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Major haplotypes of the human bitter taste receptor TAS2R41 encode functional receptors for chloramphenicol

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ABSTRACT

A complete understanding of bitterness perception requires identification of cognate bitter substances for all human bitter taste receptors (TAS2Rs). However, so far, no agonists have been identified for five of the 25 TAS2Rs, i.e., TAS2R41, TAS2R42, TAS2R45, TAS2R48 and TAS2R60. Due to substantial genetic variability several haplotypes exist for most bitter receptor genes. For some of the deorphaned TAS2Rs, haplotypes have been identified coding for proteins with severely impaired or even lacking receptor function, proposing that the use of non-functional receptor variants in previous investigations accounted for the failure to identify cognate bitter agonists for the orphan TAS2Rs. In the present report we reasoned that at least one out of the major genetically encoded TAS2R variants is functional. Therefore, we expressed the major haplotypes of the five orphan TAS2Rs in our functional assay and challenged the cells with 106 bitter compounds. Chloramphenicol was identified as agonist for TAS2R41. Further studies revealed that TAS2R41 is a 'specialist' receptor highly selective for this antibiotic. None of the other TAS2R variants responded to any of the 106 compounds, suggesting that the use of non-functional variants does not explain the failure to identify cognate agonists for the other four TAS2Rs. Probably, these TAS2Rs are highly selective for bitter substances absent in our compound library.

1. Introduction

Of the five basic taste modalities perceived by mammals bitter taste is the most complex taste quality [1]. The rejection of strongly bitter tasting chemicals is innate and important to avoid ingestion of potentially harmful substances [1,2]. The bitter taste receptor (TAS2R) family belongs to the superfamily of G protein-coupled receptors (GPCR) and consists of ~25 functional members [3] in humans. TAS2Rs are heptahelical membrane proteins expressed in a subset of oral taste receptor cells [4,5]. Somewhat in contrast to the receptors devoted to detect the other taste qualities, TAS2R genes vary tremendously in number and sequence among species reflecting a dynamic evolution [3,6]. After the initial discovery of mammalian TAS2Rs [4,7], numerous studies using functional expression assays lead to the identification of cognate bitter compounds for 20 of the 25 human TAS2Rs (for an overview see [8]). Yet, until today five TAS2Rs, TAS2R41, TAS2R42, TAS2R45, TAS2R48 and TAS2R60 resist deorphanization despite stimulation with numerous natural and synthetic bitter compounds [8]. For the difficulties associated with the functional characterization of the remaining five orphan TAS2Rs several reasons are imaginable: Firstly, in addition to the gustatory system expression of some TAS2Rs has been observed in non-gustatory tissues e.g. testis [9,10], gastrointestinal system [11,12] and respiratory epithelium [10,13]. Thus, one explanation for the persistence of orphan TAS2Rs would be their engagement in merely non-gustatory functions not involving their activation by cognate xenobiotic bitter substances. However, as all 25 TAS2Rs are expressed in taste receptor cells of human circumvallate papillae, an exclusive non-gustatory role appears unlikely [5].

Secondly, the observed receptor inactivity might be caused by technical limitations of functional expression systems such as insufficient cell surface expression, the inability to achieve formation of putative obligatory TAS2R-heteromers due to individual transfection strategies or inadequate concentrations of bitter compounds used for stimulations because of the occurrence of cellular artefacts. Yet, efficient cell surface localization is assured by the use of targeting motifs such as sst3- [14,15] or rho-tags [7] and receptor expression and membrane-localization has been repetitively shown by immunocytochemical staining [8] or cell surface biotinylation procedures [16,17]. Moreover, functional consequences of TAS2R-dimers were not observed [18]. However, due to solubility problems some bitter compounds are to be used at suboptimal concentrations.

Thirdly, most bitter compounds activate several TAS2Rs and vice versa many TAS2Rs possess more than one agonist. Breadth of tuning in TAS2Rs range from "generalist"-receptors to

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"specialists" with a majority of TAS2Rs possessing an intermediate agonist number [19]. Some of the most recently deorphaned TAS2Rs, TAS2R9 [11] and TAS2R50 [20], exhibited a narrow tuning breadth, responding to only three and two, respectively, of the numerous compounds screened. This could also be the case for the remaining orphan TAS2Rs and, perhaps, potent agonists were simply not tested thus far.

