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REGULAR ARTICLE

Expression patterns of L-amino acid receptors in the murine STC-1 enteroendocrine cell line

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Abstract

Regulation of gut function depends on the detection and response to luminal contents. Luminal L-amino acids (L-AA) are detected by several receptors including metabotropic glutamate receptors 1 and 4 (mGluR1 and mGluR4), calcium-sensing receptor (CaSR), GPRC family C group 6 subtype A receptor (GPRC6A) and umami taste receptor heterodimer T1R1/T1R3. Here, we show that murine mucosal homogenates and STC-1 cells, a murine enteroendocrine cell line, express mRNA for all L-AA receptors. Immunohistochemical analysis demonstrated the presence of all L-AA receptors on STC-1 with CaSR being most commonly expressed and T1R1 least expressed (35% versus 15% of cells); mGluRs and GPRC6a were intermediate (~ 20% of cells). Regarding coexpression of L-AA receptors, the mGluRs and T1R1 were similarly coexpressed with CaSR (10–12% of cells) whereas GPRC6a was coexpressed least (7% of cells). mGluR1 was coexpressed with GPRC6a in 11% of cells whereas coexpression between other receptors was less (2–8% of cells). CaSR and mGluR1 were coexpressed with glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) in 20–25% of cells whereas T1R1 and GPRC6a were coexpressed with GLP-1 and PYY less (8–12% of cells). Only mGluR4 showed differential coexpression with GLP-1 (13%) and PYY (21%). L-Phenylalanine (10 mM) caused a 3-fold increase in GLP-1 release, which was strongly inhibited by siRNA to CaSR indicating functional coupling of CaSR to GLP-1 release. The results suggest that not all STC-1 cells express (and coexpress) L-AA receptors to the same extent and that the pattern of response likely depends on the pattern of expression of L-AA receptors.

Keywords Enteroendocrine cells · Taste receptors · L-Amino acid receptors · STC-1 cells · Neurohumoral peptides

Introduction

Chemosensation is key to the response to the environment and maintenance of homeostasis. In recent years, it has become evident that the receptors responsible for chemosensation, although perhaps initially identified because of association with a single function, tissue, or location, are similar in a wide range of tissues. One of the main regions where chemosensation has a critical role is in the gastrointestinal tract. As ingested material enters the gut and is processed, the nutrient molecules generated become extracellular signaling molecules that activate receptors on chemoreceptive cells that line the gut from the stomach to colon. These chemoreceptive (or chemosensory) cells are the enteroendocrine cells (EECs) and brush (or tuft) cells that make up a very small proportion (< 3%) of gut mucosal cells. The population of EECs is heterogeneous in terms of morphology and the presence of a variety of gut hormones, paracrine agents and bioactive molecules released from these cells in response to activation of luminal receptors. These include peptide YY (PYY), neurotensin, cholecystokinin (CCK), glucose-dependent insulinotropic factor (GIP), glucagon-like peptide-1 and peptide-2 (GLP-1, GLP-2), somatostatin, gastrin, ghrelin and serotonin (Akiba et al. 2015; Avau et al. 2015; Bala et al. 2014; Sbarbati et al. 2010; Schneider et al. 2018; Schutz et al. 2015; Symonds et al. 2015; Young et al. 2009; Zhou and Pestka 2015). While the presence of a single bioactive agent has been used to classify EEC cell types in the past, it is becoming increasingly clear that these classical EEC cell types are themselves heterogeneous based on the colocalization and costorage of multiple and differing biological agents and presence in different regions of the gut (Egerod

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et al. 2012; Fothergill et al. 2017; Habib et al. 2012; Haber et al. 2017; Roth et al. 1990; Sutherland et al. 2007; Symonds et al. 2015). Even the enterochromaffin cell that is typically considered primarily a 5-HT containing cell is now recognized to be heterogeneous with subpopulations identified based on their chemical coding of additional hormones (Reynaud et al. 2016; Martins et al. 2017).

The ability of EECs to detect and respond to protein in the diet depends on the variety of ligands ingested and generated as protein digestion products in the lumen and the complement and selectivity of receptors for these ligands expressed on EEC. Heterogeneity of EECs also derives from expression of G protein-coupled receptors (GPCRs) on their apical or luminal surface. EECs and brush cells express receptors for a variety of luminal molecules including the TGR5 bile salt receptor, taste receptors for sweet and umami (T1R heterodimers), receptors for bitter taste (T2Rs), receptors for free fatty acids (FFARs) of differing chain lengths, nicotinic receptors (nAChRs) and receptors for microbial products and phytochemicals (Akiba et al. 2015; Avau et al. 2015; Bala et al. 2014; Sbarbati et al. 2010; Schneider et al. 2018; Schutz et al. 2015; Symonds et al. 2015; Young et al. 2009; Zhou and Pestka 2015). Umami taste is unique among the five basic tastes because the other tastes require only one receptor or channel to be activated in taste cells whereas multiple L-amino acid (L-AA) receptors likely mediate umami sensations (Pal Choudhuri et al. 2015; Palmer 2018). Protein hydrolysates and partial digestion products like peptones are sensed by the GPR92 receptor (also known as the lysophospholipid 5 (LPA5) receptor or GPR93 receptor) and the peptide transporter PepT1 (Choi et al. 2007; Diakogiannaki et al. 2013; Rettenberger et al. 2015). L-AAs have been postulated to be sensed by a variety of receptors, all of which are of the class C type of GPCRs. These include the umami taste receptor heterodimer (T1R1/T1R3), the calcium-sensing receptor (CaSR), metabotropic glutamate receptors (mGluR1 and mGluR4) and a G protein-coupled receptor family C group 6 member A (GPRC6a) receptor (Gribble and Reimann 2016, 2017; San Gabriel and Uneyama 2013; Steensels and Depoortere 2018). The presence of multiple L-AA receptors allows for responses to be elicited based on the tuning of the receptors expressed. Although each individual receptor has been shown to be present in EECs and EEC cell lines, the nature and extent of colocalization between individual receptors and between receptors and neurohumoral agents are unclear. The combination of these L-AA-responsive receptors, however, is important because it determines the response of an EEC to protein digestion products in the gut.

In the present study, we used an EEC cell line, the STC-1 cell, which was derived from a mouse intestinal tumor to identify the presence of CaSR, GPRC6a, T1R1, mGLU-1 and mGLU-4. STC-1 cells were further examined to determine the prevalence of individual and combinations of these L-AA receptors. The results indicate that the STC-1 cells express each of the L-AA receptors at differing levels with the CaSR being the most abundantly expressed and T1R1 the least abundantly expressed. The CaSR is often coexpressed with the other putative L-AA receptors. These studies also show that all putative L-AA receptor types are coexpressed with GLP-1 and PYY; however, the extent of coexpression is variable.

Materials and methods

Animals and tissue preparation

Adult mice (C57BL/6; 6–8-week-old male and female) were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in the animal facilities of the Virginia Commonwealth University with standard chow and water ad libitum. The mice were euthanized by CO_2 inhalation followed by cervical dislocation, both the whole small intestine and colon (exclusive of cecum) were removed and each region flushed to remove contents. Segments of each region were cut open longitudinally and the muscle and mucosa layers separated by blunt dissection and homogenized manually. All animal procedures were performed according to a protocol approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

Cell culture

Murine enteroendocrine cells (STC-1) were obtained from American Type Culture Collection (ATCC; Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2/95% air.

