Biochimica et Biophysica Acta 1848 (2015) 1502–1508

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

The structure–function role of C-terminus in human bitter taste receptor T2R4 signaling

Jasbir Upadhyaya [a,b], Nisha Singh [a,b], Rajinder P. Bhullar [a], Prashen Chelikani [a,b,*]

[a] Department of Oral Biology, College of Dentistry, University of Manitoba, Winnipeg, MB R3E 0W2, Canada
[b] Biology of Breathing Group, Children's Hospital Research Institute of Manitoba, University of Manitoba, Winnipeg, MB R3E 0W2, Canada

ARTICLE INFO

Article history:
Received 26 January 2015
Received in revised form 10 March 2015
Accepted 31 March 2015
Available online 6 April 2015

Keywords:
G protein-coupled receptor (GPCR)
Bitter taste receptor (T2R)
Carboxyl-terminus (C-terminus)
Constitutively active mutant (CAM)
Membrane proximal basic residue (MPBR)

ABSTRACT

Bitter taste, in humans, is sensed by 25 G protein-coupled receptors, referred to as bitter taste receptors (T2Rs). The diverse roles of T2Rs in various extraoral tissues have implicated them as a potential target for therapeutic intervention. Structure–function studies have provided insights into the role of transmembrane and loop regions in the activation mechanism of T2Rs. However, studies aimed at deciphering the role of their carboxyl-terminus (C-terminus) are limited. In this study, we identified a KLK/R motif in the C-terminus that is conserved in 19 of the 25 T2Rs. Using site-directed mutagenesis we studied the role of 16 residues in the C-terminus of T2R4. The C-terminus of T2R4 is polybasic with 6 of the 16 residues consisting of lysines, constituting two separate KK motifs. We analyzed the effect of the C-terminus mutations on plasma membrane trafficking, and characterized their function in response to the T2R4 agonist quinine. The majority of the mutants showed defective receptor trafficking with ≤50% expression on the cell surface. Interestingly, mutation of the distal Lys296 of the KLK motif in T2R4 resulted in constitutive activity. The K296A mutant displayed five-fold basal activity over wild type T2R4, while the conservative substitution K296R showed wild type characteristics. The Lys294, Leu295 and Lys296 of the KLK motif in T2R4 were found to perform crucial roles, both, in receptor trafficking and function. Results from this study provide unique mechanistic insights into the structure–function role of the C-terminus in T2R signaling.

1. Introduction

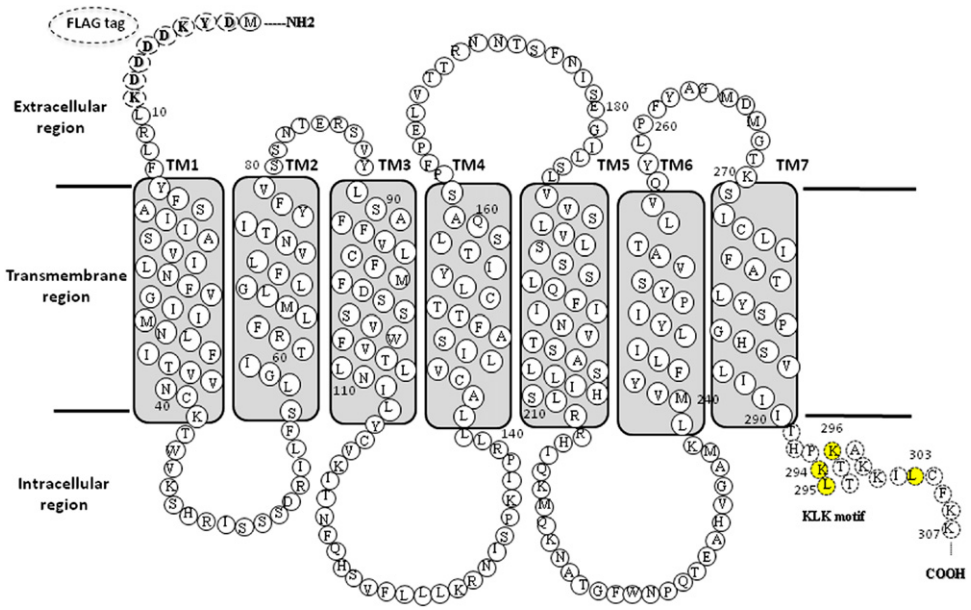
G protein-coupled receptors (GPCRs) form the largest and the most diverse superfamily of cell surface proteins [1]. The 25 human bitter taste receptors (T2Rs) belong to the GPCR superfamily. Like all GPCRs, T2Rs consist of seven transmembrane (TM) helices, three extracellular loops (ECLs) and three intracellular loops (ICLs), with a short extracellular N-terminus and an intracellular C-terminus (Fig. 1A). T2Rs are 290–333 amino acids long and are relatively divergent, showing ~30–70% amino acid identity [2]. The highly conserved amino acid residues and motifs found in most GPCRs generally have critical roles in the mechanism of receptor activation. For example, class A GPCRs have highly conserved motifs, the LxxxD motif in TM2, D/ERY motif in TM3, CWxP motif in TM6, and NPxxY motif in TM7 [3], which are absent in T2Rs. We have previously revealed 13 highly conserved residues and two conserved motifs in T2Rs, LxxxR in TM2 and LxxSL in TM5 [4,5]. These conserved residues and motifs bear no similarity to class A GPCR motifs, suggesting that T2Rs have a unique activation mechanism. Most of the structure–function studies in T2Rs are focused on determining the role of TM and loop region residues involved in T2R activation and ligand binding. The role of the C-terminus in T2R structure and function, however, remains poorly understood.