Fourthly, psychophysical studies showed different sensitivities in the bitter perception among individuals [21–25] originating from genetic variability of TAS2Rs. TAS2Rs show an unusually high level of single nucleotide polymorphisms (SNP), indels and copy number variations [23,25–27] summing up to ~150 protein coding haplotypes. Varying frequency of these haplotypes in and between ethnical groups was described [26]. TAS2R-haplotypes can encode proteins that possess extremely diverging receptor properties leading to inactive variants as shown for TAS2R38, TAS2R9 and TAS2R44 or to altered sensitivity as observed for TAS2R43 and TAS2R16 [11,21,23,25,28]. It appears conceivable that non-functional SNPs are also present in the group of orphan TAS2Rs. Several bitter compounds, not detected by the 20 deorphaned TAS2Rs, suggest yet unidentified functional receptors encoded by haplotypes of TAS2R41, TAS2R42, TAS2R45, TAS2R48 or TAS2R60 [8]. Therefore, we cloned and investigated all major haplotypes of the remaining five orphan TAS2Rs in a group of Caucasian, Asian and African subjects in calcium imaging experiments using 106 bitter compounds including 30 substances that have no cognate TAS2R.

2. Materials and methods

2.1. Bitter compounds

All used bitter compounds were either identified as bitter in psychophysical tests or analyzed in Meyerhof et al. [8]. Compounds were purchased from Sigma–Aldrich, LGC Standards or isolated by Drs. T. Hofmann (Munich) and G. Appendino (Novara). Compounds were dissolved in C1 solution (130 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM $CaCl_2$, and 10 mM glucose, pH 7.4) or in a mixture of dimethyl sulfoxide (DMSO) and C1 not exceeding final DMSO concentrations of 0.1% (v/v).

2.2. Genomic DNA samples

For identification of orphan TAS2R haplotypes, genotyping was performed in a group of three European, 12 Asian and five African subjects for TAS2R41, TAS2R45 and TAS2R60. For TAS2R42 and TAS2R48 additional 20 European subjects were analyzed. Genomic DNA was isolated from saliva with the peQGOLD Blood DNA Mini Kit (peQLab) following the manufacturer's instructions.

2.3. Genotype analysis and haplotype identification

Sequencing and haplotype identification of the orphan TAS2Rs was performed essentially as described previously [25]. Briefly, PCR amplification and sequencing was performed with primers flanking the TAS2R coding regions. Size and quality of PCR products were confirmed by agarose gel electrophoresis and purified PCR products were sequenced (Eurofins MWG). Haplotypes were determined either from homozygous individuals, by cloning and sequencing of multiple PCR-products from heterozygous individuals or inferred from genotype data using the PHASE software [29].

2.4. Generation of receptor variants

New haplotypes were amplified from genotyped genomic DNA and subcloned into pcDNA5/FRT/TO (Invitrogen) or into peak10 expression vector (EdgeBioSystems) essentially as described previously [15,30]. Briefly, vectors were composed of an 5′-flanking sequence coding for the amino acids 1–45 of the rat somatostatin type 3 receptor for cell-surface targeting [14] and a 3′-flanking sequence coding for the herpes simplex virus (HSV) glycoprotein D epitope for immunocytochemical detection of the recombinant receptors. 5′-*EcoRI* and 3′-*NotI* restriction sites added to the receptor cDNA were used for cloning. Specific amplification of TAS2R45, which is highly homologous to some other TAS2Rs, was achieved by performing nested PCR with primers complementary to *loci* flanking DNA-regions, followed by PCR. For TAS2R42 haplotypes containing an endogenous *EcoRI*-site a blunt end/*NotI* cloning strategy was used. All constructs were checked by sequencing the full coding region including terminal tags.

2.5. Calcium imaging

Functional expression studies were performed in human embryonic kidney cells (HEK 293T) stably expressing the chimeric G protein subunit Gα16gust44 [31] as described previously [8,15,30]. Briefly, cells were transiently transfected using 0.3 μl Lipofectamine 2000 (Invitrogen) and 150 ng plasmid per well of either pooled or single TAS2R constructs. Empty pcDNA5/FRT vector was used as negative control. As positive control 50 ng of a TAS2R known to respond to the corresponding test compound per well was used. After cells were loaded with the calcium sensitive-dye Fluo4-AM (Molecular Probes, 2 μM in DMEM), application of tastants and recording of fluorescence signals was performed with a fluorometric imaging plate reader ($FLIPR^{Tetra}$, Molecular Devices). The response of endogenous somatostatin receptor to 100 nM somatostatin 14 (Bachem) following application of tastants assured cell vitality.

