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The activation mechanisms of G protein-coupled receptors : the case of the adenosine A2B and HCA2/3 receptors

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Chapter 5

The role of the C-terminus of the human hydroxycarboxylic acid receptors 2 and 3 in G protein activation using G_{α} -engineered yeast cells

This chapter is based upon:

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Abstract

In the present study we focused our attention on the family of hydroxycarboxylic acid (HCA) receptors, a GPCR family of three members, of which the HCA₂ and HCA₃ receptors share 95% high sequence identity but differ considerably in C-terminus length with HCA₃ having the longest tail. The two receptors were expressed and analysed for their activation profile in *Saccharomyces cerevisiae* MMY yeast strains that have different G protein G_α subunits. The hHCA₂ receptor was promiscuous in its G protein coupling preference. In the presence of nicotinic acid the hHCA₂ receptor activated almost all G protein pathways except G_{αq} (MMY14). However, the G_α protein coupling profile of the hHCA₃ receptor was less promiscuous, as the receptor only activated G_{αi1} (MMY23) and G_{αi3} (MMY24) pathways.

We then constructed two mutant receptors by 'swapping' the short (HCA₂) and long (HCA₃) C-terminus. The differences in HCA₂ and HCA₃ receptor activation and G protein selectivity were not controlled, however, by their C-terminal tails, as we observed only minor differences between mutant and corresponding wild-type receptor. This study provides new insights into the G protein coupling profiles of the HCA receptors and the function of the receptor's C terminus, which may be extended to other GPCRs.

Introduction

G protein-coupled receptors are important drug targets. The secondary structure of these cell membrane-bound proteins is composed of seven transmembrane domains, connected via three extra- and three intracellular loops, preceded by an extracellular N-terminus and followed by an intracellular C-terminus^[1]. We have studied the role of various of these motifs in receptor and G protein activation. As an example, we learned and reviewed that the extracellular domains, while being quite distant from the G protein binding site at the receptor, contribute significantly to receptor activation^[2]. In the current study we focused our attention to the C-terminus of the receptor, which considerably differs in length between receptors, suggesting its role can be very different from one receptor to the other^[3].

Previous research has shown that the C-terminal domain is important in the regulation of intracellular trafficking^[4], internalization^[5, 6], transport^[7], photoreceptor morphogenesis^[8], blebbing phenotype^[9], desensitization and sequestration^[10], G protein coupling^[11-14] and β -arrestin interaction^[15]. However, C-terminus deletion does not affect the ability of rhodopsin to activate G proteins^[16].

In the present study we focused our attention to the family of hydroxycarboxylic acid (HCA) receptors, a GPCR family of three members, of which the HCA₂ and HCA₃ receptors share high sequence identity but differ considerably in C-terminus length with HCA₃ having the longest tail. Both HCA₂ and HCA₃ receptors have been implicated in e.g., obesity and other dyslipidemic conditions^[17].

We studied whether the C-terminus has an impact on receptor activation and G protein selectivity. We examined this research question in an engineered yeast strain^[18, 19] that we have previously studied on a number of occasions, particular with respect to adenosine receptors^[20-22]. This assay system has no endogenous GPCRs and expresses a panel of humanized G proteins, which makes it ideal for the study of receptor activation and to determine a receptor's G protein preferences.

Materials and methods

Materials

The *Saccharomyces cerevisiae* (*S. cerevisiae*) MMY strains and expression vectors, p426GPD, p426GPD_hHCA₂ and p426GPD_hHCA₃ were kindly provided by Dr. Simon Dowell (GSK, Stevenage, UK). The polymerase chain reaction (PCR) and construction of mutants were carried out with the *PfuUltra* HF DNA polymerase (Stratagene, Amsterdam, the Netherlands), dNTP mix/25mM (Bio-Connect, Huissen, the Netherlands), the restriction enzymes BstAPI (Bioke, Leiden, the Netherlands) and BlnI (Fisher Scientific, Amsterdam, the Netherlands), and T4 DNA ligase (Fisher Scientific, Amsterdam, the Netherlands). Ligands used in the liquid growth assay were nicotinic acid (Sigma-Aldrich, Zwijndrecht, the Netherlands), acifran (Tocris, Bristol, UK) and LUF7159 known as compound 6o^[23] (synthesized in house), and 3-amino-[1,2,4]-triazole (Sigma-Aldrich, Zwijndrecht, the Netherlands). The Hybond-ECL membranes and the ECL Western blotting analysis system were purchased from GE Healthcare (Eindhoven, the Netherlands). Rabbit anti-human HCA₂/HCA₃ polyclonal antibody was purchased from Sigma-Aldrich (HPA028660, Zwijndrecht, the Netherlands) and goat anti-rabbit IgG as second antibody was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Rat anti-yeast α -tubulin monoclonal antibody was used as a reference protein (GTX76511, GeneTex, Irvine, CA, USA) with goat anti-rat IgG-HRP antibody (sc-2032, Santa Cruz Biotechnology, Heidelberg, Germany) as the second antibody. The medium YNB + Nitrogen - Nicotinic acid was purchased from Sunrise Science products (San Diego, CA, USA).

C-terminus modified hHCA₂ receptor and hHCA₃ receptor constructs

The following primers were synthesized (Eurogentec, the Netherlands) and used for cloning two receptor constructs in which we swapped the C-terminus of both receptors, which were named hHCA₂+C and hHCA₃-C.