Previous studies have shown that the cytoplasmic C-terminal region of GPCRs plays an important role in receptor trafficking [6–9], intracellular signaling [8,10,11] and agonist-induced receptor internalization [12]. GPCR export from the endoplasmic reticulum (ER) represents the first step in intracellular trafficking of receptors and influences their cell-surface expression and function [13,14]. However, the definitive sequences involved in trafficking and/or signal transduction remain unclear. Sequence analysis of human GPCRs showed that basic residues are frequent in the membrane-proximal region of the C-terminus. Mutation of the C-terminal membrane-proximal basic residues (MPBRs) results in a marked reduction in the cell surface expression of multiple GPCRs [7,15–18], thus, suggesting that these residues are critically involved in mediating the anterograde trafficking of a broad range of membrane proteins, including GPCRs. The sense of taste has a significant impact on food selection, nutrition and health. It is, therefore, highly desirable to modulate bitter taste perception and T2Rs so that healthier bitter foods and medicines may be rendered more palatable. Studies in airway tissues demonstrate that endogenous T2Rs, upon activation with bitter tastants, lead to muscle relaxation

* Corresponding author at: D 319, Department of Oral Biology, College of Dentistry, 780 Bannatyne Avenue, University of Manitoba, Winnipeg, MB R3E 0W2, Canada. Tel.: +1 204 789 3539; fax: +1 204 789 3913.
E-mail address: Prashen.Chelikani@umanitoba.ca (P. Chelikani).

http://dx.doi.org/10.1016/j.bbamem.2015.03.035
0005-2736/

A



B

KLK/R motif

```
hT2R1     GNPKLKQNAKKFLLHSKCCQ------------------ 299
hT2R3     GNSKLKQTFVVMLRCESGHLKPGSKGPIFS-------- 316
hT2R4     THPKLKTTAKKILCFKK--------------------- 299
hT2R5     GIPRVKQTCQKILWKTVCARRCWGP------------- 299
hT2R7     GNNKLRHASLKVI--WKVMSILKGRKFQQHKQI----- 318
hT2R8     LNNKLRQTFVRMLTCRKIACMI---------------- 309
hT2R9     GNSKLREAFLKML--RFVKCFLRRRKPFVP-------- 312
hT2R10    GNSKLKQASLRVL--QQLKCCEKRKNLRVT-------- 307
hT2R13    GNAKLRQA---------FLLVAAKVWAKR--------- 303
hT2R14    GNKKLRQASLSVLLWLRYMFKDGEPSGHKEFRESS--- 317
hT2R16    SSPTLKRILKGKC------------------------- 291
hT2R38    GNAKLRRAVMTILLWAQSSLKVRADHKADSRTLC---- 333
hT2R39    DNPGLRRAWKRLQLRLHLYPKEWTL------------- 338
hT2R40    GNPGLRRAWKRFQHQVPLYLKGQTL------------- 323
hT2R41    SNLKLRSVFSQLLLLARGFWVA---------------- 307
hT2R43    GNKKLKQTFLSVFWQMRYWVKGEKTSSP---------- 309
hT2R44    GNKKLKQTFLSVLRQVRYWVKGEKPSSP---------- 309
hT2R45    GNKKLKQTYLSVLWQMRY-------------------- 299
hT2R46    GNKKLKQTFLSVLWHVRYWVKGEKPSSS---------- 309
hT2R47    GNKKLKQIFLSVLRHVRYWVKDRSLRLHRFTRGALCVF 319
hT2R48    GSRKLKQTFLSVLWQMTR-------------------- 299
hT2R49    GNKTLKQTFLSVLWQVTCWAKGQNQSTP---------- 309
hT2R50    RTKKLKHTFLLILCQIRC-------------------- 299
hT2R55    GNSKLRQTAVRLL--WHLRNYTKTPNPLPL-------- 314
hT2R60    SNCRLRAVLKSRRSSRCGTP------------------ 318
```

C

```
Human       THPKLKTTAKKILCFKK----------------- 299
Chimpanzee  THPKLKTTAKKILCFKK----------------- 299
Gorilla     THPKLKTTAKKILCFKK----------------- 299
Rhesus      THPKLKTTAKKILCFKK----------------- 299
Marmoset    THPKLKTTAKKILCFKK----------------- 299
Cattle      THPKLKTKAKNILCFSK----------------- 296
Dog         THPKLKTKAKKILCFNK----------------- 299
Mouse       THHKLKAKAKKIFCFYK----------------- 297
Rat         THHKLKAKAKKICCFYKLRDFVSN---------- 304
```

Fig. 1. Amino acid sequence of the bitter taste receptor T2R4. A. Two-dimensional representation of the T2R4 amino acid sequence. It comprises a short extracellular N-terminus, seven transmembrane (TM) helices, three extracellular loops (ECLs), three intracellular loops (ICLs) and a short intracellular C-terminus. The C-terminus residues are shown in dashed circles. The conserved KLK motif and Leu303 are highlighted in yellow color. Also shown is the octapeptide FLAG-epitope at the N-terminus. B. Sequence alignment of the C-terminus of the 25 human T2Rs, without any FLAG-epitope tag at the N-terminus. T2R4 is shown in red, the conserved KLK/R motif is highlighted in green. C. Sequence alignment of C-terminus of T2R4 from different species. The sequences do not include the FLAG-epitope at their N-termini. Human T2R4 is shown in red, the KLK motif is highlighted in green, and the highly conserved amino acids are in blue color. Sequence alignment was done using the ClustalW algorithm.

and bronchodilation of pre-contracted airway smooth muscle cells [19]. In view of the importance of T2R function in extraoral tissues, it becomes important to study cellular trafficking of T2Rs and to elucidate how T2R signal is desensitized.

In this study, we have examined the residues in the C-terminus of T2R4 which are required for optimal cell surface expression and receptor function. Sequence analysis identified a conserved KLK/R motif in the C-terminus of T2Rs. We pursued alanine scanning mutagenesis of the entire C-terminus of T2R4 (except for Ala299). We identified a number of residues which were responsible for proper targeting of T2R4 to the cell surface. Alanine mutations of the Lys294, Leu295 and Lys296 residues of the KLK motif showed a significant reduction in maximal Ca^{2+} responses (E_{max}). Interestingly, the EC_{50} of quinine for K294A increased almost 3-fold in comparison to the WT-T2R4. We discovered a constitutively active mutant (CAM), K296A, which displayed five-fold basal activity over the wild type receptor. In addition to the alanine mutants, residues of the KLK motif were replaced by functionally similar arginine or valine. The conservative substitution mutants, K294R, L295V and K296R, displayed E_{max} and EC_{50} values similar to WT-T2R4. Our data, based on mutagenesis and pharmacological characterization of the mutants, revealed that the KLK motif in T2R4 performs a critical functional role, and the presence of basic and hydrophobic residues in the C-terminus is important for T2R4 trafficking and activation.

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS), quinine hydrochloride, anti-FLAG mouse monoclonal antibody, 2-bromopalmitate (2-BP), and common chemicals were purchased from Sigma. Calcium sensitive dye Fluo-4 NW, cell culture media and AlexaFluor 488 goat anti-mouse antibody were from Invitrogen (Carlsbad, CA, USA). The Gα16–gust44 chimera was a gift from Dr. Takashi Ueda, Nagoya City University, Japan.