2.6. Data analysis

Data were collected from at least two independent experiments performed in duplicate. For dose–response curve calculation, the peak fluorescence change after compound addition to mock-transfected cells was subtracted from corresponding receptor-expressing cells. Signals were normalized to background fluorescence for each well ($\Delta F/F = (F-F_0)/F_0$) and baseline noise after C1 application was subtracted. Dose–response curves resulted from plotting signal amplitudes versus log of agonist concentration followed by processing with SigmaPlot 9.0. Half-maximal effective concentrations (EC_{50}) were identified by nonlinear regression of the plots to the function $f(x) = (a-d)/[1+(x/EC_{50})^{nH}]+d$, where a is the minimum, d is the maximum, x is the agonist concentration and nH is the Hill coefficient. If dose–response curves lacked saturation due to unspecific signals at higher compound concentrations the lowest activating concentration was determined as threshold value, providing information about the potency of agonist-receptor interaction.

2.7. Immunocytochemistry

To monitor receptor expression level and cell surface localization transfected cells were stained as described previously [20]. Briefly, HEK 293T-Gα16gust44 cells were seeded in 24-well plates on poly-D-lysine (10 μg/ml) coated glass coverslips. Cells were incubated with biotinylated concanavalin A (0.5 μg/ml; Sigma–Aldrich)

binding to membrane glycan structures. After fixation, cells were stained by mouse anti-HSV antiserum (Novagen, 1:15,000) followed by Alexa488-conjugated goat anti-mouse antiserum (Molecular Probes, 1:2000) and streptavidin-Alexa633 (Molecular Probes, 1:1000). Cells were examined using a confocal laser-scanning microscope TCS-SP2 (Leica).

3. Results

3.1. Identification and generation of main orphan haplotypes

Major haplotypes of the orphan bitter receptors TAS2R41, TAS2R42, TAS2R45, TAS2R48 and TAS2R60 were identified in a group of African, Asian and European subjects. Only haplotypes containing amino acid substitutions due to nonsynonymous single nucleotide polymorphisms (nsSNP) were included. Major haplotypes were defined as *TAS2R* gene variants showing a total allele-frequency of at least 5% and together represent more than 90% of sequenced alleles. We identified and further analyzed 13 major haplotypes for the orphan TAS2Rs containing together 22 nsSNPs (Table 1, bold). In addition to nucleotide polymorphisms, a copy number variation preventing amplification of receptor cDNA in three African individuals was observed for TAS2R45 corroborating previous results [23,25].

Except nsSNP G931C in TAS2R42 (TAS2R55) and C704A in TAS2R41, all observed polymorphisms and the null allele of TAS2R45 were reported previously [25,26]. TAS2R45 was the genetically most diverse receptor with four major variants plus null allele. As copy number analysis of TAS2R45 was not performed, only approximate frequencies of TAS2R45 haplotypes can be given. The polymorphism A900G in the TAS2R45 gene locus alters the stop codon to a tryptophane codon resulting in a 10 amino acid longer receptor variant. This TAS2R45 variant has the same overall length of 309 amino acid residues as the closely related receptors TAS2R43, TAS2R44, and TAS2R46 which all reside in a cluster on chromosome 12. These TAS2Rs also share the extended amino acid sequence at the carboxyl terminus with nine identical positions out of 10.

3.2. Identification of chloramphenicol as agonist for orphan receptor TAS2R41

The 13 TAS2R-variants were cloned into expression vectors encoding the rat somatostatin receptor type 3 plasma membrane targeting sequence at the amino terminus and the HSV-glycoprotein D epitope for immunodetection at the carboxy terminus of the recombinant TAS2Rs [20]. Receptor expression and cell-surface-localisation in HEK 293T cells were confirmed by immunocytochemistry for all constructs. Fig. 1 demonstrates that all TAS2R variants show comparable expression patterns. To identify cognate bitter compounds for the orphan TAS2Rs, all constructs for the same TAS2Rs were pooled and transiently transfected into HEK $293T_{G\alpha 16gust44}$-cells. Compared with our standard assays containing 150 ng DNA [8], the amount of DNA for an individual construct in the present study was only 75, 50 or 37.5 ng in mixtures of 2, 3, or 4 plasmids, respectively. Therefore, we tested the influence of the DNA amount on signal intensity before examining the construct mixtures. To this end, cells were transfected with varying amounts of DNA for the fully functional variant of TAS2R38 [21] ranging from 15 to 150 ng. Calcium imaging experiments with 100 μM phenylthiocarbamide showed no differences in signal intensity (Fig. 2A) proposing that the concentration range of 37.5 to 150 ng DNA is appropriate for testing.