RT-PCR analysis

Total RNA was extracted from homogenates of muscle and mucosa of the mouse colon and small intestine and from mouse enteroendocrine cell line STC-1 cells using TRIzol reagent. Reverse transcription (RT) was performed using a high-capacity reverse transcription kit from Thermo Fisher (Waltham, MA), followed by PCR assays and RT-PCR gels scanned by densitometry. RT-PCR was performed using PCR primers as follows: T1R1, sense primer: 5′-ctggcagcttcttcagcagatc-3′; antisense primer: 5′-ggtgtgaagctcactcgtgttg-3′. CaSR, sense primer: 5′-ggcctggcaggtcctgaaac ac-3′; antisense primer: 5′-ctcgcatctgtctcaccactgt-3′. mGluR4, sense primer: 5′-aacggaggcg tgtgcattgccc-3′; antisense primer: 5′-atcggtcaaaccctcgcacaga-3′. mGluR1 sense primer: 5′-

ccacacagaagggaattacggc-3′; antisense primer: 5′-caagctttcatttcctgtgcag-3′. GPRC6A sense primer 5′-ctttcagccatgggagctacttgc-3′; antisense primer: 5′-ggcagtgatccatatctgtctcat-3′.

Immunofluorescence and microscopy

Cells were fixed with 4% paraformaldehyde for 20 min at room temperature. After permeabilization with 0.1% Triton X-100 for 5 min and blocking with 5% goat serum for 30 min, cells were stained with primary antibodies in 5% goat serum at room temperature for 2 h, washed with buffer and incubated with fluorescent-conjugated secondary antibodies for 1 h. Nuclei were visualized with 1 mg/ml DAPI. Images were acquired with a ZEISS X-cite Series 120 microscope. Adjustments to brightness and contrast were applied to the whole image. A minimum of 1000 cells were analyzed.

Antibody against PYY (#ab22663; dilution 1:300) and mGluR4 (#ab53088; dilution 1:300) were raised in rabbit and antibody against CaSR (#ab19347; dilution 1:300) was raised in mouse. All of these antibodies were obtained from Abcam (Cambridge, UK). Antibody against GLP-1(7-37) (#H-028-13; dilution 1:250) was raised in rabbit and obtained from Phoenix Pharmaceuticals (Burlingame, CA). For costaining studies where the receptor antibody was raised in rabbit, a GLP-1(7-37) antibody raised in goat (#sc-26637; dilution 1:100) was used (Santa Cruz; Dallas, TX). Antibodies against mGluR1 (#sc-47131; dilution 1:100) were raised in goat and obtained from Santa Cruz (Dallas, TX). As needed for costaining, T1R1 antibodies raised in rabbit (#sc-50308; dilution 1:100) or raised in goat (#sc-22451; dilution 1:100) were purchased from Santa Cruz and used depending on species of origin of the other receptor antibody. Antibodies against GPRC6a receptor (#NLS-2576; dilution: 1:300) were raised in rabbit and obtained from Novus Biologicals (Littleton, CO). Because antibodies from different species were not always available, not every combination of receptors could be examined as noted in the results. Alexa Fluor 488 and Alexa Fluor 594 were obtained from Invitrogen (Carlsbad, CA).

Measurement of GLP-1 release

The functional role of the most commonly expressed L-AA receptor, CaSR, was tested by measuring the release of GLP-1 from STC-1 cells treated with siRNA for CaSR. STC-1 cells were transfected with control siRNA (SC-37007) or siRNA to CaSR (SC-44374) from Santa Cruz by incubation with siRNA at a concentration of 60 pM for 48 h. The cells were then incubated in DMEM containing protease and phosphatase inhibitors (Abcam #AB201119) with or without 10 mM L-phenylalanine for 3 h. The supernatant was collected and GLP-1 was measured by ELISA according to the directions of the manufacturer (GLP-1 EIA kit #RAB0201; Sigma Chemicals, St. Louis, MO). The ability of siRNA to suppress the CaSR expression was confirmed in two ways. STC-1 cells were collected from some wells, protein was extracted and Western blots performed to determine CaSR expression using antibody to CaSR (AB#19347; 1:1000 dilution; Abcam, Cambridge, UK). In other wells, the STC-1 were immunostained with the same CaSR antibody at a dilution of 1:300 as described above.

Results

Expression of mRNA for L-amino acid receptors

The expression of mRNA transcripts for CaSR, T1R1, GPRC6a, mGluR1 and mGluR4 was determined. T1R1 transcripts were determined instead of T1R3 because the T1R3 would not distinguish between the presence of L-AA umami receptors (T1R1/T1R3) and sweet receptors (T1R2/T1R3). Total RNA was extracted from muscle and mucosa of the mouse colon and small intestine and the STC-1 cell line and mRNA expression was analyzed by RT-PCR (Fig. 1). Each L-AA receptor was present in the small intestine and colon at the level of the mucosa and in some cases also at the level of the smooth muscle (Fig. 1a). Although quantitative RT-PCR was not done, the levels in the mucosa generally appeared greater than in muscle for most receptors. The STC-1 cells also

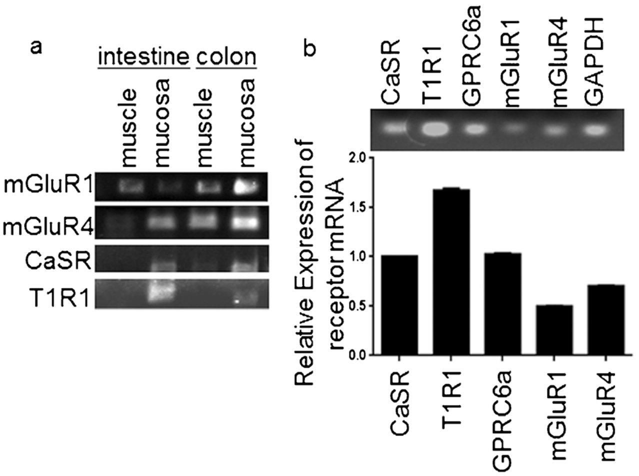


Fig. 1 **a** Expression of transcripts for CaSR, T1R1, mGluR1 and mGluR4 measured by RT-PCR in homogenates of mucosal and muscle layers of the mouse whole small intestine and colon. **b** Expression of transcripts for CaSR, T1R1, GPRC6a, mGluR1 and mGluR4 measured by RT-PCR in STC-1 cells (top) and relative quantities of transcripts for CaSR, T1R1, GPRC6a, mGluR1 and mGluR4 in STC-1 cells determined by densitometry of RT-PCR gels

expressed mRNA for all of the l-AA receptors and thus mimicked EECs of mouse gut in this regard (Fig.1b).