2.2. Molecular biology and cell culture

The synthetic N-terminus FLAG tagged TAS2R4 gene expressed in pcDNA3.1 vector has been described before [20]. Alanine, arginine and valine mutations introduced into this gene, with FLAG-tag, were synthesized commercially by GenScript Inc., USA. HEK293T cells were obtained from ATCC and maintained in 10% fetal bovine serum at 37 °C in a 95% air and 5% CO_2 chamber. The wild type and mutant TAS2R4 genes in pcDNA3.1 were transiently co-transfected with the Gα16–gust44 chimera in HEK293T cells using Lipofectamine 2000 (Invitrogen) according to previously published procedures [21].

2.3. Functional calcium assays

Wild type FLAG-T2R4 or mutant genes were co-transfected with Gα16–gust44 chimera in HEK293T cells using 3 μg DNA/1 × 10^6 cells and 6 μl Lipofectamine in six-well tissue culture plates. After 6–8 h of transfection, 1 × 10^5 viable cells/well were plated in 96-well black-walled clear bottom plates. After another 14–16 h, the cells were loaded with 100 μl/well calcium-sensitive Fluo-4 NW dye for 40 min at 37 °C and another 30 min at room temperature. Changes in intracellular calcium were measured after addition of quinine using a FlexStation-3 microplate reader (Molecular Devices), at 525 nm emission following excitation at 494 nm. Dose–response curves were generated and EC_{50} values calculated using Graph Pad Prism software.

2.4. Transformation of unstable plasmids

The T291A, H292A and K296R plasmids were unstable in common *Escherichia coli* strains including DH5α, therefore, their plasmid DNAs were transformed into the low copy number CopyCutter™ EPI400™ Electrocompetent *E. coli* cells (Epicenter Biotechnologies). To induce these clones to high copy numbers, the EPI400 *E. coli* cultures expressing these plasmids were grown overnight, and their optical density was measured at OD_{600}. The cultures were then diluted in LB media to obtain the final culture density with OD_{600} of 0.2. These cultures were then induced with 1× CopyCutter induction solution for 4 h at 37 °C with vigorous shaking. This solution raises the copy number to improve plasmid yields. DNA purification using the Qiagen Maxi-Preparation Kit was carried out after 4 h.

2.5. Flow cytometry

Cell surface expression of wild type T2R4 and mutants was determined using a BD FACS Canto flow cytometer. HEK293T cells, in a 6-well tissue culture plate, were transfected with 3 μg DNA/1 × 10^6 cells. Following 24 h of transfection, 1 × 10^6/ml viable cells were collected and washed with cold PBS buffer and incubated with mouse monoclonal anti-FLAG M2 primary antibody (1:500 dilution in PBS) for 1 h on ice. After 2 to 3 washings, cells were incubated with AlexaFluor 488 goat anti-mouse secondary antibody (1:1000 dilution). Following washing, cells were resuspended in 300 μl PBS buffer. The fluorescence signals of 10,000 cells/tube were measured by the BD FACS Canto using single-color analysis. The results were analyzed using the FACS Diva and FlowJo software programs. The cell surface expression was calculated in terms of Mean Fluorescence Intensity (MFI) as percentage increase over control, i.e., expression of wild type T2R4, which was set at 100%.

2.6. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) with *Tukey's* or *Dunnett's* post-hoc test from a minimum of 3 independent experiments to determine the statistical significance where applicable. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

3. Results

Studies have demonstrated the importance of the C-terminal region of GPCRs for a variety of receptor functions, including cell surface expression, signaling pathways, dimerization, agonist-induced internalization, and desensitization. The role of the C-terminus of T2Rs in

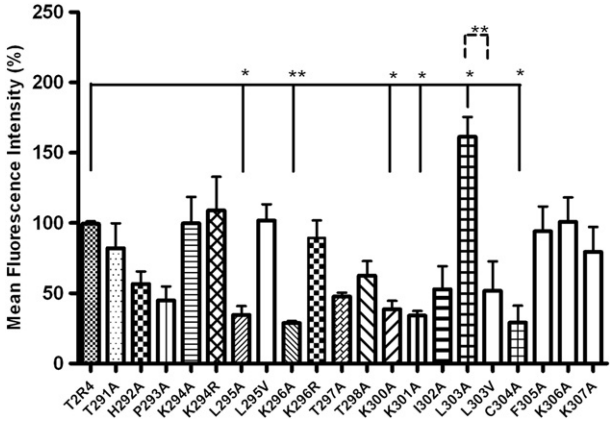


Fig. 2. Cell surface expression of the T2R4 and C-terminus mutants. Analysis of cell surface expression was performed by flow cytometry using the monoclonal mouse anti-FLAG antibody (1:500 dilution), which detects the FLAG-sequence at the N-terminus of the T2R4 and mutant receptors. The primary antibody was detected by goat anti-mouse AlexaFluor 488 antibody (1:1000 dilution). The mean fluorescence intensities of mutants were normalized to those of WT-T2R4 and expressed in percentage. Some of the mutants showed statistically significant reduced surface expression, whereas, L303A showed significantly increased expression compared to wild type. Data is representative of 5–7 independent experiments. Significance was analyzed using one way ANOVA with *Dunnett's* post-hoc test, *$p < 0.05$ and **$p < 0.01$.

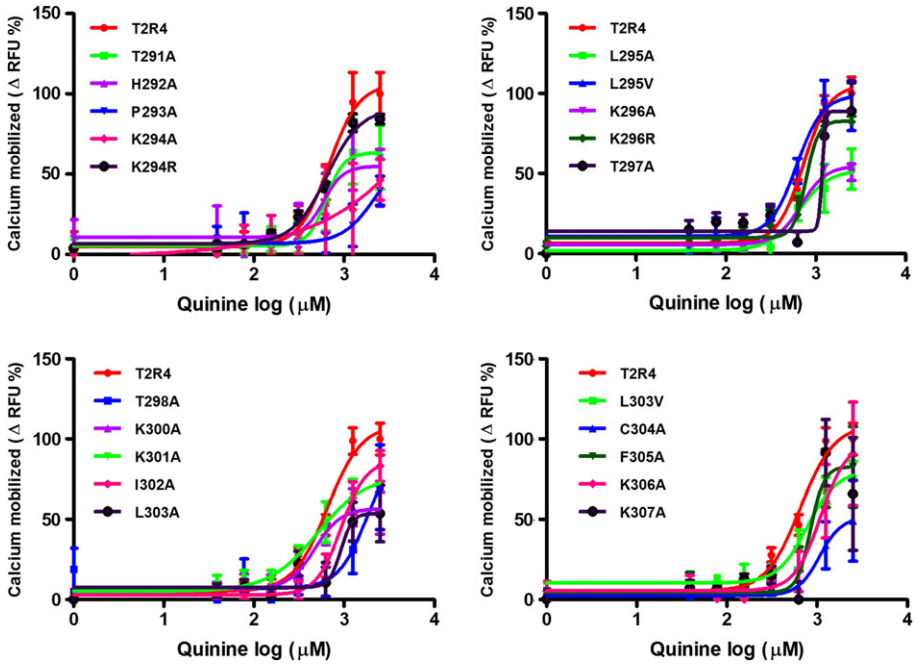


Fig. 3. Functional characterization of T2R4 C-terminus mutants. Concentration-dependent changes in intracellular calcium $[Ca^{2+}]_i$ induced by the bitter compound quinine in HEK293T cells transfected with T2R4 or mutants. Data is representative of 3–4 independent experiments performed in triplicates. After subtracting the responses of quinine-induced mock-transfected cells, dose–response curves were generated and EC_{50} values were calculated by non-linear regression analysis using Graph Pad Prism software. RFU, relative fluorescence units.