Functional experiments were performed with a set of 106 natural and synthetic bitter compounds, representing numerous chemical classes (Supplementary Table 1). The collection contained

Table 1
Haplotypes of orphan TAS2Rs with nsSNPs (nt), resulting amino acid (aa) exchanges and allele-frequencies are shown. Only major haplotypes (bold) were functionally analyzed. TAS2R variants used in a previous study are indicated ([§])[8].

TAS2R41	nt	C380T	C704A							
	aa	P127L	S235Y	%						
	H1	**P**	**S**	**68.4**						
	H2[§]	**L**	**S**	**29**						
	H3	P	Y	2.6						
TAS2R42 (TAS2R55)	nt	T524A	C587T	T763G	G782A	A794G	G875A	G931C		
	aa	F175Y	S196F	W255G	W261Stop	Y265C	R292Q	A311P	%	
	H1	**F**	**S**	**W**	**W**	**Y**	**R**	**P**	**68**	
	H2	**Y**	**F**	**G**	**W**	**Y**	**R**	**A**	**21**	
	H3[§]	**F**	**S**	**W**	**W**	**Y**	**R**	**A**	**7**	
	H4	Y	F	W	W	C	Q	P	2.6	
	H5	Y	F	G	Stop	Y	R	A	1.4	
TAS2R45	nt	A227G	G394A	G509A	G630C	T703C	T712C	G893C	A900G	
	aa	Y76C	V132M	R170H	Q210H	F235L	C238R	R298T	Stop300 W	%
	H0	**–**	**–**	**–**	**–**	**–**	**–**	**–**	**–**	**~15**
	H1	**C**	**M**	**R**	**H**	**L**	**C**	**T**	**W**	**~42**
	H2	**Y**	**V**	**R**	**Q**	**F**	**R**	**R**	**Stop**	**~25**
	H3	**Y**	**V**	**R**	**Q**	**F**	**C**	**R**	**Stop**	**~11**
	H4	**C**	**M**	**H**	**H**	**L**	**C**	**T**	**W**	**~2.5**
	H5	C	M	R	H	L	C	R	W	~2.5
TAS2R48 (TAS2R19)	nt	G94A	A376C	C895T						
	aa	V32I	K126Q	R299C	%					
	H1[§]	**V**	**K**	**R**	**49**					
	H2	**V**	**K**	**C**	**45**					
	H3	**I**	**K**	**R**	**4**					
	H4	V	Q	R	2					
TAS2R60	nt	C251T	A595T							
	aa	P84L	M199L	%						
	H1[§]	**P**	**M**	**95**						
	H2	L	L	2.5						
	H3	P	L	2.5						