Expression and coexpression of l-amino acid receptors on STC-1 cells

Expression of the l-AA receptors was examined by immunohistochemical identification in STC-1 cells. Cultured STC-1 cells were exposed to a specific antibody for each of the l-AA receptor proteins (CaSR, GPRC6a, mGluR1, mGluR4 and T1R1) and visualized by Alexa 488 and Alexa 594 dyes. DAPI staining was used for nuclear localization (Fig. 2a-a’”’). A total of 1000 cells were identified by counting the DAPI stained nuclei in successive fields and the number of STC-1 cells staining positive for a given l-AA receptor quantified and expressed as a percentage of the total number of cells (Fig. 2b). As noted for mRNA presence, all receptors were expressed in cultures of STC-1 cells; however, not all STC-1 cells expressed all receptors. Although it appeared that mRNA for the T1R1 was abundant in both intestinal homogenates and the STC-1 cultures, the T1R1 protein was the least expressed receptor of the l-AA receptors (15 ± 2% of total). In contrast, the CaSR was the most expressed l-AA receptor in STC-1 cells (35 ± 5% of total). The mGluR 1 and 4 and the GPRC6a receptors were intermediate and similar in their levels of expression (20–23% of total).

Since CaSR was expressed more frequently than other l-AA receptors in STC-1 cells, we next determined whether CaSR was colocalized with other receptors and the extent of colocalization. Costaining for CaSR along with GPRC6a, T1R1, mGluR1, or mGluR4 was done in separate cultures of STC-1 cells. The costaining of CaSR with GPRC6a, T1R1, mGluR1 and mGluR4 was quantified by counting 1000 cells in successive fields and expressed as percent of cells expressing CaSR and one of the other receptors (Fig. 3). This result demonstrated a similar degree of coexpression (range 10–12% coexpression; Fig. 3b) of CaSR with T1R1, mGluR1 and mGluR4. GPRC6a was coexpressed somewhat less frequently with the CaSR (7 ± 1% coexpression) than the other receptors. The ability to determine coexpression of other l-AA receptors was somewhat limited by availability of antibodies raised in differing species. Where

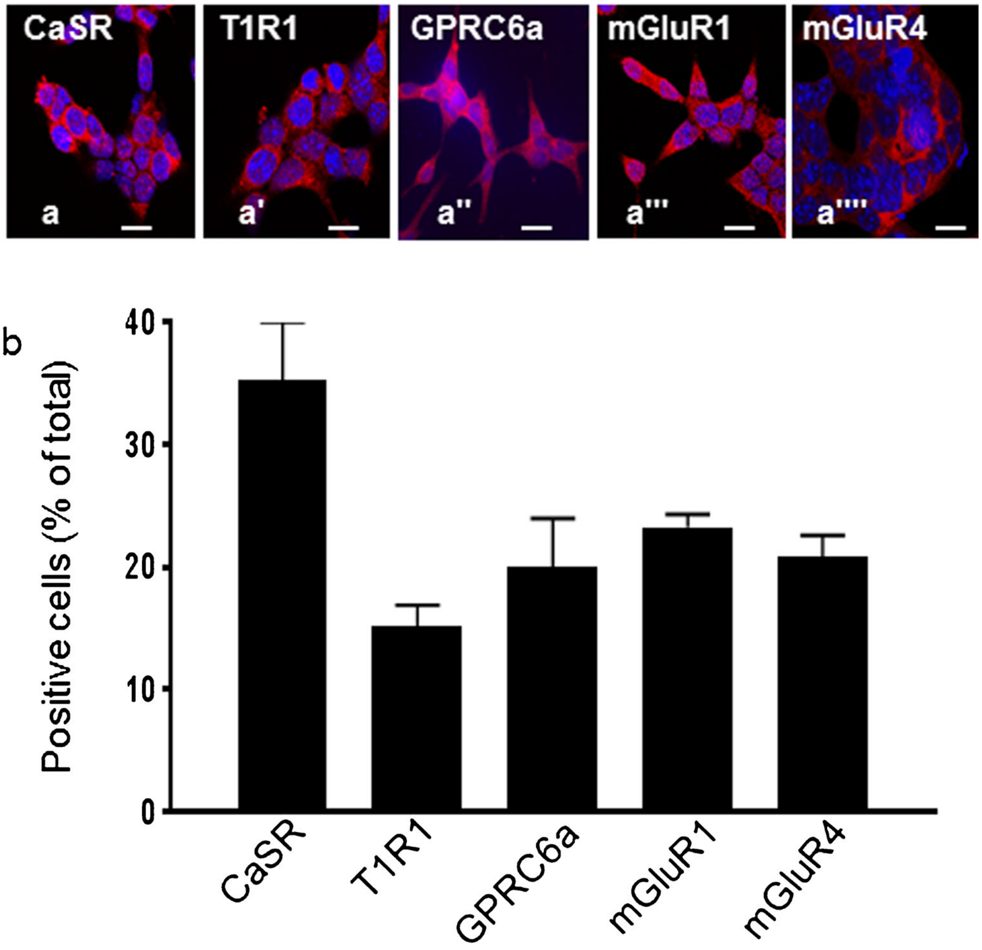


Fig. 2 Immunofluorescence staining for CaSR (**a**), T1R1 (**a’**), GPRC6a (**a”**), mGluR1 (**a’”**) and mGluR4 (**a’”’**) protein in STC-1 cells using Alexa 594-coupled secondary antibody. DAPI staining was used for nuclear localization. **b** Quantitative analysis of cells stained for CaSR, T1R1, GPRC6a, mGluR1 and mGluR4. One thousand cells were counted for each group. Values are mean ± SD. Bars in **a** indicate 10 μm