receptor function remains to be elucidated. Previously, using TM prediction algorithms we proposed a secondary structure for T2R4 [20]. T2R4 has a short C-terminus which consists of 17 amino acids, one of them being alanine (Fig. 1A). Six of these residues are lysines and one is histidine, indicating the polybasic nature of this cytoplasmic region. Human T2Rs have multiple basic residues in the C-terminus. The number of basic residues varies with the length of the C-termini, ranging from 3 in T2R41 and T2R49 to 12 in T2R47 (Fig. 1B). This indicates that these residues might have a role of functional importance in regulating receptor signaling. Thus, we performed an alanine scan mutagenesis of all C-terminus residues (except Ala299) of T2R4, and characterized the role of these mutations on cell surface expression and receptor function. The residues were mutated to an alanine, with the expectation that this substitution will have a minimal effect on receptor folding and ligand binding. In addition to the alanine mutations, conservative substitutions such as lysine to arginine, and leucine to valine for the residues of the KLK motif and for Leu303, which is conserved in 18 of the 25 T2Rs, were made (Fig. 1B). The KLK motif is highly conserved, while Leu303 is conserved in 7 of the 9 species analyzed or 78% conserved (Fig. 1C).

Table 1
Pharmacological characterization of WT-T2R4 and C-terminus mutants. Functional characterization was performed by measuring changes in intracellular calcium after application of different concentrations of quinine. Cell surface expression was determined by flow cytometry.

Receptor	EC_{50} (μM)	E_{max} (%)	Cell surface expression (%)
WT-T2R4	715 ± 264	100 ± 14	100 ± 2
T291A	647 ± 141	61 ± 20	82 ± 17
H292A	758 ± 477	48 ± 17**	56 ± 8
P293A	NS	42 ± 9**	45 ± 10
K294A	2132 ± 700**	53 ± 17*	100 ± 20
K294R	693 ± 249	84 ± 3	109 ± 19
L295A	712 ± 277	52 ± 12*	30 ± 6
L295V	680 ± 304	92 ± 15	102 ± 10
K296A	776 ± 341	50 ± 5**	29 ± 1
K296R	817 ± 245	82 ± 3	89 ± 11
T297A	NS	89 ± 17	48 ± 2
T298A	NS	60 ± 26	63 ± 10
K300A	513 ± 195	54 ± 13*	39 ± 6
K301A	591 ± 299	69 ± 2	34 ± 4
I302A	935 ± 102	82 ± 4	53 ± 16
L303A	1281 ± 891	53 ± 4*	161 ± 14
L303V	853 ± 343	75 ± 10	52 ± 21
C304A	1209 ± 100	50 ± 14**	29 ± 10
F305A	874 ± 212	82 ± 25	94 ± 17
K306A	1194 ± 454	91 ± 32	101 ± 18
K307A	NS	65 ± 30	80 ± 17

The data represent the mean ± SEM of three to five independent experiments performed in triplicates. NS — not saturated, concentration-dependent increase in calcium not observed with quinine, E_{max} — the maximum possible effect of quinine. Significance was checked using one-way ANOVA and *Dunnett's* post-hoc test with *$p < 0.05$ and **$p < 0.01$.

3.1. Role of the C-terminus residues in cell surface expression

Having the relatively conserved C-terminal MPBRs in GPCRs, we wanted to investigate whether these MPBRs are also required for cell surface trafficking of T2Rs. For this, amino-terminal FLAG-tagged WT-T2R4 or the mutants were transiently expressed in HEK293T cells and cell surface expression, of the non-permeabilized cells, was measured by flow cytometry. Our data revealed that alanine mutations of many C-terminus residues resulted in reduced surface expression when compared to WT-T2R4 (Fig. 2). Mutants L295A, K296A, K300A, K301A and C304A showed significantly reduced surface expression levels amounting to a reduction of 60%–70% (Fig. 2). Of these residues, Leu295 and Lys296 form part of the conserved KLK motif, at positions 294 to 296, towards the amino end of the C-terminus in T2Rs. Lys294 in the KLK motif is the least conserved residue in this triplet at 76%, Leu295 is at 96% and Lys296 or Arg is 100% conserved among T2Rs. Leucine at position 295 is replaced by a valine in 4% or one T2R. The K294A mutant showed cell surface expression similar to WT-T2R4, indicating that the Leu295 and Lys296 residues of this conserved motif are critical for proper targeting of T2R4 to the cell surface. We further mutated these residues to functionally similar valine or arginine to mimic the T2Rs that contain these residues at the indicated positions. Mutants K294R, L295V and K296R behaved similar to the wild type receptor in terms of cell surface expression (Fig. 2). For other residues replaced with alanine, the cell surface expression was comparable to that of WT-T2R4 as no statistically significant results were obtained. In contrast, Leu303, which is conserved in 18 T2Rs (72%), after substitution with alanine showed an increase of 61 ± 14% in surface expression