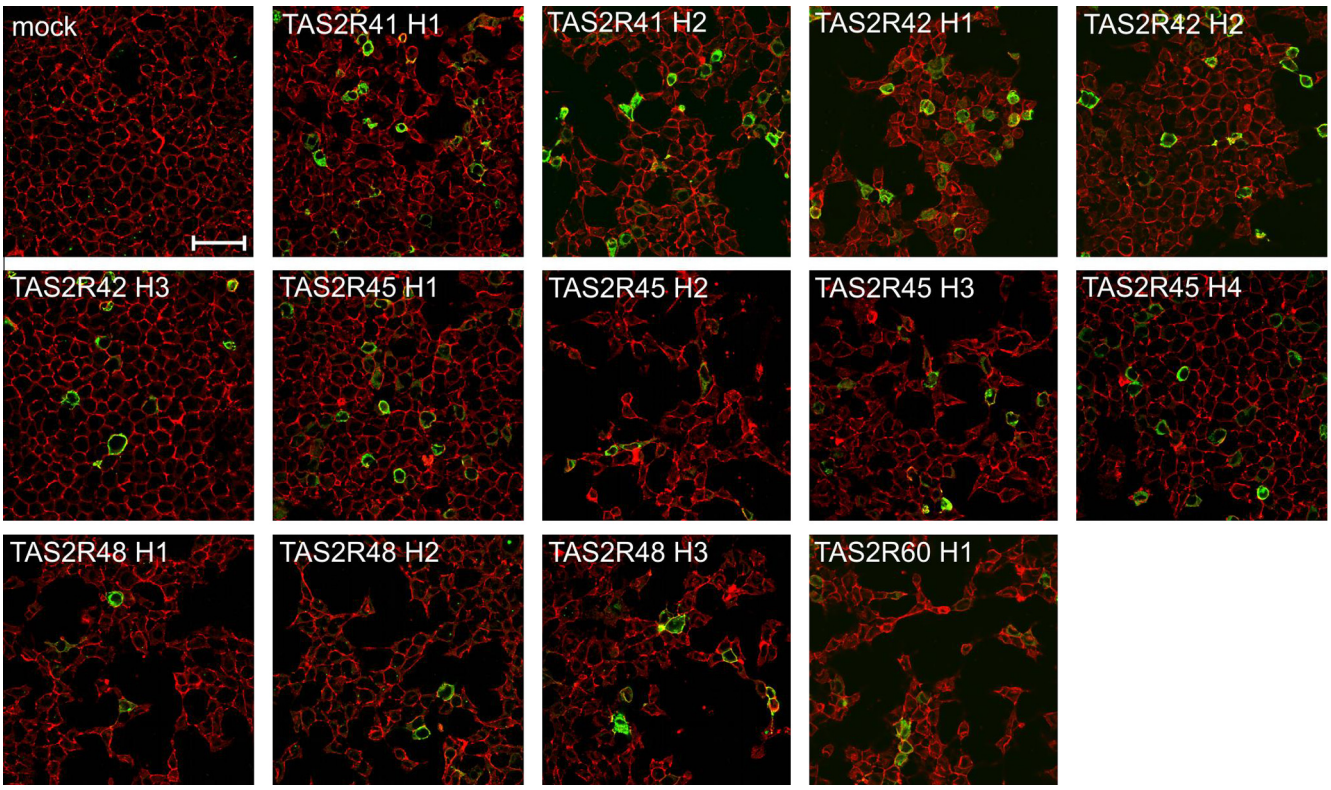

Fig. 1. Confocal images of HEK 293T cells expressing haplotypes (H) coding for the orphan receptors TAS2R41, TAS2R42, TAS2R45, TAS2R48, and TAS2R60. As negative control, cells transfected with empty vector (mock) are shown. Receptor expression is visualized by a mouse anti-HSV glycoprotein D antibody combined with an Alexa488-labeled anti-mouse antibody (green). Biotinylated concanavalin A, binding to plasma membrane carbohydrate moieties, in combination with streptavidin-Alexa633 (red) enabled cell surface staining. Scale bar = 50 μm.

