimester villous and decidual basal plate, as well as trophoblast cell lines. The protein was localized to

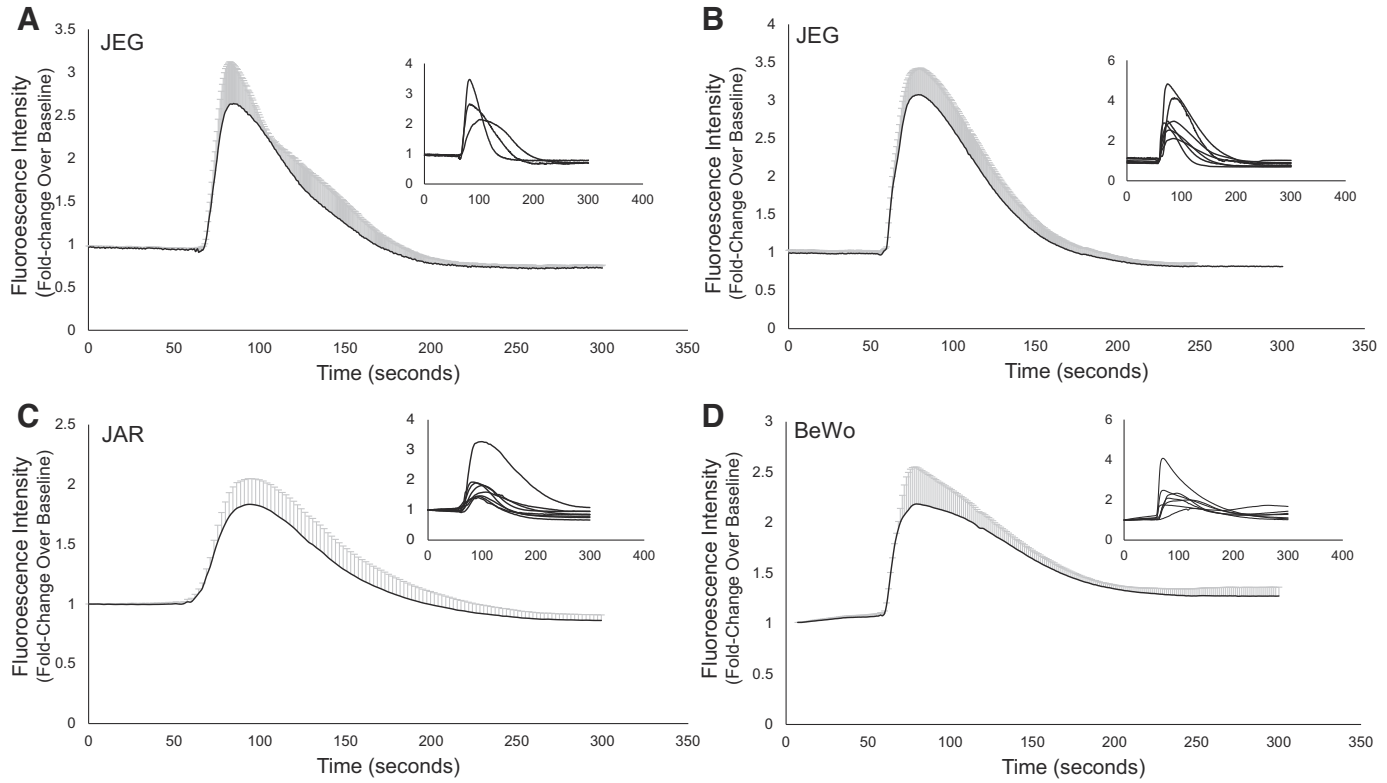

Fig. 7. Intracellular calcium of trophoblast-derived choriocarcinoma cells in response to cholecystokinin (CCK). Values are means ± SE of dishes analyzed depicted in the main figure. *Insets*: results for each dish, in which intracellular calcium was averaged for only responding cells. *A*: JEG cells ($n = 3$ dishes) treated with 10 nM nonsulfated CCK. The percentage of responding cells was 86.2 ± 7.0%. *B*: JEG cells ($n = 7$ dishes) treated with 10 nM sulfated CCK. Responding cells: 86.2 ± 4.9%. *C*: JAR cells ($n = 8$ dishes) treated with 1.0 μM sulfated CCK. Responding cells: 38.6 ± 6.9%. *D*: BeWo cells ($n = 7$ dishes) treated with 1.0 μM sulfated CCK. Responding cells: 31.4 ± 9.1%.

ST and EVT in term and early-trimester placentas, although further study of early-trimester placenta is needed due to limited availability of these tissues for TAS2R14 immunohistochemistry. Many, but not all EVT coexpressed CCK and TAS2R14 in the term and early-gestation placentas. Because TAS2R14 is a specific human taste receptor, we did not investigate mouse placenta in parallel as we did for CCK, CCKAR, and –BR and gastrin. To our knowledge, expression of TAS2R14 in the human placenta has not been previously reported. Nevertheless, sweet and bitter taste receptors outside of the oral cavity are being identified in a growing number of organs including the kidney, thyroid, and heart; however, the physiological and pathophysiological consequences remain incompletely understood or speculative (23).

FFA (10 nM-30 μM) and CHLX (0.1–1.0 μM) specifically increase intracellular calcium expression in HEK-293T cells transfected with a plasmid harboring TAS2R14, but not with other human taste receptors (24). It should be pointed out, however, that the maximum concentrations of FFA and CHLX which activate TAS2R14 without increasing intracellular calcium nonspecifically, i.e., independently of TAS2R or activating other TAS2R