5'-ATGAATCGGCACCATCTGCAGGATCACTTTCTG-3'

5'-TCCAGATAATTCTGGCTGAGCAGAACAGGATGATG-3'

Restriction sites in the primers, *Bst*API and *B*lpI, are in italics and bold. The hHCA₂+C mutant was generated by switching part of the hHCA₂ receptor, which was cloned by PCR from p426GPD_hHCA₂, to the ligation vector p426GPD_hHCA₃ (for details see Fig. S1A). The hHCA₃-C mutant was generated by switching part of the hHCA₃ receptor, which was cloned by PCR from p426GPD_hHCA₃, to the ligation vector p426GPD_hHCA₂ (for details see Fig. S1B). Plasmids were amplified using DH5 α *E. Coli* competent cells (Invitrogen, San Diego, CA, USA). This procedure yielded a total of four plasmids, which were all confirmed by DNA sequencing (LGTC, the Netherlands). Sequence alignment was performed using CLUSTALW^[24].

Transformation in *S. cerevisiae* strains

The p426GPD, p426GPD_hHCA₂, p426GPD_hHCA₃, p426GPD_hHCA₂+C and p426GPD_hHCA₃-C plasmids were transformed according to the Lithium-Acetate procedure^[25] into a panel of engineered *S. cerevisiae* yeast strains expressing different Gpa1p/G α chimeras. The series of yeast strains are derived from the MMY11 strain and further adapted to communicate with mammalian GPCRs. The difference between these integrated Gpa1p/G α chimeras is that the last five amino acids of the endogenous Gpa1p C-terminus have been replaced with the same five amino acids sequence motif of mammalian G α proteins^[18, 19, 26] (Table 1). The positive clones with an expression plasmid were selected in YNB-UL-NA medium (YNB + adenine + tryptophan + histidine, lacking uracil, leucine and also nicotinic acid).

Liquid growth assay

The degree of receptor activation was measured by the growth rate of the yeast on histidine-deficient medium via the *FUS1-HIS3* reporter gene induction, which has been described in our previous research except that nicotinic acid was omitted from the medium mix. The assays were performed in the absence or presence of the indicated concentrations of nicotinic acid, acifran or LUF7159, the structures of which are shown in Fig. 1A. Stock solutions of all compounds

Table 1. The genotypes of the humanized yeast strains used for transformations^[18, 19, 26]. All these strains grow in YNB-L medium (YNB without leucine), while they do not grow in YNB-UL-NA medium (without uracil, leucine and nicotinic acid) unless the expressed receptors are present or activated.

Strain	Genotype	The five C-terminal residues of G _α
MMY11	MATa <i>his3 ade2 leu2 trp1 ura3 can1 fus1:FUS1-HIS3 FUS1-lacZ:LEU2 far1Δ:ura3Δgpa1Δ:ADE2Δsst2Δ:ura3 Δste2Δ:G418^R</i>	
MMY12 (G _{αWT})	MMY11TRP1: <i>GPA1</i>	KIGII ^{COOH}
MMY14 (G _{αq})	MMY11TRP1: <i>Gpa1/G_{αq}</i> (5)	EYNLV ^{COOH}
MMY16 (G _{α16})	MMY11TRP1: <i>Gpa1/G_{α16}</i> (5)	EINLL ^{COOH}
MMY19 (G _{α12})	MMY11TRP1: <i>Gpa1/G_{α12}</i> (5)	DIMLQ ^{COOH}
MMY20 (G _{α13})	MMY11TRP1: <i>Gpa1/G_{α13}</i> (5)	QLMLQ ^{COOH}
MMY21 (G _{α14})	MMY11TRP1: <i>Gpa1/G_{α14}</i> (5)	EFNLV ^{COOH}
MMY23 (G _{αi1})	MMY11TRP1: <i>Gpa1/G_{αi1}</i> (5)	DCGLF ^{COOH}
MMY24 (G _{αi3})	MMY11TRP1: <i>Gpa1/G_{αi3}</i> (5)	ECGLY ^{COOH}
MMY25 (G _{αz})	MMY11TRP1: <i>Gpa1/G_{αz}</i> (5)	YIGLC ^{COOH}
MMY28 (G _{αs})	MMY11TRP1: <i>Gpa1/G_{αs}</i> (5)	QYELL ^{COOH}

were made in DMSO. Yeast cells with the hHCA₂ receptor or the hHCA₂+C receptor obtained from an overnight culture were diluted to around 2×10⁴ cells/ml (OD₆₀₀ ≈ 0.001) and 50 μl was added into each well (approx. 1,000 cells/well). The yeast cells with the hHCA₃ receptor or the hHCA₃-C receptor were diluted to approx. 2×10⁵ cells/ml (OD₆₀₀ ≈ 0.01) and 50 μl was added into each well (approx. 1×10⁴ cells/well). Cell suspensions were aliquotted over a 96-well plate that was then incubated for 35 h at 30 °C in a Genios plate reader (Tecan, Durham, NC) measuring the absorption at a wavelength of 595 nm. The experiments presented in Table 2 (cells in suspension by shaking every 10 min at 300 rpm for 1 min) and Table 3 (without shaking), respectively, were done slightly different, such that EC₅₀ values for both nicotinic acid and acifran are not fully comparable for

the MMY24 strain. Results were obtained from three independent experiments, performed in duplicate.