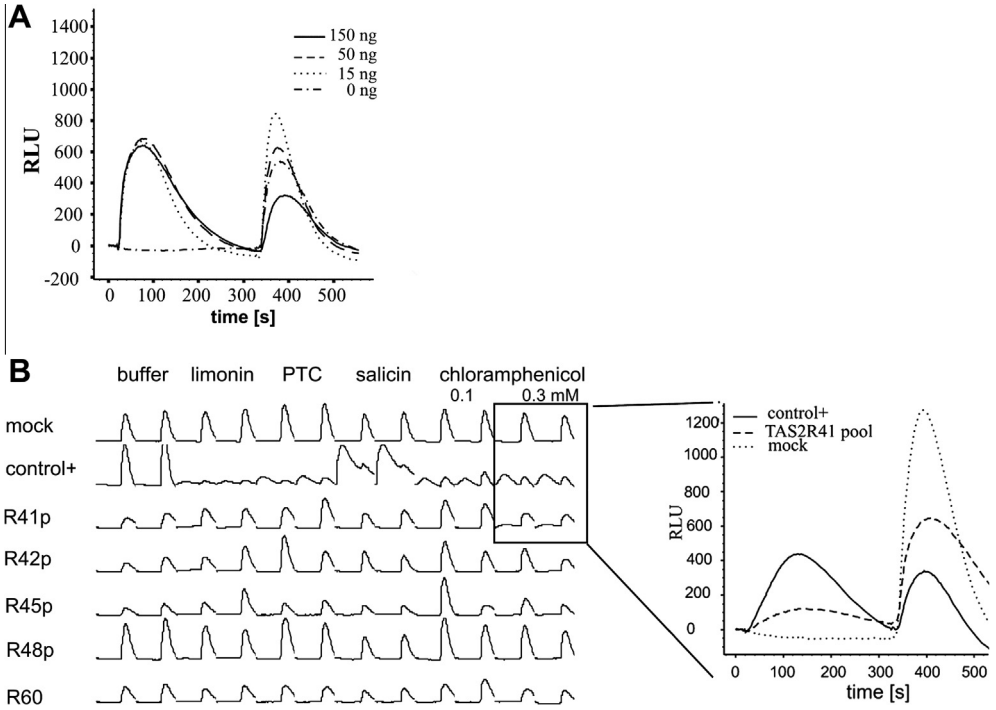

Fig. 2. Functional screening of mixtures of constructs encoding TAS2R variants. (**A**) Calcium traces of HEK 293T-G16gust44 cells transfected with increasing amounts of TAS2R38 upon application of 100 μM phenylthiocarbamide (PTC) followed by 100 nM somatostatin-14 as control for cell vitality are shown. As negative control cells transfected with empty vector (=0 ng receptor construct) were used; (**B**) Calcium imaging traces of HEK 293T cells transfected with the different TAS2R-pools upon application of buffer, 0.3 mM limonin, 0.1 mM PTC, 10 mM salicin, 0.1 and 0.3 mM chloramphenicol followed by 100 nM somatostatin-14 are shown. As positive control cells expressing responding TAS2Rs and as negative control cells transfected with empty vector (mock) were used.

30 bitter compounds for which no responding TAS2R has been identified. Cells transfected with empty vector (mock) were used to control for TAS2R-independent non-specific signals. For bitter compounds known to activate deorphaned TAS2Rs [8], responsive receptors were used as positive control.

Of the 106 bitter compounds only the antibiotic chloramphenicol evoked calcium responses specifically in cells transfected with the mixture of TAS2R41 constructs (Fig. 2B). This pool consisted of DNA for two variants, TAS2R41-P127 and TAS2R41-L127. For further analysis constructs for the two TAS2R41 variants were transfected individually and dose–response relations monitored. Both TAS2R41 variants responded to chloramphenicol in a concentration-dependent manner. At concentrations higher than 1 mM chloramphenicol induced signals in the absence of receptor constructs, precluding establishment of complete dose–response function and calculation of EC_{50} values. However, inspection of the data clearly revealed that the calcium traces of TAS2R41-P127 showed higher signal amplitudes and a lower threshold concentration than those of TAS2R41-L127 (Fig. 3). Thus, the two major TAS2R41-haplotypes encode receptor variants with different sensitivities.

As we did not observe activation of constructs of the other eleven haplotypes (not shown), receptors TAS2R42, TAS2R45, TAS2R48 and TAS2R60 still remain orphan. Furthermore, for the 30 tested bitter compounds with unknown bitter receptor, no responding TAS2R variant was identified.

3.3. Application of structural analogs of chloramphenicol

Of the structurally diverse bitter compounds used for our screening only chloramphenicol activated TAS2R41 suggesting that TAS2R41 belongs to the group of narrowly tuned receptors. In order to test the putatively high selectivity for the chemical structure of its agonist, we tested structural analogs of chloramphenicol for their ability to activate the two TAS2R41 variants. For this purpose we selected the closely related antibiotics thiamphenicol and florfenicol as well as the structurally less complex compounds p-nitro cinnamic acid and caffeic acid (Fig. 4).

Functional experiments were performed on cells expressing either TAS2R41-P127 or TAS2R41-L127 (Fig. 4). Each panel shows calcium traces of cells stimulated with one substance. As negative control cells transfected with empty vector were used. For florfenicol and thiamphenicol (Fig. 4C and D) cells expressing TAS2R46, a broadly tuned chloramphenicol-responsive receptor, served as control. As no TAS2R has been identified for p-nitro cinnamic and caffeic acid, we could not set up a positive control assay for these compounds (Fig. 4A and B).

Application of caffeic acid, florfenicol or thiamphenicol did not activate any of the TAS2R41 variants (Fig. 4B–D). Only TAS2R46 used as control responded to florfenicol and thiamphenicol. However, application of 2 mM p-nitro cinnamic acid elicited weak calcium responses in cells expressing the sensitive variant, TAS2R41-P127 (Fig. 4A).

Somewhat surprisingly, thiamphenicol did not activate TAS2R41, although the only difference to chloramphenicol is the presence of a methylsulfonyl-group instead of a nitro group. The same was observed for the fluorinated form, florfenicol, whereas both compounds, like chloramphenicol itself, activated TAS2R46. The weak agonist p-nitro cinnamic acid was the only substance also possessing the nitro group at the phenyl ring, pointing to the importance of this group for TAS2R41 activation. These observations underscore the assumed specificity of TAS2R41 for a narrow agonist spectrum characterized by defined structural features.