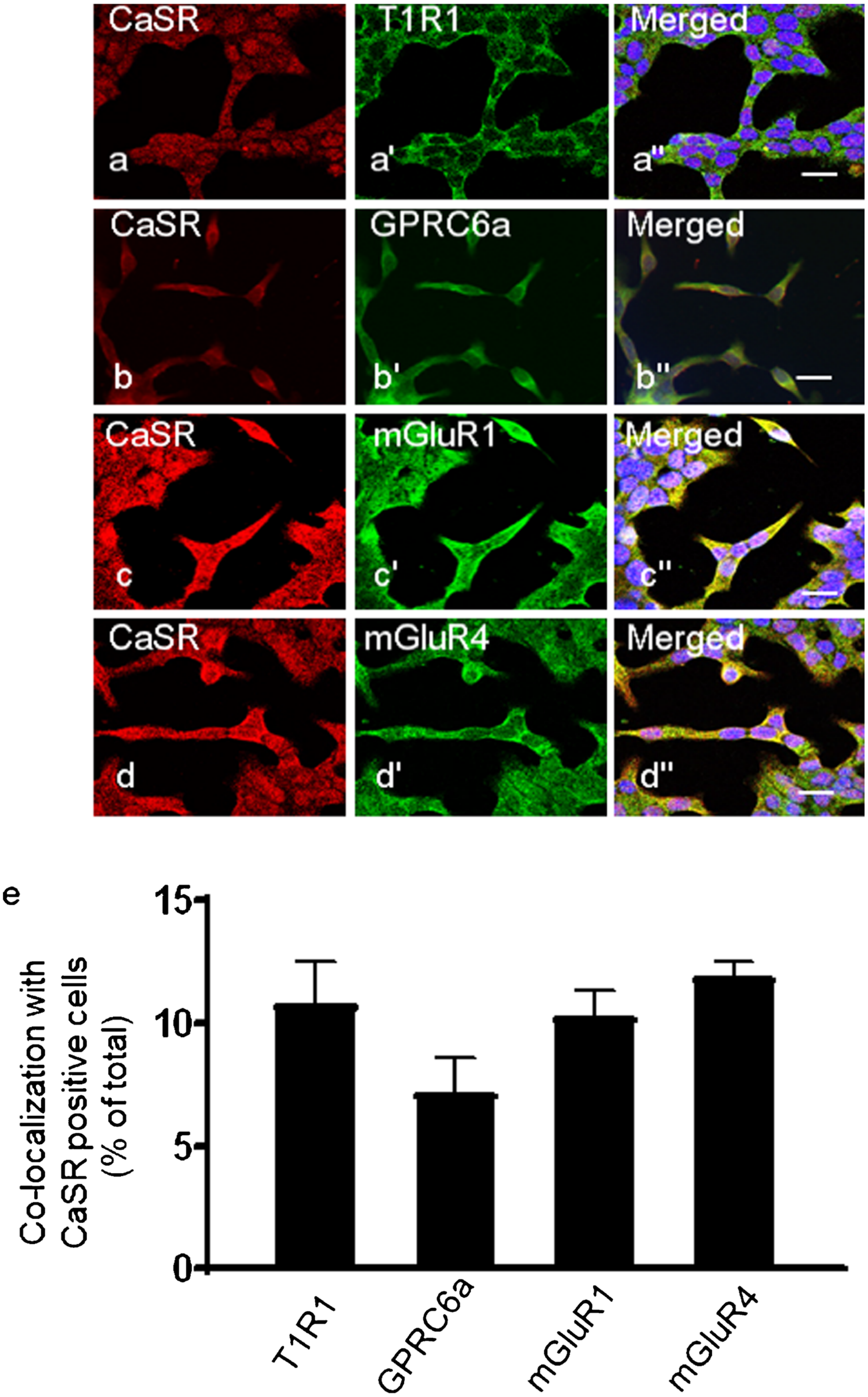


Fig. 3 **a** Immunofluorescence staining for CaSR (**a**, **b**, **c**, **d**) and T1R1 (**a'**), GPRC6a (**b'**), mGluR1 (**c'**) and mGluR4 (**d'**) in separate preparations of STC-1 cells. The right panel is the merged image of the left and middle panels. DAPI staining was used for nuclear localization (**a**'', **b**'', **c**'', **d**''). **e** Quantitative analysis of cells costained for CaSR and each of the other receptors (T1R1, GPRC6a, mGlu1 and mGluR4). One thousand cells were counted for each pairing. Values are mean ± SD. Bars in **a**'', **b**'', **c**'', and **d**'' indicate 10 μm

possible, coexpression of the other receptors was examined and quantified (Fig. 4). The mGluR1 was colocalized most often with GRPC6a (11 ± 1%), which is similar to the extent of coexpression of mGluR1 with CaSR. The colocalization of mGluR1 with T1R1 and mGluR4 was less frequent (7–8% colocalization) (Fig. 4a). The

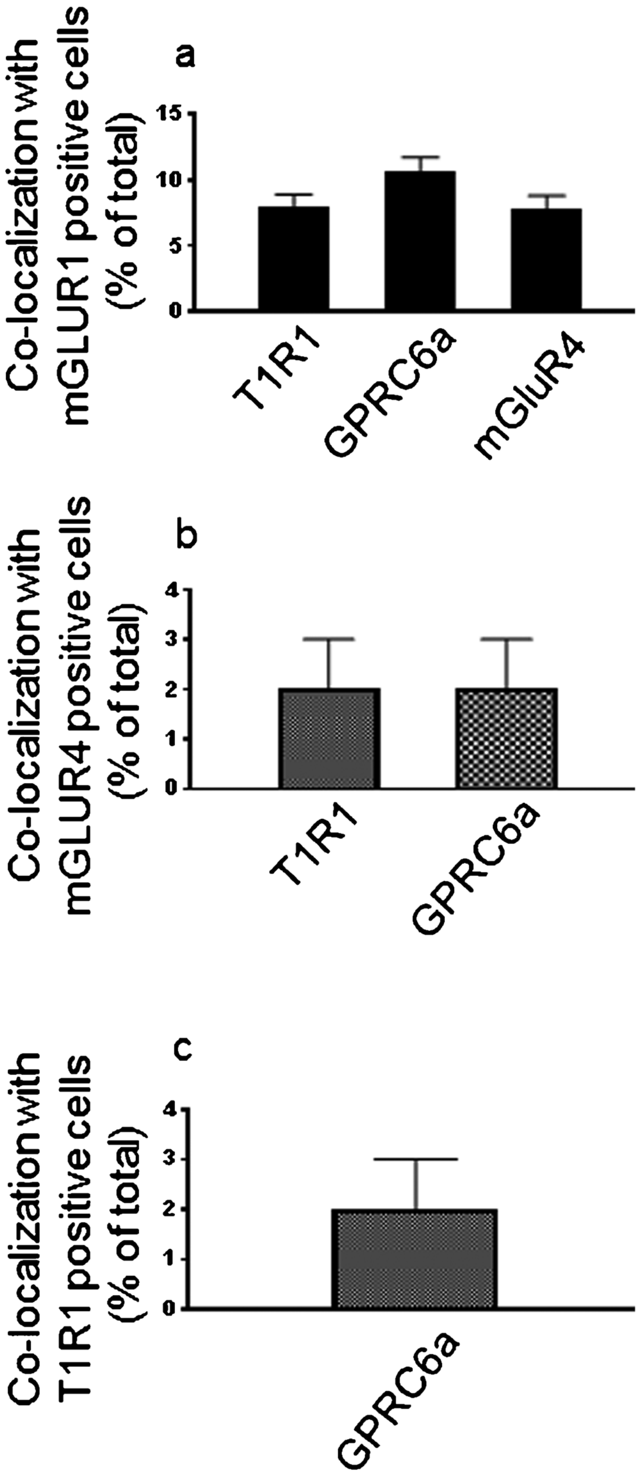


Fig. 4 **a** Quantitative analysis of STC-1 cells costained for mGluR1 and each of the other receptors (T1R1, GPRC6a and mGluR4). **b** Quantitative analysis of STC-1 cells costained for mGluR4 and each of the other receptors (T1R1 and GPRC6a). **c** Quantitative analysis of STC-1 cells costained for T1R1 and GPRC6a. One thousand cells were counted for each pairing. Values are mean ± SD

colocalizations of mGluR4 with T1R1 or GPRC6a (Fig. 4b) and the colocalization of T1R1 with GPRC6a (Fig. 4c) were much lower (about 2% for each pairing).