Yeast protein extraction and immunoblotting

Protein extractions were done according to the trichloroacetic acid (TCA) method from the Clontech Yeast Protocols Handbook 2001. More details were described in our previous research^[21]. Target proteins were analyzed by immunoblotting using a rabbit anti-human HCA₂/HCA₃ polyclonal antibody at 0.3 µg/ml with Tris-buffered saline containing 0.05% tween 20 (TBST) (pH 7.6) containing 5% milk powder for all these four receptors. The immunogenic amino acid sequence of the receptors used was NRCLQRKMTGEPDNNRSTSVELTGDPNKTRGAPEALMANSGEPSYLG. After thorough removal of unbound antibody from the membranes by washing them three times with TBST, the membranes were incubated with 1:2,500 diluted HRP-conjugated goat anti-rabbit IgG for 1 h. The membranes were washed twice with TBST and once with TBS. The specific signal of the hHCA₂/hHCA₃ receptor was probed with the ECL Western blotting analysis system (GE Healthcare, Eindhoven, the Netherlands) using enhanced chemiluminescence (ChemiDox XRS, Bio-Rad, Hercules, CA, USA). A reference protein (α -tubulin) was analyzed simultaneously using rat anti-yeast α tubulin monoclonal antibody at 1 µg/ml and then using goat anti-rat IgG-HRP antibody at 0.16 µg/ml as the second antibody.

Statistical analysis

EC₅₀ values and E_{max} values of the liquid assay were analyzed using the nonlinear regression package available in Prism 5.0 software (GraphPad Software Inc., San Diego, CA). Differences were examined for significance by a two-tailed homoscedastic Student's *t*-test, yielding *p*-values.

Expression of the receptors was quantified using Quantity One imaging software from Bio-Rad after correction for the background; it was calculated as density (OD/mm²). The α -tubulin, at approx. 130 kDa, was used as loading control and the specific hHCA₂ receptor protein bands were approx. at 29 kDa and 52 kDa. The ratio was determined between the density of the specific hHCA₂

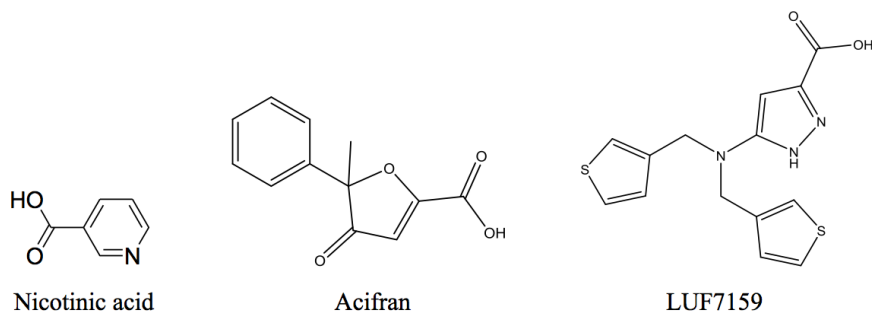
bands and the density of loading control band. MMY12 carrying the wild-type hHCA₂ receptor, was set at 100%. Results were obtained from two independent experiments and all expression levels of the hHCA₂ receptor in different MMY strains were normalized to the hHCA₂ receptor in the MMY12 strain on the same blot.

Results

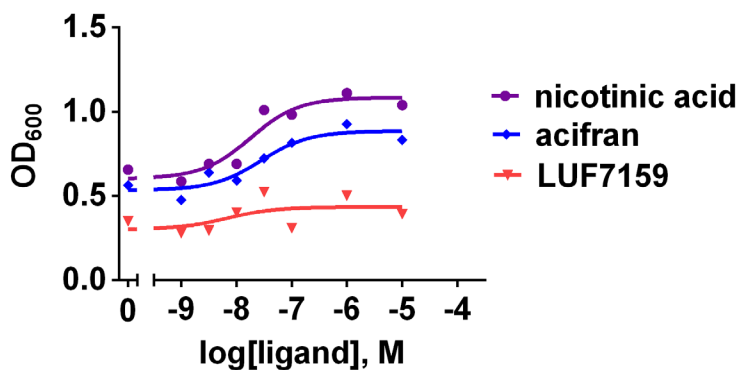
Characterization of the wild-type hHCA₂ and hHCA₃ receptors expressed in the MMY24 yeast strain with three agonist ligands

To characterize the activation of the hHCA₂ and hHCA₃ receptor in the yeast system, we first expressed the yeast expression plasmids: p426GPD, P426GPD_hHCA₂, p426GPD_hHCA₃ in a yeast *S. cerevisiae* strain, MMY24 (with G_{αi}). The hHCA₂ receptor was activated by both nicotinic acid (EC₅₀ = 35 nM) and acifran (EC₅₀ = 56 nM), but not by LUF 7159, an agonist selective for the HCA₃ receptor^[23]. Nicotinic acid's E_{max} value was slightly higher than for acifran, while the receptor itself displayed significant constitutive activity (CA) under these conditions (Fig. 1B and Table 2). The hHCA₃ receptor was activated by both LUF7159 (EC₅₀ = 671 nM) and acifran (EC₅₀ = 13,600 nM), but not by nicotinic acid. LUF7159 showed a 20-fold higher potency than acifran, while the E_{max} values for both ligands were the same. The HCA₃ receptor displayed lower constitutive activity than the HCA₂ receptor (Fig. 1C and Table 2).