receptors besides TAS2R14, were not clearly delineated in the report of Meyerhof et al. (24). Nevertheless, we found that for FFA, intracellular calcium was increased in the choriocarcinoma cell lines and in HTR-8/SVneo cells using concentrations that fell within the range of concentrations reported to be specific for TAS2R14 (vide supra). For CHLX, however, a higher concentration of 10 μM was required to elicit an increase in intracellular calcium. Inexplicably, the pattern of change in intracellular calcium was not consistent for the two TAS2R14 agonists in JEG and HTR-8/SVneo cells with FFA eliciting the usual robust increase, in contrast to CHLX, which produced a gradual rise.

As intimated above, the TAS2R14 agonists are likely to have off-target effects, e.g., FFA may exert other actions (8). Nevertheless, we used two other agonists of the TAS2R14 receptor, CHLX and diphenhydramine (15, 16, 24, 33), and these also increased cytosolic calcium in trophoblast-derived cells. Although we cannot exclude additional alternative mechanisms for raising intracellular calcium, one target that all three agonists do have in common is the TAS2R14 receptor.

As with CCKAR- and BR, the physiological or pathophysiological roles of trophoblast TAS2R14 are presently unknown and require exploration in future investigations. By analogy to other systems, TAS2R14 may detect harmful chemicals in the circulation that otherwise would produce a "bitter taste" in the oral cavity, although it is difficult to conceive of how such compounds could reach sufficient concentrations in the maternal blood to be effective. Nevertheless, activation of TAS2R38 expressed by enteroendocrine cells in the gut, presumably by bitter taste compounds that are not completely expectorated, leads to secretion of CCK that, in turn, upregulates ABCB1 transporters in neighboring enteric cells, leading to extrusion of the noxious compound into the gut lumen, thereby limiting intestinal absorption (13). Conceivably, a similar mechanism, albeit autocrine and not paracrine, operates in trophoblasts,