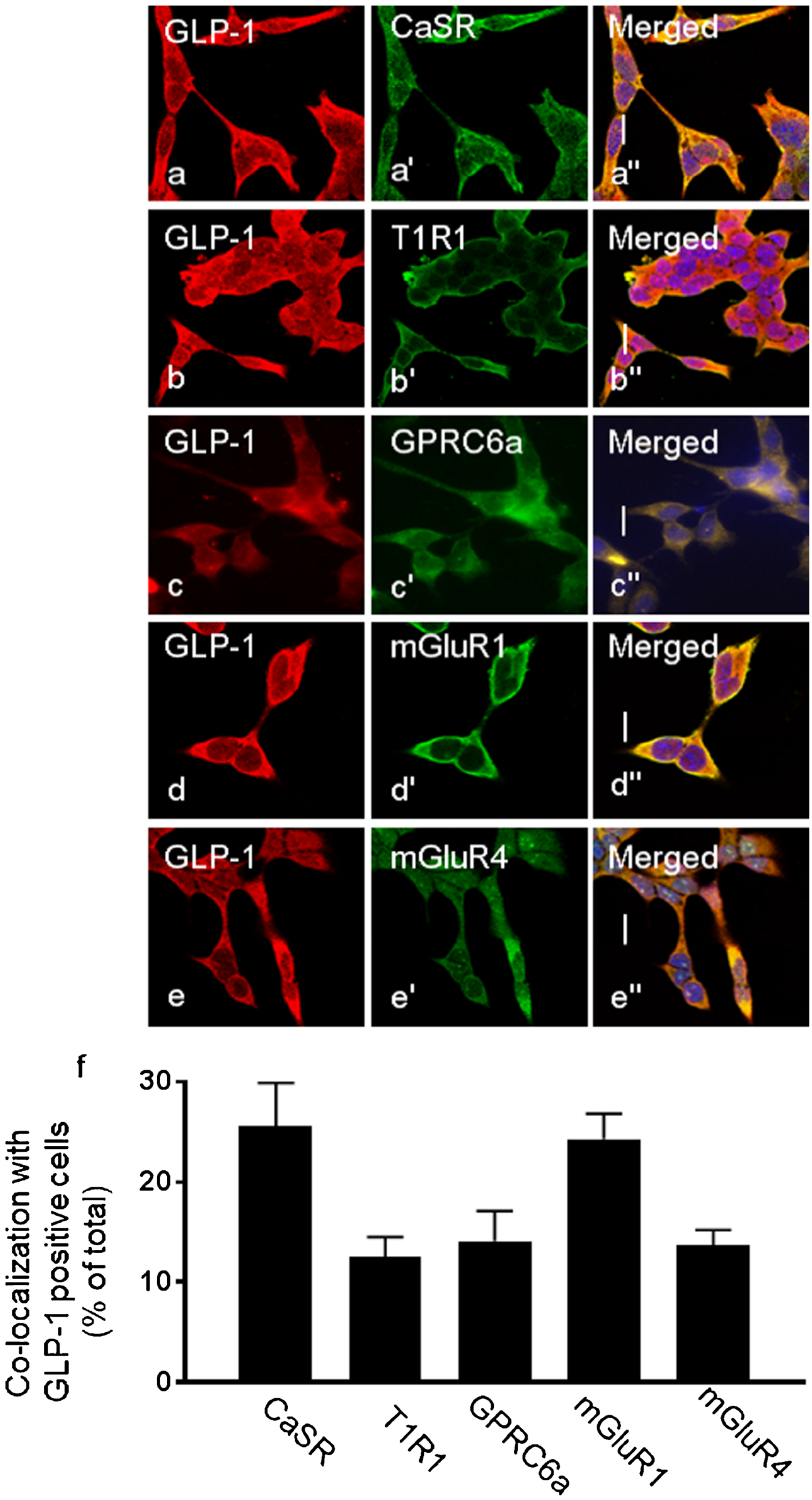
Coexpression of L-amino acid receptors with GLP-1 or PYY in STC-1 cells

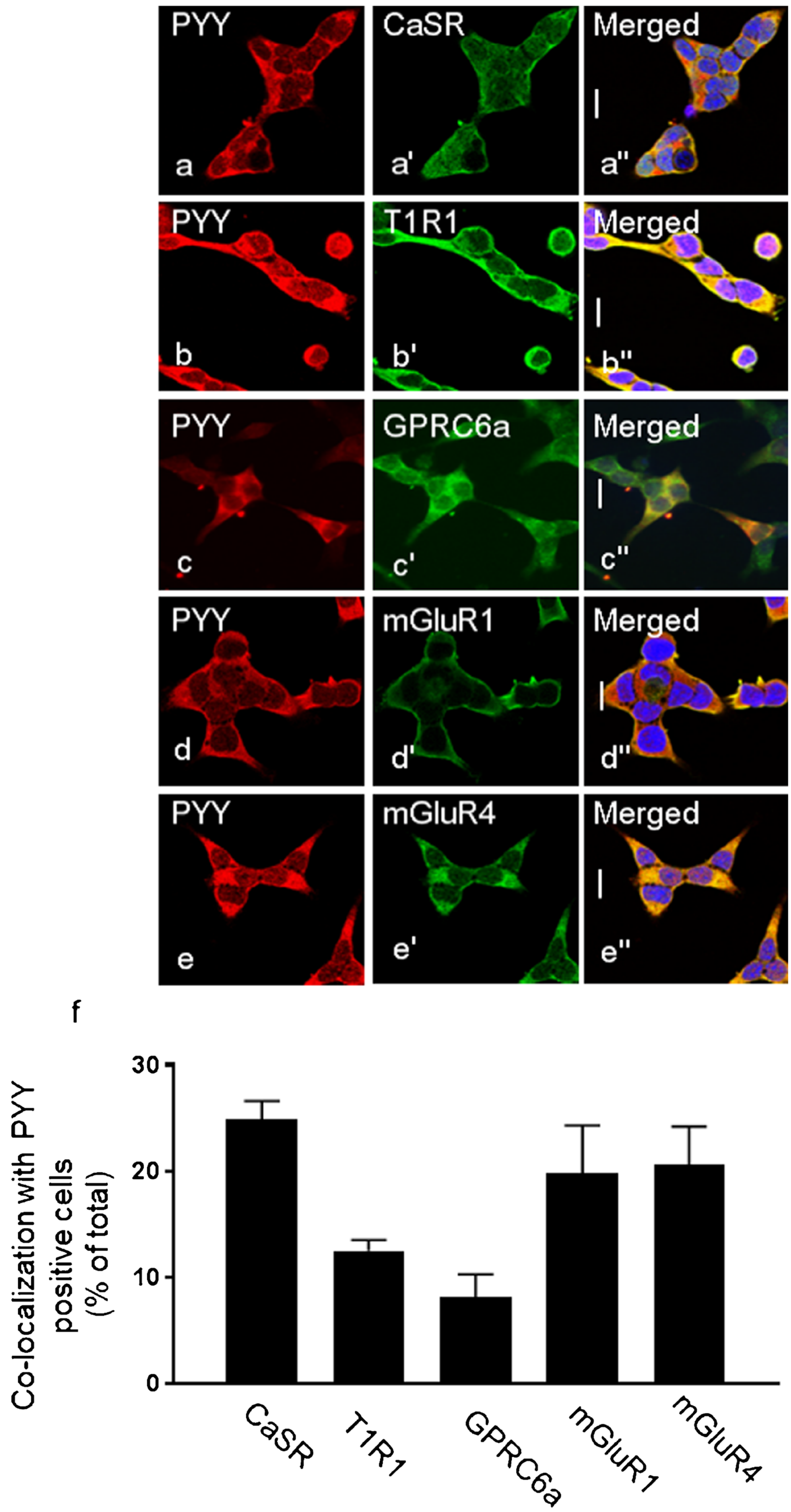
Since the STC-1 are derived from a murine enteroendocrine cell, we examined the coexpression of the L-AA receptors with two major enteric hormones known to be involved in regulation of glucose homeostasis and appetite, GLP-1 and PYY (Gribble and Reimann 2016, 2017; McCarthy et al. 2015; Steensels and Depoortere 2018). Typically, these hormones were associated with the cells designated as L-cells, although, as noted in the discussion, these classifications have become less stringent recently. GLP-1-expressing STC-1 cells were found to express each of the L-AA receptors although the extent of colocalization varied with the receptor type (Fig. 5). The CaSR and mGlu1 receptors were most commonly expressed on GLP-1-expressing STC-1 cells with coexpression levels of 26 ± 4% and 24 ± 5%, respectively, whereas the T1R1, GPRC6a and mGlu4 receptors were coexpressed less frequently with GLP-1-expressing STC-1 cells (12–14% coexpression). With regard to PYY-expressing STC-1 cells, the CaSR was the most frequently expressed receptor (25 ± 2%), with each mGlu receptor coexpressed slightly less frequently (20–21%). The T1R1 (12 ± 1%) and GRPC6a (8 ± 2%) were coexpressed with PYY least often (Fig. 6).