A



B



C

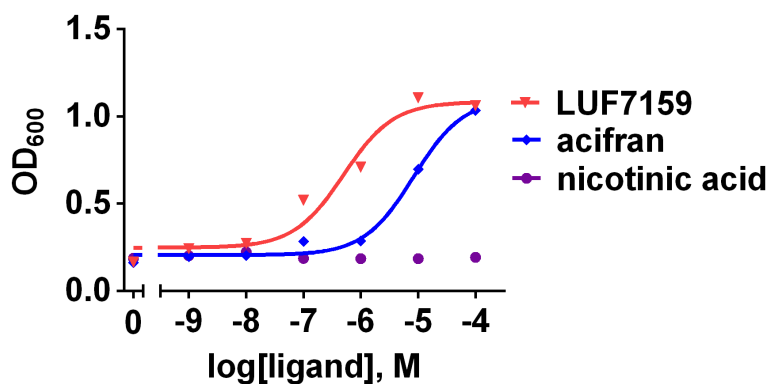


Fig. 1. (A) Chemical structures of ligands from left to right: nicotinic acid, acifran, and LUF7159. Concentration-effect curves of the wild-type receptor hHCA₂ (B) and hHCA₃ (C) in the MMY24(G_{α13}) strain responding to nicotinic acid, acifran and LUF7159. The assay was performed in YNB-ULH-NA medium. A representative experiment is shown (of a total of n = 3).

Table 2. EC₅₀, constitutive activity (CA) and E_{max} values of the wild-type hHCA₂ receptor, the hHCA₂+C mutant receptor, the wild-type hHCA₃ receptor and the hHCA₃-C mutant receptor in MMY 24 (G_{αi3}) strain in response to nicotinic acid, acifran and LUF7159. Results were obtained from 3 independent experiments, performed in duplicate.

hHCA ₂			
	EC ₅₀ (nM)	CA	E _{max}
nicotinic acid	35 ± 11	0.6 ± 0.03	1.0 ± 0.07
acifran	56 ± 37	0.4 ± 0.06	0.8 ± 0.07
LUF7159	-	-	-
hHCA ₂ +C			
	EC ₅₀ (nM)	CA	E _{max}
nicotinic acid	46 ± 27	0.5 ± 0.07	1.3 ± 0.13
acifran	71 ± 37	0.5 ± 0.08	1.1 ± 0.16
LUF7159	-	-	-
hHCA ₃			
	EC ₅₀ (nM)	CA	E _{max}
nicotinic acid	-	-	-
acifran	13600 ± 5700	0.2 ± 0.04	1.2 ± 0.02
LUF7159	671 ± 91	0.2 ± 0.05	1.1 ± 0.04
hHCA ₃ -C			
	EC ₅₀ (nM)	CA	E _{max}
nicotinic acid	-	-	-
acifran	4900 ± 1800	0.3 ± 0.07	1.2 ± 0.05
LUF7159	285 ± 136	0.3 ± 0.06	1.1 ± 0.05

Characterization of the different G protein coupling profiles of the hHCA₂ and hHCA₃ receptors

To investigate the activation mechanism of the hHCA₂ receptor and hHCA₃ receptor at the interface with the C terminus of the G protein G_α subunit, we expressed the yeast plasmid p426GPD, p426GPD_hHCA₂ and p426GPD_hHCA₃ in a panel of yeast *S. cerevisiae* strains with humanized G proteins. The difference between these ten integrated Gpa1p/G_α chimeras is that the last five amino acids of the endogenous Gpa1p C-terminus have been replaced with the same five amino acids sequence motif as that from mammalian G_α proteins^[18, 19] (Table 1). Corresponding to the replaced last five C-terminal residues of the mammalian G_α subunit, they were classified into five families:

$G_{\alpha_{WT}}$ (MMY12), G_{α_s} (MMY28), G_{α_i} (MMY23, MMY24 and MMY25), $G_{\alpha_{i2}}$ (MMY19 and MMY20), and G_{α_q} (MMY14, MMY16 and MMY21) (Table 1).

Concentration-effect curves of nicotinic acid ($0, 10^{-9} - 10^{-4}$ M) on the wild-type $hHCA_2$ receptor in all strains except MMY20 and MMY28 are shown in Fig. 2A. The two latter strains, when only carrying the 'empty' plasmid p426GPD (without any receptor), already showed substantial growth of the yeast cells, and were excluded from further examination although they expressed the receptors well (see also Fig. 5). Please, note that the experiments in Table 2 were performed slightly differently (see M&M) from the ones in Table 3, yielding somewhat different EC_{50} values for nicotinic acid and acifran in case of the MMY24 strain.

We found the three different G_{α_i} protein pathways MMY23 ($G_{\alpha_{i1}}$), MMY24 ($G_{\alpha_{i3}}$) and MMY25 (G_{α_z}) to show varying degrees of enhancement of both the efficacy and potency of nicotinic acid compared to the wild-type $hHCA_2$ in the wild-type yeast G_{α} strain MMY12 ($G_{\alpha_{WT}}$). The most efficient yeast strains, MMY23 ($G_{\alpha_{i1}}$) and MMY24 ($G_{\alpha_{i3}}$) showed a significant increase in potency of the agonist nicotinic acid (71-fold or 42-fold, respectively) and a 2-fold increase in intrinsic activity, while the other G_{α_i} strain, MMY25 (G_{α_z}), showed a significant 5-fold enhancement in potency, concurrent with the highest E_{max} value. The other strains showed less efficient G protein coupling. MMY14 (G_{α_q}) showed no activation at all; MMY16 ($G_{\alpha_{i6}}$) showed a 2-fold decrease in potency of nicotinic acid. MMY21 ($G_{\alpha_{i4}}$) showed a 3-fold increase in potency and a similar efficacy of nicotinic acid. The $G_{\alpha_{i2}}$ protein pathway was less responsive, as MMY19 ($G_{\alpha_{i2}}$) showed a 3-fold decrease of potency and similar efficacy of the agonist nicotinic acid (Table 3).