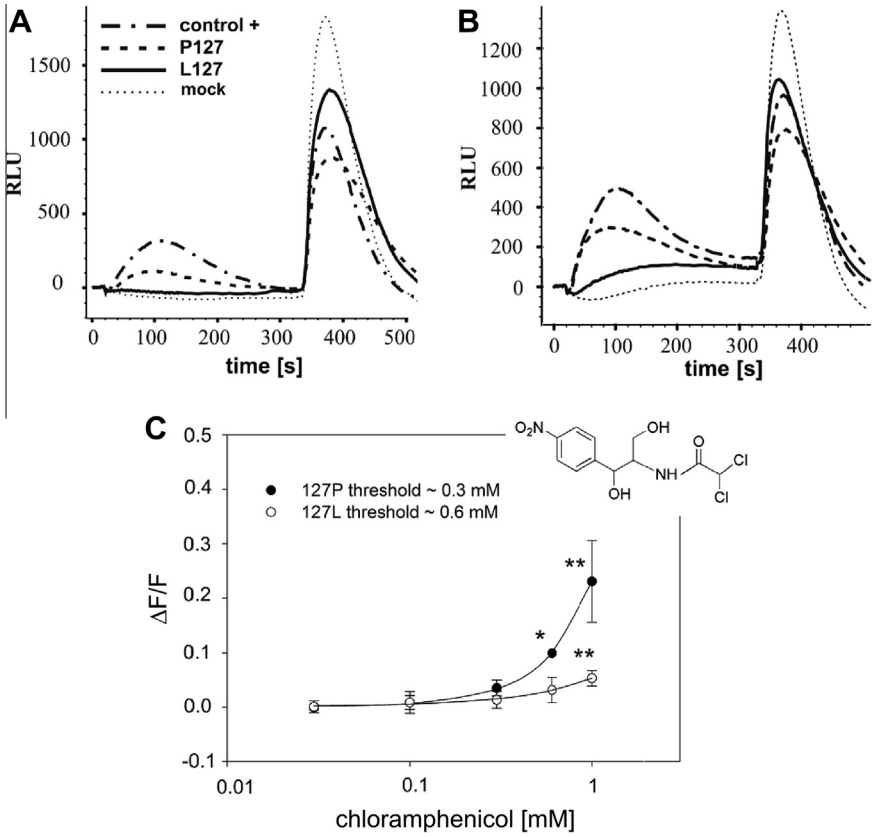

Fig. 3. HEK 293T cells expressing either TAS2R41-P127 or TAS2R41-L127 were stimulated with increasing concentrations of chloramphenicol. Calcium traces are shown for 0.3 mM (**A**) and 1 mM (**B**) chloramphenicol. The resulting dose–response curves and the corresponding standard deviations are shown (**C**). For statistical significance of receptor signals compared to cells transfected with empty vector (mock), *t*-test was performed.