Release of GLP-1 by L-phenylalanine

Since the most commonly expressed L-AA receptor was the CaSR, we tested the functional coupling of this receptor to release of GLP-1 using siRNA to deplete cells of this receptor. The siRNA to CaSR caused a significant reduction in the expression of CaSR as measured by immunofluorescence staining (Fig. 7a-a"') or by Western blot (Fig. 7b) as compared with STC-1 cells treated with control siRNA. Addition of 10 mM L-phenylalanine, a known agonist of CaSR (Wallendorph et al. 2009), caused a significant 3-fold increase in GLP-1 release in STC-1 cells treated with control siRNA for CaSR. In contrast, the release of GLP-1 in response to L-phenylalanine was strongly inhibited in STC-1 cells treated with siRNA to CaSR (Fig. 7c). There was no difference between basal GLP-1 release in the cells treated with control siRNA or siRNA to CaSR.

Fig. 5 **a** Immunofluorescence staining for GLP-1 (**a**, **b**, **c**, **d**, **e**) and each of the other L-AA receptors (CaSR (**a'**), T1R1 (**b'**), GPRC6a (**c'**), mGluR1 (**d'**) and mGluR4 (**e'**)) in STC-1 cells. In each case, the GLP-1 image was merged with that of another L-AA receptor to determine colocalization and DAPI staining was used for nuclear localization (**a"**, **b"**, **c"**, **d"**, **e"**). **f** Quantitative analysis of STC-1 cells costained for GLP-1 and each of the other receptors (CaSR, T1R1, GPRC6a, mGluR1, or mGluR4). One thousand cells were counted for each pairing. Values are mean ± SD. Bars in **a"**, **b"**, **c"**, **d"**, and **e"** indicate 10 μm ▶


GLP-1
CaSR
Merged
a
a'
a''
GLP-1
T1R1
Merged
b
b'
b''
GLP-1
GPRC6a
Merged
c
c'
c''
GLP-1
mGluR1
Merged
d
d'
d''
GLP-1
mGluR4
Merged
e
e'
e''
f
Co-localization with GLP-1 positive cells (% of total)
0
10
20
30
CaSR
T1R1
GPRC6a
mGluR1
mGluR4


PYY
CaSR
Merged
a
a'
a''
PYY
T1R1
Merged
b
b'
b''
PYY
GPRC6a
Merged
c
c'
c''
PYY
mGluR1
Merged
d
d'
d''
PYY
mGluR4
Merged
e
e'
e''
f
Co-localization with PYY positive cells (% of total)
30
20
10
0
CaSR
T1R1
GPRC6a
mGluR1
mGluR4

◀ **Fig. 6** **a** Immunofluorescence staining for PYY (**a**, **b**, **c**, **d**, **e**) and each of the other L-AA receptors (CaSR (**a**’), T1R1 (**b**’), GPRC6a (**c**’), mGluR1 (**d**’), and mGluR4 (**e**’)) in STC-1 cells. In each case, the PYY image was merged with that of another L-AA receptor to determine colocalization and DAPI staining was used for nuclear localization (**a**”, **b**”, **c**”, **d**”, **e**”). **f** Quantitative analysis of STC-1 cells costained for PYY and each of the other receptors (CaSR, T1R1, GPRC6a, mGluR1, or mGluR4). One thousand cells were counted for each pairing. Values are mean ± SD. Bars in **a**”, **b**”, **c**”, **d**”, and **e**” indicate 10 μm

Discussion

The ability of the body to respond to nutrients and maintain homeostasis depends on a complex system that requires the ability to sense specific components and either activate neurons or release appropriate hormones in response. The process begins in the oral cavity with the taste buds containing specialized cells which recognize the 5 basic tastes and respond by activation of sensory nerves (cranial nerves VII, IX and X). It has recently become clear that the taste system is only the “tip of the iceberg” and that chemosensation, using similar receptors, signaling cascades and response mechanisms, exist throughout the body including the pulmonary and gastrointestinal systems (Sbarbati et al. 2010). In these systems, the chemosensitive cells are not organized into structures like the taste bud but rather are dispersed throughout the epithelial cell layer as individual chemosensitive cells. In the gut, these can be brush cells (often also called tuft cells) (Schneider et al. 2018; Schutz et al. 2015) or they can be EECs that share many characteristics with lingual taste cells including the same unique intracellular signaling mechanisms such as the taste G protein $G\alpha_{gustducin}$, phospholipase C-β2 (PLC-β2) and calcium-activated nonselective cation channel (transient receptor potential cation channel subfamily M member 5 or TRPM5) (Bezencon et al. 2007; Gribble and Reimann 2016; Sbarbati et al. 2010; Steensels and Depoortere 2018; Sutherland et al. 2007; Young et al. 2009). Taste receptors