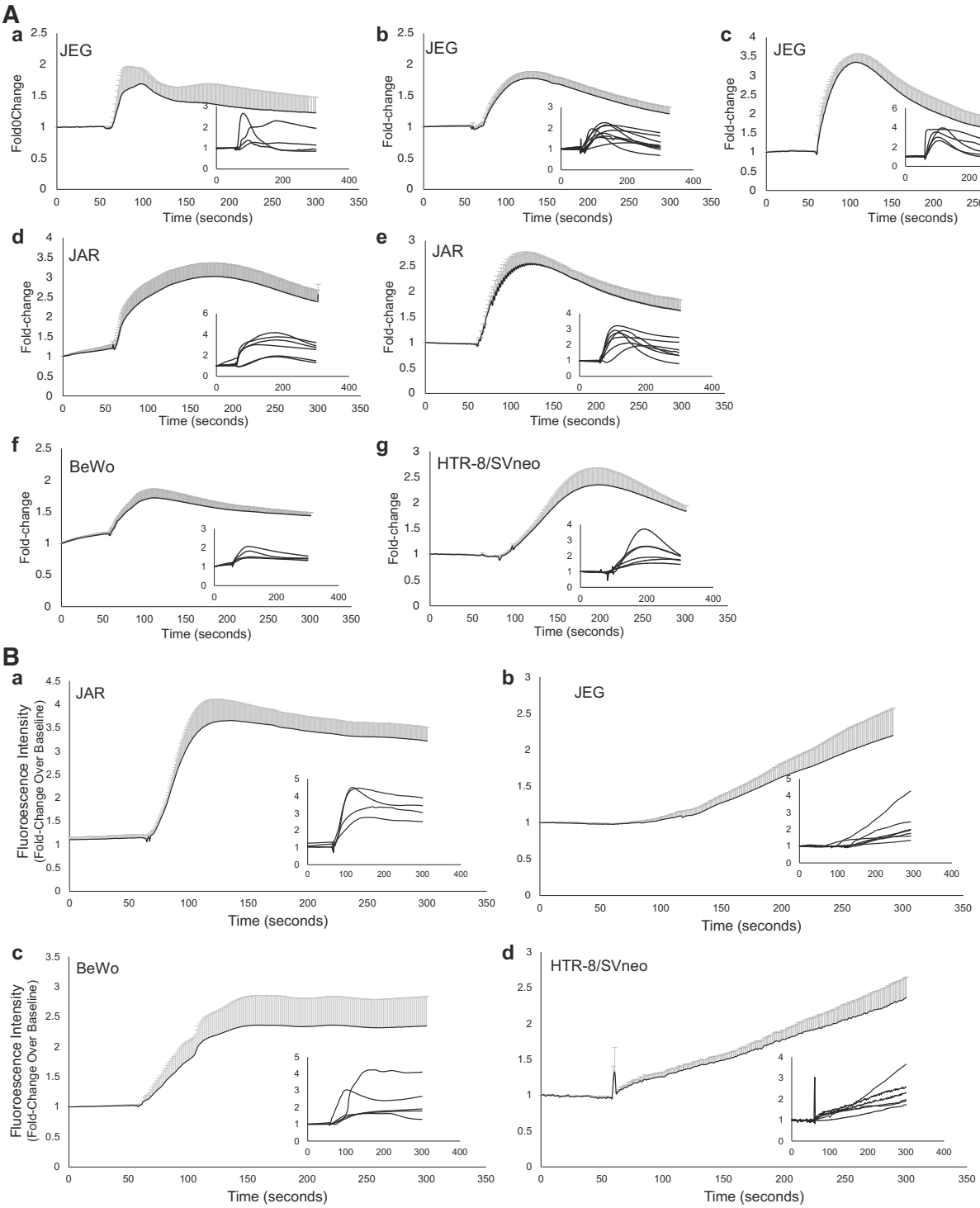

Fig. 8. *A*: intracellular calcium of trophoblast-derived choriocarcinoma cells in response to the TAS2R14 receptor agonist flufenamic acid (FFA). Values are means ± SE of dishes analyzed as depicted in the main figure. *Insets*: results for each dish, in which intracellular calcium was averaged for only responding cells. JEG cells were treated with FFA in *a*, 10 μM, $n = 4$ dishes; *b*: 30 μM, $n = 9$ dishes; and *c*: 100 μM, $n = 5$ dishes. Percentages of responding cells were 49.5 ±9.9, 76.8 ± 9.2, and 94.4 ± 4.9%, respectively. JAR cells were treated with FFA in *d*, 30 μM, $n = 6$ dishes and *e*: 100 μM, $n = 7$ dishes. Percentages of responding cells were 24.1 ± 8.5 and 94.3 ± 4.1%, respectively. BeWo and HTR8 cells, respectively, were treated with FFA in *f*, 30 μM, $n = 4$ dishes and *g*, 100 μM, $n = 6$ dishes. Percentages of responding cells were 53.8 ± 10.3 and 93.9 ± 2.4%, respectively. *B*: intracellular calcium of trophoblast-derived choriocarcinoma cells in response to the TAS2R14 receptor agonist chlorhexidine (CHLX; 10 μM). Values are means ± SE of all dishes analyzed as depicted in the main figure. *Insets*: results for each dish, in which intracellular calcium was averaged for only responding cells. *a*: JAR cells ($n = 4$ dishes). Percentage of responding cells was 87.0 ± 5.0%. *b*: JEG cells ($n = 7$ dishes). Responding cells: 70.2 ± 8.6%. *c*: BeWo cells ($n = 5$ dishes). Responding cells: 63.6 ± 7.3%. *d*: HTR8 ($n = 6$ dishes). Responding cells: 59.5 ± 5.3%. No responses were observed with lower concentrations of 1.0 or 3.0 μM.

ultimately protecting the placenta, and thus, the fetus from potentially harmful chemicals. Indeed, the placenta is a rich source of ABC transporters (2, 44). Other possible roles relate to immunological function. For example, TAS2R14 activation was shown to increase antimicrobial peptide secretion in the human airway (20). By analogy, a similar pathway may exist