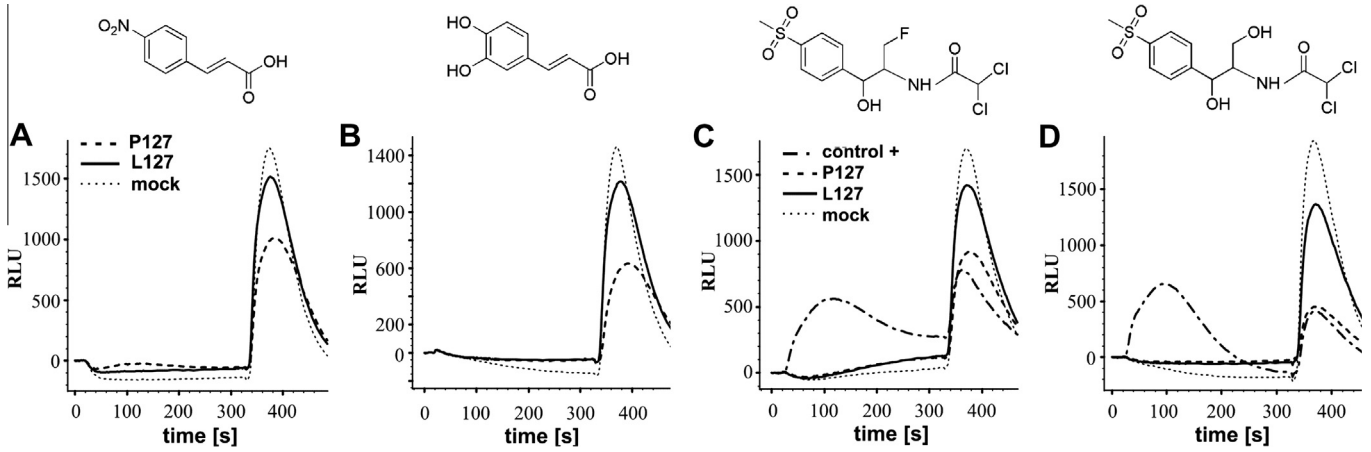

Fig. 4. Calcium traces of HEK 293T-G16gust44 cells transfected with TAS2R41-P127 or TAS2R41-L127 after application of 2 mM p-nitro cinnamic acid (**A**), 3 mM caffeic acid (**B**), 4 mM florfenicol (**C**) or 8 mM thiamphenicol (**D**) are shown. Chemical structures of compounds are shown above each panel. Cells transfected with empty expression vector (mock) were used as negative control. For florfenicol and thiamphenicol (**C and D**) cells expressing TAS2R46 were used as positive control. The highest possible concentrations were used for all compounds.

4. Discussion

In this study receptor variants encoded by the major haplotypes for the five orphan TAS2Rs were stimulated with 106 bitter compounds and functional experiments were performed. We identified a single compound, chloramphenicol, as TAS2R41 agonist indicating that this TAS2R is a narrowly tuned 'specialist' receptor. This was confirmed by testing substances structurally similar to chloramphenicol. These experiments also suggested that a $-NO_2$ group present as substituent at the phenylring is a critical component for TAS2R41 activation.

The two major variants TAS2R41-P127 and TAS2R41-L127 showed clear differences in threshold concentrations and signal amplitudes (Fig. 3). Particularly, the ~10fold difference in signal amplitude, which likely prevented the deorphanization of the TAS2R41-L127 variant used in our previous study [8], proposes that the functional variance could result in different taste phenotypes in subjects. Interestingly, all African subjects in our sample were homozygous for the sensitive variant TAS2R41-P127. This finding is in accordance with the results of Kim et al. [26] who found greatest TAS2R diversity in African populations and may reflect local adaptive processes as already shown for TAS2R16 [28].

The major haplotypes of TAS2R42, TAS2R45, TAS2R48 and TAS2R60 that we found in our sample have been observed before [25,26] with the exception of the TAS2R42 variants with nsSNP G931C. Interestingly, only five of 38 genotyped subjects in the present study were heterozygous for TAS2R42. As we identified five haplotypes for TAS2R42 this apparent homogeneity seems surprising and needs further analyses.

As no responsive receptor variant was identified for TAS2R42, TAS2R45, TAS2R48 and TAS2R60, these receptors remain orphan. It appears unlikely that only the rare variants of these TAS2Rs with frequencies below 5% are functional. Most likely, other reasons account for the unresponsiveness of these four TAS2Rs. Since we were able to demonstrate expression of all TAS2Rs variants by immunocytochemistry, insufficient receptor expression in the heterologous system is also unlikely to be responsible for the lack of responsiveness. It might, however, be possible that cofactors [16], important receptor modifications [17] or essential components of the native signalling pathway are missing in the heterologous expression system for these TAS2Rs.

Psychophysical studies found an association between variants of TAS2R48 (TAS2R19) and bitterness of grapefruit juice [32] or high concentrations (180 μM) of the bitter substance quinine [24] whereas our assays were limited to using 30 μM quinine. No activation of the TAS2R48-variants was observed for quinine at this concentrations or for bitter components of grapefruits such as limonin and naringin in this and a previous study [8]. In the previous study, seven TAS2Rs sensitive to quinine have been identified with threshold concentrations of 10 μM, which is around the human threshold concentration suggesting that TAS2R48 is likely not a sensitive quinine receptor.

Whereas many TAS2Rs respond to several or even numerous agonists, some receptors are very specialized to detect few agonists [19]. The recently deorphaned receptors TAS2R9 [11], TAS2R50 [20] and TAS2R41 in this study, seem to be specific for single or very few compounds. This may also hold true for TAS2R42, TAS2R45, TAS2R48 and TAS2R60. Most likely, agonists for these receptors were simply not present in our collection of compounds leaving these receptors orphan.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.04.066.

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