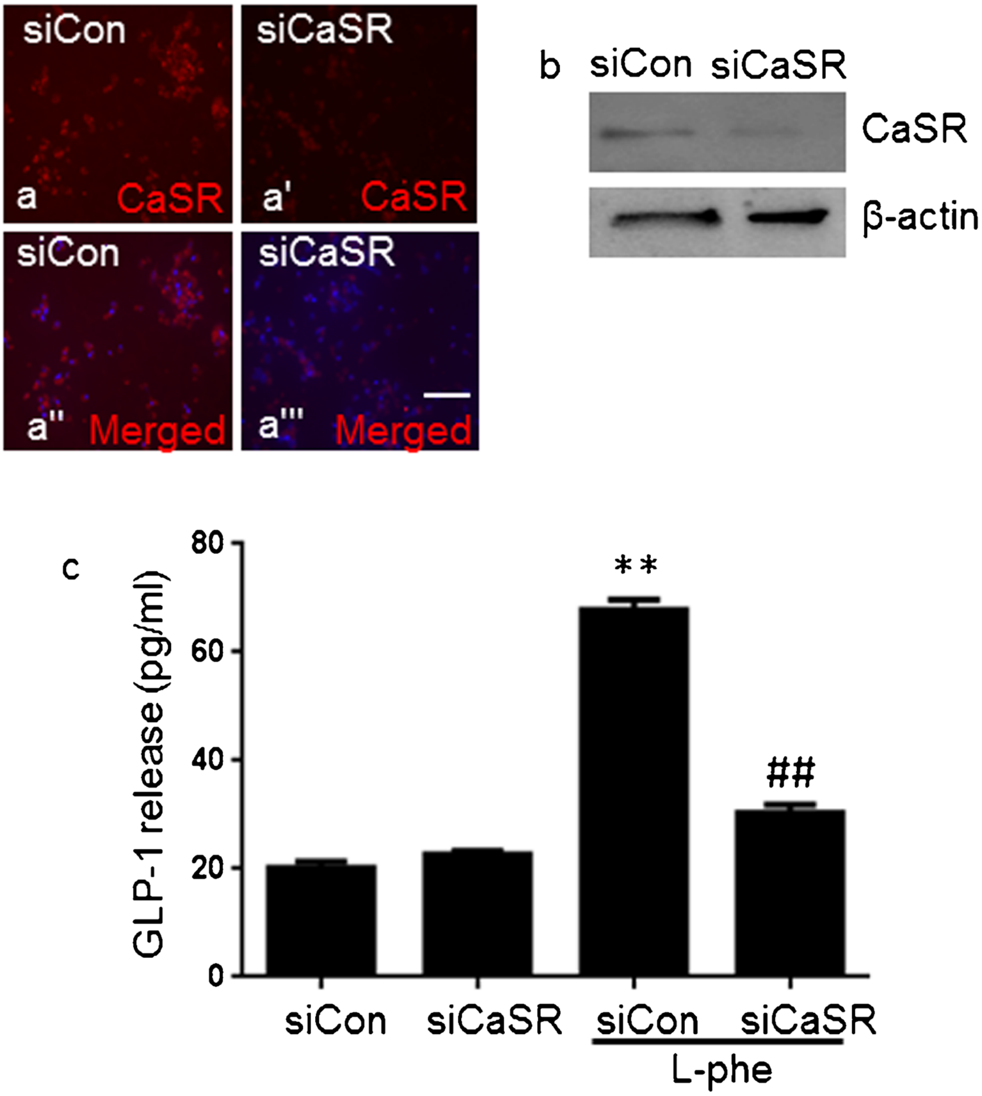


Fig. 7 Immunofluorescence staining for CaSR in STC-1 cells treated with 60 pM control siRNA (**a**) or siRNA to CaSR (**a**’). Cells were costained with DAPI for nuclear identification of cells (**a**”, **a**”’). Bar indicates 20 μm. **b** Western blot of CaSR protein in homogenates of STC-1 cells treated with 60 pM control siRNA or siRNA to CaSR. **c** Release of GLP-1 in response to 10 mM L-phenylalanine in STC-1 cells treated with 60 pM control siRNA or siRNA to CaSR. ** indicates significant difference of $p < 0.01$ from basal release. ## indicates significant difference of $p < 0.01$ from L-phenylalanine-induced release in STC-1 cells treated with control siRNA

for sweet (T1R2/T1R3), umami (T1R1/T1R3) and bitter (TR2s) are present on EECs; however, unlike taste cells where individual cells are specifically "tuned" to one taste modality as a result of expressing a singular receptor type, the gut EECs are known to express receptors for all the basic tastes, as well as a multitude of receptors for other ligands such as fatty acids, bile salts, phytochemicals and products of the microbiome. Like some cells of the taste bud, EEC contain secretory granules, release hormones and transmitters when appropriately stimulated (Cox 2016; Gribble and Reimann 2016; Kaji and Kaunitz 2017; Steensels and Depoortere 2018) and appear to have synaptic-like connections to sensory neurons and/or subepithelial glial cells (Bohorquez et al. 2015; Bellono et al. 2017).

Both EECs and brush cells have been difficult to study for many reasons including their solitary and dispersed nature and their low density (1–3% of mucosal cells) and the fact that they have been difficult to isolate and grow in culture beyond 24–48 h. As a result, several cell lines have been successfully used to study receptor expression, signaling pathways and hormone expression and release. Most notably among these are the GLUTag cells, which were derived from a colonic tumor cell and the STC-1 cell line that were derived from an intestinal tumor cell. Both mimic the GLP-1-secreting or classically described L-cell (Alamshah et al. 2017; Diakogiannaki et al. 2013; Kuhre et al. 2016; McCarthy et al. 2015; Oya et al. 2013; Zhou and Pestka 2015). These and other cell lines used to mimic EEC have also been shown to release a variety of additional neurohormonal peptides depending on conditions; these include GLP-2, oxyntomodulin, CCK, GIP, secretin and brain-derived neurotrophic factor (BDNF) among others (Bala et al. 2014; Gribble and Reimann 2017; Kuhre et al. 2016; McCarthy et al. 2015; Qian et al. 2016; Zhou et al. 2015). While no cell line is a perfect match for a specific class of native EEC, it has been suggested that GLUTag cells might be a better mimic of L-cells than the STC-1 cells (Kuhre et al. 2016). However, a recent review by Gribble and Reimann (2017) suggested that the pluripotential nature of the STC-1 cells makes them a valid model of plurihormonal native small intestinal EEC in general. Recent studies indicate that EECs are also pluripotent on an individual cell basis and are now recognized to secrete more hormones and paracrine agents and in more variable patterns than previously thought (Egerod et al. 2012; Fothergill et al. 2017; Gribble and Reimann 2017; Habib et al. 2012; Haber et al. 2017; Martins et al. 2017; Reynaud et al. 2016; Roth et al. 1990; Sutherland et al. 2007). This may be the result of the ability of individual EECs to respond to local signals in their immediate milieu and alter their hormonal expression accordingly. A recent paper showed that EECs respond to BMP4 signals along the crypt-villus axis during migration and change their hormone expression reflective of greater phenotypic flexibility (Beumer et al. 2018). While the influence of the local environmental cues is lost in cell culture, the variability of individual STC-1 cells in the expression of multiple L-AA receptors evident in our study supports the notion that STC-1 cells show individual phenotypic variation and are generally a valid model of small intestinal EECs.

While STC-1 cells have been shown to express individual L-AA receptors in earlier studies, there has been no determination of the level of expression or degree of colocalization. The results of the present study indicate that (i) mRNAs for all L-AA receptors are expressed in the small intestine and colon; (ii) mRNA for L-AA receptors is more evident in mucosa than smooth muscle homogenates; (iii) in STC-1 cells, the most widely expressed L-AA receptor is the CaSR receptor and the least widely expressed is the T1R1 (umami taste) receptor; (iv) the range of coexpression of L-AA receptors was from 2 to 12%; (v) CaSR coexpression with other receptors was most common whereas coexpression among other receptors was less with lowest (about 2%) coexpression among the umami taste receptor T1R1, mGluR4 and GPRC6a; (vi) all L-AA receptors were coexpressed with the hormones GLP-1 and PYY in a generally similar pattern; and (vii) the release of GLP-1 in response to L-phenylalanine was shown to be mediated by CaSR.