in the placenta, which is a rich source of antimicrobial peptides (17). In addition, there is an emerging literature suggesting that bitter taste receptors are targets of microbial products leading to stimulation of the innate immune system and other protective mechanisms (19, 43, 45). Thus, again by analogy, TAS2R14 and other potential taste receptors expressed by placental ST and EVT might recognize bacterial quorum-sensing molecules, thereby contributing to interkingdom communication within the placenta (11, 34).

In summary, we identified CCK, gastrin, and their receptor(s) in both human and mouse placenta. Furthermore, bitter taste receptor TAS2R14 was also observed in the human placenta. We demonstrated expression of these molecules by ST and extravillous trophoblasts. These conclusions were based upon parallel mRNA and protein expression by RT-PCR and immunohistochemistry, respectively, and demonstration of calcium signaling using CCK and TAS2R14 agonists in human trophoblast cell lines. The precise functions of these ligands and receptors, as well as their potential interactions in the normal and pathological placenta are currently unknown, but the possibilities are numerous and intriguing. This study opens new and unexplored avenues of placental investigation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

K.P.C. conceived and designed research; S.T., Y.B., L.C., V.J.C., M.C.-M., J.C.B., B.D., M.T.B., and K.P.C. performed experiments; S.T., Y.B., L.C., V.J.C., M.C.-M., J.C.B., B.D., M.T.B., M.B.R., E.D.P.U., and K.P.C. prepared figures; S.T., Y.B., L.C., V.J.C., M.C.-M., J.C.B., B.D., M.T.B., and K.P.C. drafted manuscript; S.T., Y.B., L.C., V.J.C., M.C.-M., J.C.B., B.D., M.T.B., M.B.R., E.D.P.U., and K.P.C. edited and revised manuscript; M.B.R., E.D.P.U., and K.P.C. analyzed data; M.B.R., E.D.P.U., and K.P.C. interpreted results of experiments; K.P.C. approved final version of manuscript.

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