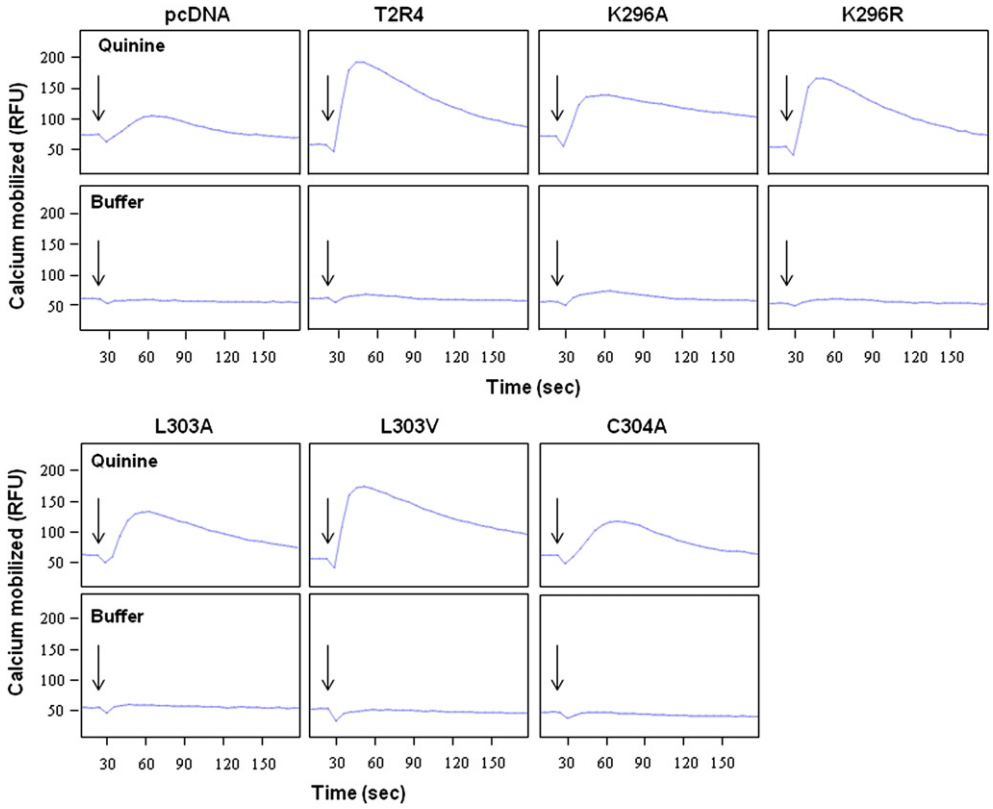


Fig. 4. Representative calcium traces of T2R4 and select mutants transfected in HEK293T cells and induced with 2.5 mM quinine or buffer. The first panel represents cells expressing vector pcDNA3.1 (control or mock transfected). The top panel in each row shows calcium traces of transfected cells stimulated with 2.5 mM quinine, whereas, the lower panel in each row shows the calcium response of cells exposed to assay buffer. Quinine was added to the cells by the automated microplate reader at a time point of 20 s, which is indicated by arrows. The calcium mobilized, which was represented as relative fluorescence units (RFU), was detected by the calcium-sensitive Fluo-4 NW dye after application of a saturating concentration of quinine or buffer and measured by the Flex Station III microplate reader.

(Fig. 2). Interestingly the conservative substitution, L303V, displayed expression comparable to WT-T2R4. The surface expression was significantly different between the two mutants with $^{**}p < 0.01$.

3.2. Functional characterization

After assessing the involvement of C-terminus in optimizing the cell surface expression of T2R4, we next examined whether these residues have a role in stimulating calcium mobilization and receptor function. HEK293T cells transiently transfected with WT-T2R4 or the mutants were stimulated with the agonist quinine, and calcium mobilization was measured as reported previously [4,20,22]. All mutants displayed varied levels of calcium mobilization upon quinine stimulation (Fig. 3). Alanine mutations of the Lys294, Leu295 and Lys296 residues of the KLK motif showed a statistically significant reduction in maximal Ca^{2+} responses (E_{max} of 53 ± 17%, 52 ± 12% and 50 ± 5% respectively, with a significance of $^{*}p < 0.05$ for both K294A and L295A, and $^{**}p < 0.01$ for K296A, Fig. 3 and Table 1). Interestingly, the EC_{50} of quinine for K294A increased almost 3-fold in comparison to the WT-T2R4 which was statistically significant with $^{**}p < 0.01$ (Fig. 3, Table 1). There was no remarkable change in the EC_{50} values of L295A and K296A compared to WT-T2R4, despite their reduced E_{max} values. In contrast, the conservative substitution mutants, K294R, L295V and K296R, displayed E_{max} and EC_{50} values similar to those of WT-T2R4. Fig. 4 shows representative calcium traces for select mutants and mock-transfected cells stimulated with a saturating concentration of 2.5 mM quinine or with buffer (control). The calcium signal of mutants P293A, T297A, T298A and K307A did not saturate even at the highest concentration of quinine (2.5 mM). The P293A and H292A mutants displayed a significantly reduced E_{max} compared to WT-T2R4 with $^{**}p < 0.01$ (Table 1). A right-shift was observed in the dose–response curves of T297A and T298A, but despite the low surface expression, the E_{max} of T297A was similar to WT-T2R4. The EC_{50} values of K300A and K301A were within the normal range. However, K300A showed a statistically reduced E_{max} in comparison to WT-T2R4 with a significance of $^{*}p < 0.05$ (Fig. 3, Table 1). A right-shift was observed in the dose–response curves of I302A, L303A, C304A, F305A, K306A and K307A. L303A showed a statistically significant reduction in E_{max} (~45%) despite an increase in surface expression (Figs. 2, 3, Table 1). However, the L303V phenotype behaved similarly to WT-T2R4. C304A showed significantly reduced maximal calcium responses ($^{**}p < 0.01$) and a marginal increase in EC_{50}, which corresponded with its reduced surface expression.

3.3. Characterization of basal signaling

Recently, we characterized several CAMs in T2R4 [5,20]. Constitutive activity locks a receptor in an active conformation and allows it to signal even in the absence of an agonist [23,24]. This activity, which has been observed for more than 60 GPCRs, is known to have important pathophysiological roles in human disease [25]. To characterize the agonist-independent (or basal) signaling of the C-terminal mutants, we measured changes in intracellular calcium with assay buffer alone (basal activity). This response was then normalized to receptor cell surface expression. Of the 20 mutants analyzed, K296A and C304A displayed more than a 2-fold increase over WT-T2R4 basal activity (Fig. 5A).