Concentration-effect curves of acifran ($0, 10^{-7} - 10^{-3}$ M) on the wild-type $hHCA_3$ receptor in MMY23 ($G_{\alpha_{i1}}$) and MMY24 ($G_{\alpha_{i3}}$) are shown in Fig. 2B, and the resulting parameters in Table 3. The EC_{50} values for acifran were in the 10-100 micromolar range. However, no activation was observed in any of the other MMY strains.

Table 3 EC₅₀, constitutive activity (CA) and E_{max} values of wild-type hHCA₂ receptor in response to the ligand nicotinic acid (0, 10⁻⁹ M to 10⁻⁴ M) and hHCA₃ receptor in response to acifran (0, 10⁻⁷ M to 10⁻³ M) in all examined MMY strains. Results were obtained from three independent experiments, performed in duplicate. “-”: no activation

	hHCA ₂ receptor			hHCA ₃ receptor		
	EC ₅₀ (μM)	CA	E _{max}	EC ₅₀ (μM)	CA	E _{max}
MMY12 (G _{αWT})	10 ± 4.7	0.07 ± 0.002	0.6 ± 0.03	-		
MMY14 (G _{αq})	-	0.07 ± 0.01	0.1 ± 0.03	-		
MMY16 (G _{α16})	21.5 ± 6.9	0.09 ± 0.015	1.5 ± 0.03	-		
MMY19 (G _{α12})	30 ± 6	0.1 ± 0.03	0.5 ± 0.18	-		
MMY21 (G _{α14})	3 ± 0.58	0.08 ± 0.008	0.6 ± 0.06	-		
MMY23 (G _{α11})	0.14 ± 0.04	0.5 ± 0.04	1.3 ± 0.02	68.7 ± 21.4	0.2 ± 0.01	1.2 ± 0.13
MMY24 (G _{α13})	0.24 ± 0.15	0.3 ± 0.03	1.1 ± 0.08	88.2 ± 17.4	0.1 ± 0.03	1.2 ± 0.05
MMY25 (G _{αz})	2.25 ± 0.48	0.14 ± 0.014	1.8 ± 0.02	-		

Construction of the hHCA₂+C and hHCA₃-C mutant receptors

The main difference between the hHCA₂ and hHCA₃ receptors is that the hHCA₂ has a shorter C-terminus with the hHCA₃ carrying 24 more amino acid residues at its C-terminus. Although the two receptors have high overall sequence homology, there are also 17 different amino acid residues in other parts of the two receptors that mainly cluster around ECL1 and ECL2 (Fig. 3). To investigate the importance of the C-terminus of the hHCA₂ and hHCA₃ receptor in G protein coupling, we constructed two mutants by ‘swapping’ the C-terminus, which were named the hHCA₂+C receptor (the wild-type hHCA₂ receptor with the extra C terminus of the wild-type hHCA₃ receptor) and the hHCA₃-C receptor (the wild-type hHCA₃ receptor with the shorter C terminus of the wild-type hHCA₂ receptor) (Fig. 3 and S1).

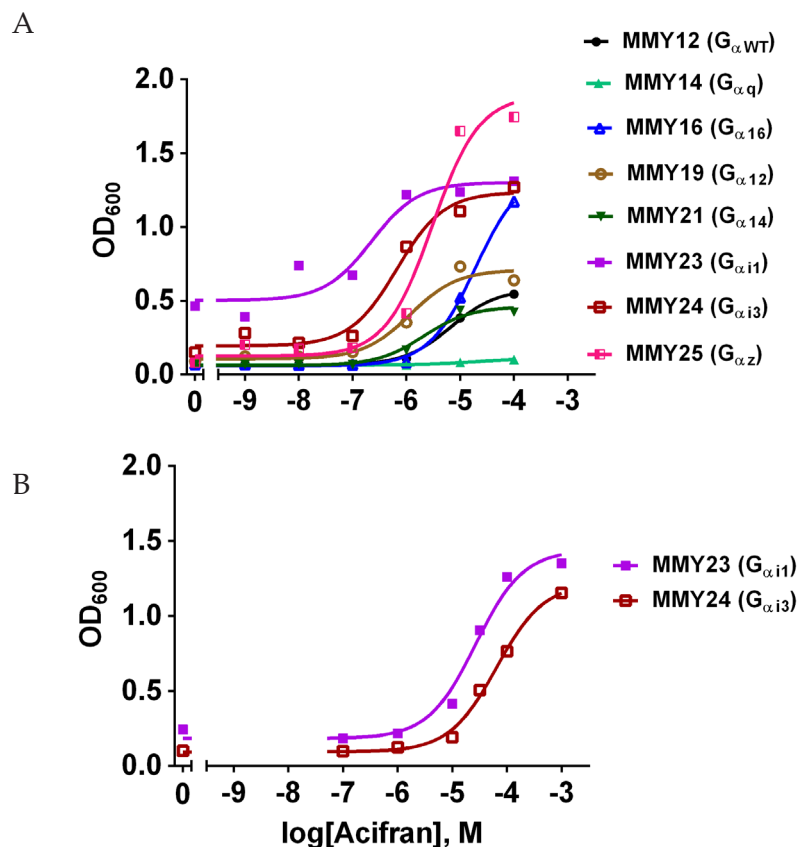


Fig. 2. Concentration-effect curves from liquid assay experiments. (A) Curves are shown of the wild-type receptor hHCA₂ in the different yeast strains: MMY25 (G_{α_z}), MMY23 (G_{α_{i1}}), MMY24 (G_{α_{i3}}), MMY16 (G_{α₁₆}), MMY19 (G_{α₁₂}), MMY12 (G_{α_{WT}}), MMY21 (G_{α₁₄}), MMY14 (G_{α_q}) responding to the hHCA₂ agonist nicotinic acid. (B) The wild-type receptor hHCA₃ in the different strains: MMY23 (G_{α_{i1}}) and MMY24 (G_{α_{i3}}) responding to the hHCA₂/hHCA₃ agonist acifran. The hHCA₃ receptor was not activated in other strains, and, hence, no further curves are shown. The assays were performed in YNB-NA-ULH medium. A representative experiment is shown (of a total of n = 3).