All of the putative L-AA receptors are members of the Class C GPCRs, which are characterized by a large extracellular Venus flytrap domain (VFD) motif containing the L-AA binding site (Wellendorph and Brauner-Osborne 2009; Wellendorph et al. 2009). Although they share basic structural characteristics, each differs in selectivity for individual L-AAs and allosteric modulators. Our data suggest that the CaSR is the most frequently expressed L-AA receptor on STC-1 cells. This is noteworthy since the other receptors are directly activated by L-AAs and positively modulated by Ca^{2+} or require Ca^{2+} whereas the CaSR is directly activated by Ca^{2+} and positively modulated by L-AAs. With regard to L-AAs, the CaSR receptor is considered broadly tuned and responds mainly to aromatic AAs such as L-Phe, with lesser sensitivity to aliphatic and polar L-AAs. The CaSR is least sensitive or insensitive to acidic and basic L-AA and the branched chain AAs (Wellendorph and Brauner-Osborne 2009; Wellendorph et al. 2009). Generally, the next most common receptors, which were also most commonly coexpressed with CaSR, were the mGluRs. These receptors are least closely related to the CaSR and have a relatively fine-tuned sensitivity being responsive to only L-glutamate. While they are related to the mGluR expressed in the brain, those expressed in the taste buds and gut and which play a role in umami taste sensation have a truncated N-terminal extracellular domain (San Gabriel and Uneyama 2013; San Gabriel et al. 2005; Yasumatsu et al. 2015). Phylogenetically, the GPRC6a receptor is most closely related to the CaSR and responds primarily to basic AAs such as L-Arg and L-Lys and to a lesser extent to small and polar amino acids but not aromatic amino acids (Wellendorph and

Brauner-Osborne 2009; Wellendorph et al. 2009). The GPRC6a was coexpressed least often with the CaSR and this may reflect the similar broad tuning of both receptors. Like other receptors in this class, the response is augmented by divalent cations especially Ca^{2+}; however, the GPRC6aR is unique in that it also responds to the hormone testosterone and the peptide osteocalcin (Clemmensen et al. 2014; Pi et al. 2017; Wellendorph and Brauner-Osborne 2009; Wellendorph et al. 2009). The T1R1/T1R3 receptor, as identified by T1R1 immunostaining, was the least frequently expressed L-AA receptor by STC-1 cells in our study. The T1R1/T1R3 responds primarily to L-Glu and L-Asp in humans. Its specificity in responding to monosodium glutamate (MSG) in humans led to it being classified as the umami receptor responsible for the taste of foods like meat, soy sauce, fish and mushrooms. In contrast to humans, in mice it is broadly tuned and responds to all of the 20 natural L-AAs (Li et al. 2002; Nelson et al. 2002; San Gabriel and Uneyama 2013; Wellendorph and Brauner-Osborne 2009; Wellendorph et al. 2009). The T1R1/T1R3 receptor heterodimer is unique among the L-AA receptors in its characteristic potentiation by 5′-ribonucleotides like inosine monophosphate (IMP) (Kusuhara et al. 2013; Yamaguchi 1970; Zhang et al. 2008; Zhao et al. 2003).

The presence of receptors for a wide range of nutrients in cell lines and in gut EECs and their potential role in gut hormone release and function has been the subject of many recent reviews (Cox 2016; Gribble and Reimann 2016, 2017; Kaji and Kaunitz 2017; Latorre et al. 2016; Steensels and Depoortere 2018). The detection of specific L-AA by individual receptors has been shown to have an important role in gut function and in the regulation of the expression and release of individual hormones. In the guinea pig small intestine, activation of inhibitory reflexes to circular muscle in response to L-Phe was inhibited by an antagonist of CaSR whereas similar inhibitory reflexes activated by L-Glu were unaffected by the same antagonist. This suggests that the same or similar reflexes can be activated by different L-AA and L-AA receptor pairs (Gwynne and Bornstein 2007; Gwynne et al. 2017). In contrast, in the rat colon, the T1R1/T1R3 heterodimer has been demonstrated on EEC and shown to mediate the increased activation of the peristaltic reflex and increased colonic pellet propulsion induced by MSG plus IMP and L-Cys but not L-Trp consistent with the activation profile of T1R1/T1R3 (Kendig et al. 2014). The T1R1/T1R3 receptor has also been shown to mediate the release of CCK from the mouse small intestine and STC-1 cells in response to L-Phe, L-Leu and L-Glu, whereas release induced by L-Trp was completely blocked by a CaSR antagonist. The CaSR antagonist also partially inhibited CCK release induced by L-Phe (Daly et al. 2013). Studies on purified CCK-expressing EEC from the mouse intestine also suggest a role of CaSR in mediating the effects of L-Trp and L-Phe (Wang et al. 2011). These studies indicate that the response to individual L-AA depends on one or more L-AA receptors but does not indicate the extent to which each receptor might be involved. The final response would depend on the difference in sensitivity of individual receptors to a given L-AA as well as the extent of colocalization of L-AA receptors.

In the present study, we used STC-1 cells to quantify the colocalization of each of the L-AA receptors with GLP-1 and PYY, two key hormones released from EEC in distal gut and important in the regulation of motility and secretion in the proximal gut and in the regulation of appetite. Our result shows a similar but not identical, pattern of receptor coexpression with GLP-1 and PYY. The CaSR was most often coexpressed with GLP-1 and PYY followed by a similar level of expression of mGlu1. T1R1/T1R3 and GPRC6a were least expressed. The low level of T1R1 is consistent with the finding of low level of expression of T1R1 and T1R3 in tongue and in mouse L-cells (Dyer et al. 2005; Reimann et al. 2008). The exception to the pattern was mGluR4, which had a lower coexpression with GLP-1 than with PYY in STC-1 cells.

Depending on the species, the cell type and location and the stimulus, each L-AA receptor has been postulated to mediate the release of GLP-1 and/or PYY solely or in combination with another L-AA receptor. However, consistent with our finding of the CaSR receptor being the most frequently expressed receptor on STC-1 cells and the ability of siRNA to CaSR to inhibit L-Phe-induced GLP-1 release, most other studies also implicate CaSR in the release of GLP-1 and PYY (Diakogiannaki et al. 2013; Pais et al. 2016; Alamshah et al. 2017). With regard to the release of GLP-1 and PYY, two interesting studies suggest that the coupling of L-AA receptor to hormone release may not be identical with regard to CaSR. In the human colon, L-Phe and L-Trp which most potently activate the CaSR, caused a significant increase in release of GLP-1 but not PYY or the costored biogenic amine serotonin (Symonds et al. 2015). Similarly, recent studies in rat demonstrated that L-Trp-induced secretion of GLP-1 but not PYY was inhibited by a CaSR antagonist (Acar et al. 2018). This suggests differential coupling of the L-AA receptors to GLP-1 and PYY release and points to the importance of knowing the relative complement of different L-AA receptors on EEC and STC-1 cells.

In conclusion, the present study indicates that mRNA for all putative L-AA receptors are expressed by mucosal and to a lesser extent muscle cells of the mouse whole small intestine and colon. The difficulty in isolation and examination of EECs has led to the use of cell lines to mimic EECs for examination of receptors, signaling pathways and release of hormones and bioactive agents. We demonstrated that one of these lines, the STC-1 cell, also expresses mRNA and protein for all of the putative L-AA receptors. Quantitation of these receptors on STC-1 demonstrated that CaSR is the most commonly expressed receptor overall and is the most commonly expressed L-AA receptor in STC-1 cells that also express GLP-1 and PYY. Consistent with this finding, the studies also show release of GLP-1 induced by L-Phe is largely mediated by CaSR. Our studies also show that STC-1 cells coexpress the other L-AA receptors in variable combinations and degrees. This variability in L-AA receptor expression may explain in part why the release of different hormones and other agents depends on multiple receptors and may point the way to understanding the degree of participation of a given receptor in mediating physiological responses and release of specific hormones.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national and institutional guidelines for the care and use of animals were followed. All animal procedures were performed according to a protocol approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

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