Next, to assess the constitutive activity of K296A and C304A in detail, we analyzed the effect of receptor density on basal calcium mobilization (Fig. 5B). HEK293T cells were transiently transfected with increasing amounts of WT-T2R4 or mutant DNA (2 μg, 4 μg and 6 μg DNA per 10^6 cells). The rationale was that an increase in DNA concentration would increase the surface expression of receptors and as a result their activity. This method of identifying CAMs has been validated in our previous

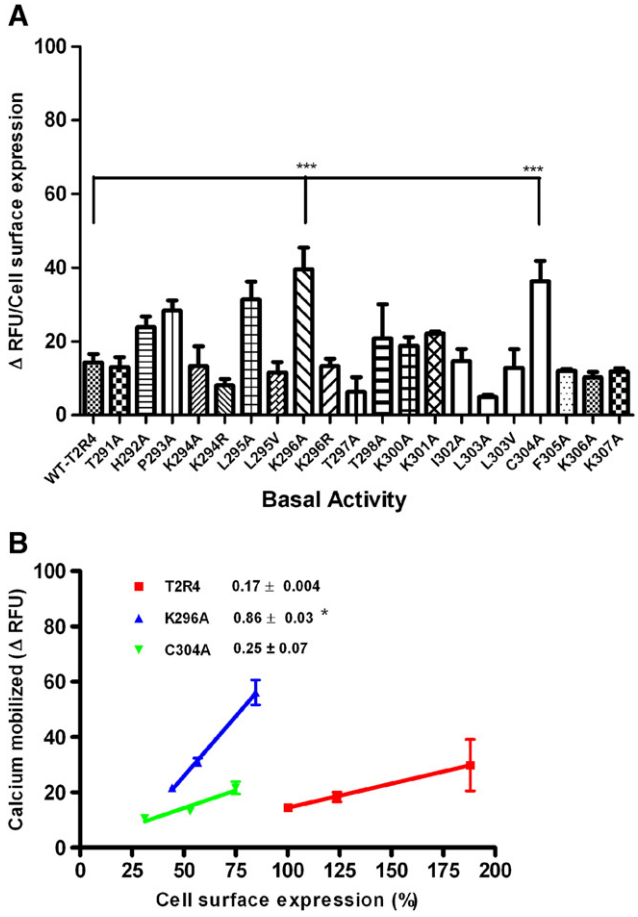


Fig. 5. Pharmacological characterization of the basal activity of WT-T2R4 and C-terminus mutants. A. The agonist-independent or basal activity of all the C-terminus mutants. Changes in calcium mobilized, represented by ΔRFU, were normalized to their cell surface expression as analyzed by flow cytometry. Significance was determined using one way ANOVA with *Dunnett's* post-hoc test, ***$p < 0.001$. Mutants K296A and C304A showed increased basal activity in comparison to wild type receptor. Data is representative of 3–4 independent experiments. B. Effect of receptor density on basal calcium mobilization. WT-T2R4 (red line), K296A (blue line) and C304A (green line) constructs were transiently expressed in HEK293T cells at different receptor densities by varying amounts of DNA used in each transfection (2 μg, 4 μg, and 6 μg DNA per 10^6 cells). Receptor expression levels were determined by flow cytometry and normalized to WT-T2R4 transfected with 2 μg DNA per 10^6 cells, which was taken as 100%. The slope of WT-T2R4, which was calculated by linear regression analysis, was 0.17 ± 0.01, whereas that of K296A and C304A was 0.86 ± 0.03 and 0.25 ± 0.07, respectively. The slope value of K296A was statistically significant, with *$p < 0.05$, when compared to that of WT-T2R4. Significance was determined using one-way ANOVA with *Dunnett's* post-hoc test. This shows that K296A displays five-fold increase over basal WT-T2R4 activity.

publications [5,26]. The effect of receptor density on Ca^{2+} mobilization was calculated from the slope of expression vs. basal activity of K296A and C304A, which was compared to that of WT-T2R4. The slope of WT-T2R4 was 0.17 ± 0.01, whereas, that of K296A and C304A was 0.86 ± 0.03 and 0.25 ± 0.07 respectively (Fig. 5B). This data shows that K296A exhibits true constitutive activity, which is five-fold higher than that of WT-T2R4, with a significance of *$p < 0.05$.

4. Discussion

Sequence analysis of the human membrane protein database demonstrated the prevalence of MPBRs in the C-terminal tail in GPCRs and other receptor proteins [15]. These MPBRs were reported to be involved in anterograde trafficking in a limited number of proteins. For example, the basic domain in C-terminus of chemokine receptor CCR5 supports optimal surface expression by co-operating with the adjacent downstream cysteine cluster [7]. The C-terminal MPBRs were also required for both cell surface expression and signaling of melanin-concentrating hormone receptor 1, MCH1R [16]. The positive role of MPBRs in anterograde trafficking was reported for the HIV coreceptor GPR15 and Golgi-resident glycosyltransferases [15,17]. The C-terminal 10–12 residues following the TM segment in human GPCRs are considered the membrane proximal residues, with +1 being the most proximal to the TM [15,27]. A majority of the mutants which displayed reduced surface expression in our study fall in the MPBR of T2R4 C-terminus, with Thr291 being the most proximal and Leu303 the most distal residue (Fig. 1A and B). Interestingly L303A is the only mutant that showed increased cell surface expression but most of it is not functional as indicated by its reduced E_{max} (Table 1). This shows that in addition to the hydrophobic property, the bulkiness or the branched nature of the amino acid at position 303 is important for proper function. To determine whether a structurally and functionally amino acid similar to leucine is required at this position, we made the L303V mutation. As expected, the conservative substitution L303V showed wild type characteristics. The L295A and K296A mutants form part of the conserved KLK/R motif in T2Rs. Conservative substitution of these residues with valine or arginine rescued the cell surface expression equivalent to WT-T2R4, suggesting that basic and hydrophobic residues are required at the indicated positions for proper trafficking of the receptor.

Di-lysine (KK) motif in GPCRs is one of the common sorting signals, which leads to ER retention [28,29]. There are two such motifs, Lys300, Lys301 and Lys306, Lys307 in the C-terminus of T2R4 but they are not conserved among T2Rs (Fig. 1B). In human T2Rs, Lys300 (24% in T2Rs) is replaced by either a leucine or valine in 60% of T2Rs, whereas, Lys301 (20% in T2Rs) is replaced by an arginine in 20% of T2Rs. Interestingly, only the first KK motif of Lys300 and Lys301 is highly conserved among T2R4s in different species (Fig. 1C). Our results suggest that this KK motif plays a role in trafficking of T2R4 (Table 1). The KLK/R motif is highly conserved in ICL3 within the class B secretin/glucagon GPCR subfamily [30,31]. This motif is reported to be involved in G-protein coupling for GLP-1 and secretin receptor. Alanine mutation of Lys296, an important residue of the KLK motif in T2Rs, displayed constitutive activity five-fold higher than that of WT-T2R4, indicating that this residue stabilizes an active conformation of T2R4. However, in the absence of a crystal structure of any T2R it is difficult to predict the structural mechanism(s) by which this CAM stabilizes an active conformation. CAMs in the C-terminus of Class A GPCRs, such as the 5-HT4 receptors, dopamine receptors, and P_2Y_{12} have been identified [32–34]. The CAMs in the C-terminus were suggested to have different kinetics of receptor activation compared to CAMs in the ICL3 for the 5-HT4 receptors [32]. Several naturally occurring and disease-causing GPCR mutants with increased constitutive activity have been identified [25]. However, naturally occurring T2R variants with high constitutive activity are yet to be reported.