Characterization of the hHCA₂+C and hHCA₃-C mutant receptors expressed in the MMY24 yeast strain with three agonist ligands

To characterize the activation of the hHCA₂+C and hHCA₃-C mutant receptors in the yeast MMY24 (G_{α_{i1}}) strain we followed the same protocol as for the wild-type HCA₂ and HCA₃ receptors. The hHCA₂+C receptor was activated by nicotinic acid and acifran with similar potency and efficacy as WT hHCA₂, and could not be activated by the HCA₃-selective agonist LUF7159. The hHCA₃-C receptor

was only activated by acifran and LUF7159 with 3-fold and 2-fold increases in potency, respectively, with no changes in efficacy. The hHCA₃-C receptor could not be activated by nicotinic acid.

The role of the C terminus in G protein activation and selectivity

To investigate the role of the C terminus of both the hHCA₂ receptor and the hHCA₃ receptor at the interface of the C terminus of the G protein G_α subunit, we expressed the yeast plasmid p426GPD_hHCA₂+C and p426GPD_hHCA₃-C in a panel of 6 yeast *S. cerevisiae* strains with humanized G proteins. We did not include the MMY16 and MMY25 strains, as these two strains displayed a very low expression level of the HCA₂ receptor. The efficacy of these two mutants, hHCA₂+C and hHCA₃-C, compared to the two wild-type receptors, hHCA₂ and hHCA₃, are shown in Fig. 4, in the absence or presence of a saturating concentration of nicotinic acid (Fig. 4A and B) or acifran (Fig. 4C and D). The wild-type hHCA₂ significantly activated four of the six pathways examined, MMY12 (G_{αWT}), MMY21 (G_{α14}), MMY23 (G_{α11}) and MMY24 (G_{α13}) (Fig. 4A). The hHCA₂+C receptor activated all six pathways (Fig. 4B), although the constitutive activity in MMY23 was so high that the response to nicotinic acid did not differ significantly from the basal value. The hHCA₂+C receptor tended to show higher activation levels, but otherwise behaved like the wild-type receptor in MMY12 (G_{αWT}), MMY21 (G_{α14}), and MMY24 (G_{α13}), while it was additionally active in MMY14 (G_{αq}). A comparison of the hHCA₃-C receptor (Fig. 4D) with the wild-type hHCA₃ receptor (Fig. 4C) revealed that the hHCA₃-C activates three rather than two pathways: MMY19 (G_{α12}) was activated with half the efficacy compared to activation in MMY23 (G_{α11}) or MMY24 (G_{α13}), while the basal activity of hHCA₃-C in MMY23 (G_{α11}) was increased.

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      10      20      30      40      50      60      70
HCA2  MNRHHLQDHFLEIDKKNCVFRDDFIVKVLPPVGLGFI FGLLGNGLALWIFCFHLKSWKSSRIFLFNLA 70
HCA3  MNRHHLQDHFLEIDKKNCVFRDDFIAKVLPPVGLGFI FGLLGNGLALWIFCFHLKSWKSSRIFLFNLA 70
HCA2+C MNRHHLQDHFLEIDKKNCVFRDDFIVKVLPPVGLGFI FGLLGNGLALWIFCFHLKSWKSSRIFLFNLA 70
HCA3-C MNRHHLQDHFLEIDKKNCVFRDDFIAKVLPPVGLGFI FGLLGNGLALWIFCFHLKSWKSSRIFLFNLA 70
      *

      80      90      100     110     120     130     140
HCA2  VADFLLIICLPFLMDNYVRRWDWKFQDIPCRMLFMLAMNRQGSIIFLTVAVDVRYFRVPHHALNKIS 140
HCA3  VADFLLIICLPFLMDNYVRRSDWNFGDIPCRVLVLFMFAMNRQGSIIFLTVAVDVRYFRVPHHALNKIS 140
HCA2+C VADFLLIICLPFLMDNYVRRWDWKFQDIPCRMLFMLAMNRQGSIIFLTVAVDVRYFRVPHHALNKIS 140
HCA3-C VADFLLIICLPFLMDNYVRRSDWNFGDIPCRVLVLFMFAMNRQGSIIFLTVAVDVRYFRVPHHALNKIS 140
      * * * * * * *

      150     160     170     180     190     200     210
HCA2  NRTAAIISCLLWGITIGLTVHLLKMKMPIQGGANLCSFSFISICHTFQWHEAMFLEFFLLPLGIIIFCSAR 210
HCA3  NRTAAIISCLLWGITVGLTVHLLKMKLLIQNGPANVCISFSICHTFRWHEAMFLEFFLLPLGIIIFCSAR 210
HCA2+C NRTAAIISCLLWGITIGLTVHLLKMKMPIQGGANLCSFSFISICHTFQWHEAMFLEFFLLPLGIIIFCSAR 210
HCA3-C NRTAAIISCLLWGITVGLTVHLLKMKLLIQNGPANVCISFSICHTFRWHEAMFLEFFLLPLGIIIFCSAR 210
      * * * * * * *