Studies on G-proteins, both heterotrimeric and monomeric have shown that positive charge conferred by a polybasic domain consisting of six lysines or arginines in the C-terminus in conjunction with a second signal, such as a CAAX lipid anchoring motif (palmitoylation or isoprenylation) is sufficient to target the G-protein to the plasma membrane [35,36]. Even in Class A GPCRs, the CAAX motif, which tethers C-terminus to the plasma membrane forming a 4th intracellular loop, is highly conserved. However, there is no CAAX consensus motif in T2Rs. There is only one cysteine, Cys304, present in the C-terminus of T2R4. Surprisingly, there are no conserved cysteines, and many of the T2Rs have no cysteines in the C-terminus (Fig. 1B). The C304A showed a significantly reduced surface expression and E_{max} value (Fig. 2, Table 1). A right-shift in the dose–response curves was observed for distal residue mutants of C-terminus, i.e., I302A, C304A, F305A, K306A and K307A. We speculate that conformational changes in the receptor brought about by these mutations might have affected receptor activation and/or ligand binding.

Another common motif found in the C-termini of GPCRs is the PDZ binding motif, which is recognized by PDZ containing proteins [37]. A potentially large number of mammalian family A GPCRs have the ability to engage PDZ domain-containing proteins. The first member of this family reported to contain a PDZ motif was the β2AR, which in addition to its signaling function was found to mediate an essential trafficking function [38]. Many T2Rs possess a PDZ binding motif, but no motif is recognizable from the sequence analysis of the T2R4 C-terminus. The absence of conserved cysteines and a PDZ binding motif in C-terminus, thus, depict the disparity among T2Rs. Studying the factors responsible for this low sequence conservation can provide the basis for understanding the vast diversity of the exogenous bitter ligands recognized by T2Rs and their desensitization mechanisms.

5. Conclusion

This study identified both conserved and receptor specific motifs in the C-terminus of T2Rs. Residues in these motifs play different roles in receptor function. The majority of the mutants, which displayed reduced surface expression fall in the MPBR of T2R4 C-terminus. A conserved KLK/R motif in T2Rs, is important for receptor trafficking and activation. Of the two dibasic KK motifs present in the C-terminus of T2R4, the KK motif present at the proximal end and made of Lys300 and Lys301 is important for cell surface expression of T2R4. Additionally, our results revealed a CAM, K296A, indicating that Lys296 of the KLK motif is important for stabilizing the active conformation of T2Rs. Taken together, the results from this study provide novel insights into the role of conserved residues and MPBRs in the C-terminus of T2Rs in receptor expression and activation.

Conflict of interest

None.

Acknowledgements

This work was supported by a Discovery grant (RGPIN-2014-04099) from the Natural Sciences and Engineering Research Council of Canada (NSERC) to PC. A graduate studentship from Research Manitoba and the Children's Hospital Research Institute of Manitoba to JDU, and a Manitoba Medical Service Foundation Dr. Allen Rouse Career Award to PC.