      220     230     240     250     260     270     280
HCA2  IIWSLRQRQMDRHAKIKRAITFIMVVAIVFVICFLPSVVRIRIFWLLHTSGTQNCVYRSDLAFFITL 280
HCA3  IIWSLRQRQMDRHAKIKRAITFIMVVAIVFVICFLPSVVRIRIFWLLHTSGTQNCVYRSDLAFFITL 280
HCA2+C IIWSLRQRQMDRHAKIKRAITFIMVVAIVFVICFLPSVVRIRIFWLLHTSGTQNCVYRSDLAFFITL 280
HCA3-C IIWSLRQRQMDRHAKIKRAITFIMVVAIVFVICFLPSVVRIRIFWLLHTSGTQNCVYRSDLAFFITL 280

      290     300     310     320     330     340     350
HCA2  SFTYMN SMLDPVVYFSSPFPNFFSTLINRCLQRKMTGEPDNNRSTSVELTGDPNKTRGAPEALMANS 350
HCA3  SFTYMN SMLDPVVYFSSPFPNFFSTLINRCLQRKMTGEPDNNRSTSVELTGDPNKTRGAPEALMANS 350
HCA2+C SFTYMN SMLDPVVYFSSPFPNFFSTLINRCLQRKMTGEPDNNRSTSVELTGDPNKTRGAPEALMANS 350
HCA3-C SFTYMN SMLDPVVYFSSPFPNFFSTLINRCLQRKMTGEPDNNRSTSVELTGDPNKTRGAPEALMANS 350

      360     370     380
HCA2  EPWSPSYLGPTSP. 364
HCA3  EPWSPSYLGPTSNNHKKGHCHQEPASLEKQLGCCIE. 388
HCA2+C EPWSPSYLGPTSNNHKKGHCHQEPASLEKQLGCCIE. 388
HCA3-C EPWSPSYLGPTSP. 364
      *****

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Fig. 3. Sequence alignment of the wild-type hHCA₂ receptor (GenBank: AAN71621.1), the wild-type hHCA₃ receptor (GenBank: BAA01721.1), the hHCA₂+C receptor and the hHCA₃-C receptor. The non-conserved residues are marked as *.

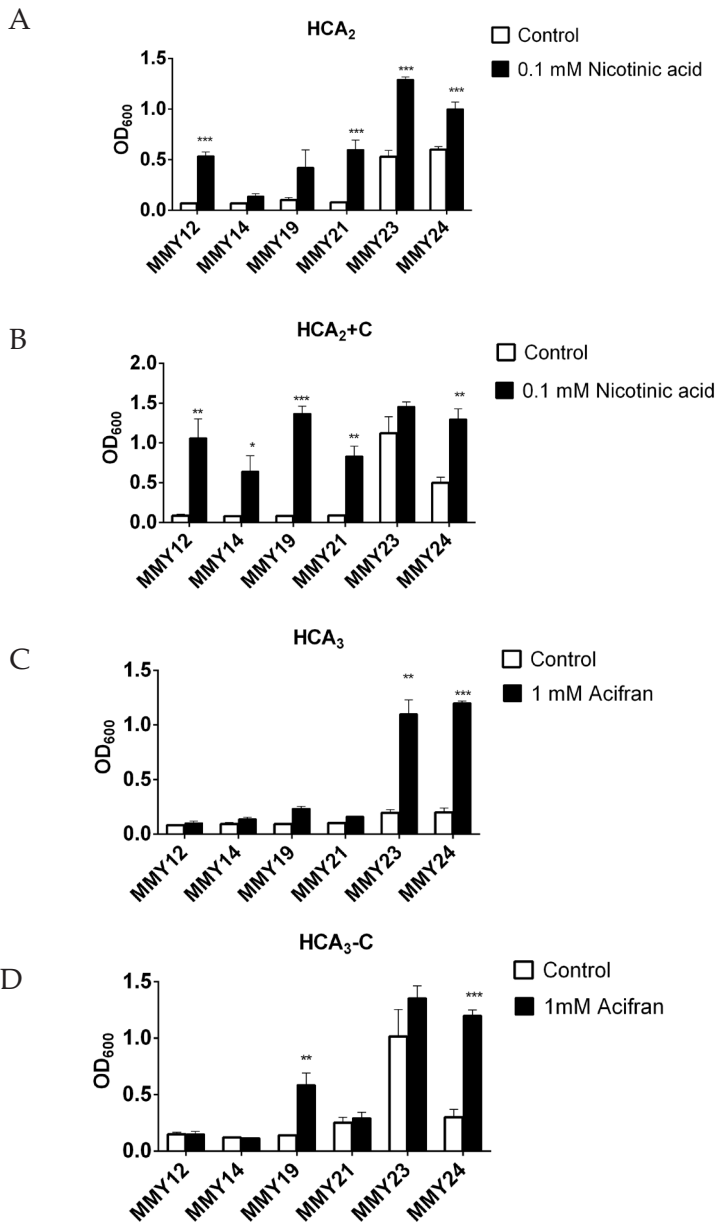


Fig. 4. Liquid assay experiments with the wild-type receptor hHCA₂ (A) and hHCA₂+C receptor (B) in the absence or presence of the agonist nicotinic acid (0.1 mM), and of the wild-type receptor hHCA₃ (C) and hHCA₃-C receptor (D) in the absence or presence of the agonist acifran (1 mM) in the different yeast strains. The assay was performed in YNB-ULH-NA medium. A representative experiment is shown (of a total of n = 2). Differences in receptor activation in the absence or presence of ligand in each strain were examined for significance by a two-tailed homoscedastic Student's *t*-test, yielding *p*-values, indicated as follows: *: *p* < 0.05, **: *p* < 0.01, ***: *p* < 0.001.