References

[1] K.L. Pierce, R.T. Premont, R.J. Lefkowitz, Seven-transmembrane receptors, Nat. Rev. Mol. Cell Biol. 3 (2002) 639–650.
[2] M. Behrens, W. Meyerhof, Bitter taste receptors and human bitter taste perception, Cell. Mol. Life Sci. 63 (2006) 1501–1509.
[3] M. Arakawa, R. Chakraborty, J. Upadhyaya, M. Eilers, P.J. Reeves, S.O. Smith, P. Chelikani, Structural and functional roles of small group-conserved amino acids present on helix-H7 in the beta(2)-adrenergic receptor, Biochim. Biophys. Acta 1808 (2011) 1170–1178.
[4] S.P. Pydi, T. Sobotkiewicz, R. Billakanti, R.P. Bhullar, M.C. Loewen, P. Chelikani, Amino acid derivatives as bitter taste receptor (T2R) blockers, J. Biol. Chem. 289 (2014) 25054–25066.
[5] S.P. Pydi, N. Singh, J. Upadhyaya, R.P. Bhullar, P. Chelikani, The third intracellular loop plays a critical role in bitter taste receptor activation, Biochim. Biophys. Acta 1838 (2014) 231–236.
[6] J.C. Bermak, M. Li, C. Bullock, Q.Y. Zhou, Regulation of transport of the dopamine D1 receptor by a new membrane-associated ER protein, Nat. Cell Biol. 3 (2001) 492–498.
[7] S. Venkatesan, A. Petrovic, M. Locati, Y.O. Kim, D. Weissman, P.M. Murphy, A membrane-proximal basic domain and cysteine cluster in the C-terminal tail of CCR5 constitute a bipartite motif critical for cell surface expression, J. Biol. Chem. 276 (2001) 40133–40145.
[8] H. Pankevych, V. Korkhov, M. Freissmuth, C. Nanoff, Truncation of the A1 adenosine receptor reveals distinct roles of the membrane-proximal carboxyl terminus in receptor folding and G protein coupling, J. Biol. Chem. 278 (2003) 30283–30293.
[9] D. VanLeeuwen, M.E. Steffey, C. Donahue, G. Ho, R.G. MacKenzie, Cell surface expression of the melanocortin-4 receptor is dependent on a C-terminal di-isoleucine sequence at codons 316/317, J. Biol. Chem. 278 (2003) 15935–15940.
[10] T. Namba, Y. Sugimoto, M. Negishi, A. Irie, F. Ushikubi, A. Kakizuka, S. Ito, A. Ichikawa, S. Narumiya, Alternative splicing of C-terminal tail of prostaglandin E receptor subtype EP3 determines G-protein specificity, Nature 365 (1993) 166–170.
[11] R.M. Lyu, P.M. Germano, J.K. Choi, S.V. Le, J.R. Pisegna, Identification of an essential amino acid motif within the C terminus of the pituitary adenylate cyclase-activating polypeptide type I receptor that is critical for signal transduction but not for receptor internalization, J. Biol. Chem. 275 (2000) 36134–36142.
[12] S.K. Bohm, L.M. Khitin, S.P. Smeekens, E.F. Grady, D.G. Payan, N.W. Bunnett, Identification of potential tyrosine-containing endocytic motifs in the carboxyl-tail and seventh transmembrane domain of the neurokinin 1 receptor, J. Biol. Chem. 272 (1997) 2363–2372.
[13] C. Dong, G. Wu, Regulation of anterograde transport of adrenergic and angiotensin II receptors by Rab2 and Rab6 GTPases, Cell. Signal. 19 (2007) 2388–2399.
[14] U.E. Petaja-Repo, M. Hogue, A. Laperriere, P. Walker, M. Bouvier, Export from the endoplasmic reticulum represents the limiting step in the maturation and cell surface expression of the human delta opioid receptor, J. Biol. Chem. 275 (2000) 13727–13736.
[15] Y. Okamoto, J.D. Bernstein, S. Shikano, Role of C-terminal membrane-proximal basic residues in cell surface trafficking of HIV coreceptor GPR15 protein, J. Biol. Chem. 288 (2013) 9189–9199.
[16] M. Tetsuka, Y. Saito, K. Imai, H. Doi, K. Maruyama, The basic residues in the membrane-proximal C-terminal tail of the rat melanin-concentrating hormone receptor 1 are required for receptor function, Endocrinology 145 (2004) 3712–3723.
[17] C.G. Giraudo, H.J. Maccioni, Endoplasmic reticulum export of glycosyltransferases depends on interaction of a cytoplasmic dibasic motif with Sar1, Mol. Biol. Cell 14 (2003) 3753–3766.
[18] C. Dong, C.D. Nichols, J. Guo, W. Huang, N.A. Lambert, G. Wu, A triple arg motif mediates alpha(2B)-adrenergic receptor interaction with Sec24C/D and export, Traffic 13 (2012) 857–868.
[19] D.A. Deshpande, W.C. Wang, E.L. McIlmoyle, K.S. Robinett, R.M. Schillinger, S.S. An, J.S. Sham, S.B. Liggett, Bitter taste receptors on airway smooth muscle bronchodilate by localized calcium signaling and reverse obstruction, Nat. Med. 16 (2010) 1299–1304.
[20] S.P. Pydi, R.P. Bhullar, P. Chelikani, Constitutively active mutant gives novel insights into the mechanism of bitter taste receptor activation, J. Neurochem. 122 (2012) 537–544.
[21] J. Upadhyaya, S.P. Pydi, N. Singh, R.E. Aluko, P. Chelikani, Bitter taste receptor T2R1 is activated by dipeptides and tripeptides, Biochem. Biophys. Res. Commun. 398 (2010) 331–335.
[22] W. Meyerhof, C. Batram, C. Kuhn, A. Brockhoff, E. Chudoba, B. Bufe, G. Appendino, M. Behrens, The molecular receptive ranges of human TAS2R bitter taste receptors, Chem. Senses 35 (2010) 157–170.
[23] R.A. Cerione, J. Codina, J.L. Benovic, R.J. Lefkowitz, L. Birnbaumer, M.G. Caron, The mammalian beta 2-adrenergic receptor: reconstitution of functional interactions between pure receptor and pure stimulatory nucleotide binding protein of the adenylate cyclase system, Biochemistry 23 (1984) 4519–4525.
[24] T. Costa, A. Herz, Antagonists with negative intrinsic activity at delta opioid receptors coupled to GTP-binding proteins, Proc. Natl. Acad. Sci. U. S. A. 86 (1989) 7321–7325.
[25] R. Seifert, K. Wenzel-Seifert, Constitutive activity of G-protein-coupled receptors: cause of disease and common property of wild-type receptors, Naunyn Schmiedeberg's Arch. Pharmacol. 366 (2002) 381–416.
[26] R. Chakraborty, R.P. Bhullar, S. Dakshinamurti, J. Hwa, P. Chelikani, Inverse agonism of SQ 29,548 and Ramatroban on Thromboxane A2 receptor, PLoS ONE 9 (2014) e85937.
[27] M. Conner, M.R. Hicks, T. Dafforn, T.J. Knowles, C. Ludwig, S. Staddon, M. Overduin, U.L. Gunther, J. Thome, M. Wheatley, D.R. Poyner, A.C. Conner, Functional and biophysical analysis of the C-terminus of the CGRP-receptor; a family B GPCR, Biochemistry 47 (2008) 8434–8444.
[28] R.D. Teasdale, M.R. Jackson, Signal-mediated sorting of membrane proteins between the endoplasmic reticulum and the Golgi apparatus, Annu. Rev. Cell Dev. Biol. 12 (1996) 27–54.
[29] F. Letourneur, S. Hennecke, C. Demolliere, P. Cosson, Steric masking of a dilysine endoplasmic reticulum retention motif during assembly of the human high affinity receptor for immunoglobulin E, J. Cell Biol. 129 (1995) 971–978.
[30] K.Y. Chan, R.T. Pang, B.K. Chow, Functional segregation of the highly conserved basic motifs within the third endoloop of the human secretin receptor, Endocrinology 142 (2001) 3926–3934.
[31] S. Takhar, S. Gyomorey, R.C. Su, S.K. Mathi, X. Li, M.B. Wheeler, The third cytoplasmic domain of the GLP-1[7-36 amide] receptor is required for coupling to the adenylyl cyclase system, Endocrinology 137 (1996) 2175–2178.
[32] S. Claeysen, M. Sebben, C. Becamel, M.L. Parmentier, A. Dumuis, J. Bockaert, Constitutively active mutants of 5-HT4 receptors are they in unique active states? EMBO Rep. 2 (2001) 61–67.
[33] B. Zhang, A. Albaker, B. Plouffe, C. Lefebvre, M. Tiberi, Constitutive activities and inverse agonism in dopamine receptors, Adv. Pharmacol. 70 (2014) 175–214.
[34] Z. Ding, S. Kim, S.P. Kunapuli, Identification of a potent inverse agonist at a constitutively active mutant of human P2Y12 receptor, Mol. Pharmacol. 69 (2006) 338–345.
[35] J.F. Hancock, K. Cadwallader, H. Paterson, C.J. Marshall, A CAAX or a CAAL motif and a second signal are sufficient for plasma membrane targeting of ras proteins, The EMBO journal 10 (1991) 4033–4039.
[36] F.L. Zhang, P.J. Casey, Protein prenylation: molecular mechanisms and functional consequences, Annu. Rev. Biochem. 65 (1996) 241–269.
[37] J. Saras, C.H. Heldin, PDZ domains bind carboxy-terminal sequences of target proteins, Trends Biochem. Sci. 21 (1996) 455–458.
[38] T.T. Cao, H.W. Deacon, D. Reczek, A. Bretscher, M. von Zastrow, A kinase-regulated PDZ–domain interaction controls endocytic sorting of the beta2-adrenergic receptor, Nature 401 (1999) 286–290.