Determination of the expression levels of the hHCA₂ and hHCA₃ receptors in different yeast strains

In Fig. 5A a Western blot analysis is shown of the expression levels of the hHCA₂ receptor in all strains. A more quantitative bar graph analysis is depicted in Fig. 5B. Expression levels of the hHCA₂ receptor in most strains were quite comparable except for MMY16, MMY25 and MMY28.

Expression of the hHCA₃ receptor was detected in all other strains as well (Fig. 5D), but a more quantitative determination of hHCA₃ expression levels could not be performed due to the absence of its expression in the control MMY12 strain.

Unfortunately, the hHCA₂+C and hHCA₃-C mutant receptors failed to show in Western blot experiments, although these receptors showed concentration-effect curves with high E_{\max} values in a number of strains (Fig. 4). Apparently the antibody developed for the two wild-type receptors did not react with the constructs.

Discussion

One-GPCR-one-G protein yeast system and MMY strains

Our previous research has shown that our one-GPCR-one-G protein yeast (*S. cerevisiae*) screening is very well suited to study a G protein-coupled receptor with respect to its G protein preference. The main principle is activation of the G protein pathway by a GPCR agonist making the yeast cells grow in medium lacking histidine through the *FUS1-HIS3* reporter gene^[27, 28] in which the growth of the yeast cells quantitatively reflects the activation of the expressed receptor by the agonist. The advantage of this engineered yeast system over other (mammalian) systems is that it has zero background of G protein activation, which makes it suitable to detect the G protein profiles of individual receptor activation^[19, 29, 30].

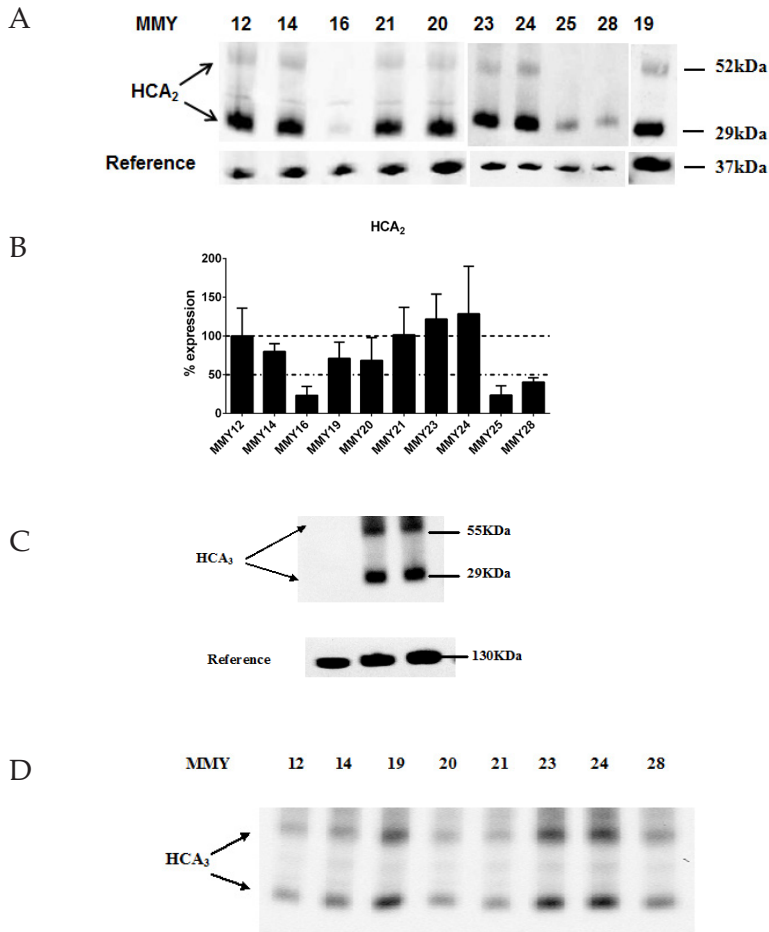


Fig. 5. Western blot analysis of the wild-type hHCA₂ and hHCA₃ receptor. (A) Representative blots of the hHCA₂ receptor in all strains in upper panel, i.e. MMY12, MMY14, MMY16, MMY21, MMY20, MMY23, MMY24, MMY25, MMY28, MMY12 and MMY19, all carrying the p426GPD-hHCA₂ transcript. The hHCA₂ receptor specific bands are located at 29 kD and 52 kD, which were absent in MMY12 carrying p426GPD without receptor (mock, not shown); α -tubulin (lower panel) is located at approx. 130kDa, which was used as loading control/reference (it also appeared in MMY12 carrying p426GPD without receptor). (B) Bar graph analysis of hHCA₂ receptor expression in all strains, as calculated from a densitometric analysis of the blots. The ratio was determined between the density of the specific bands and that of the nonspecific band that was always present on the blots. MMY12 carrying wild-type hHCA₂ was set as 100%. All expression levels of the hHCA₂ receptor in different MMY strains were normalized against a hHCA₂ receptor in the MMY12 strain on the same blot. The experiment was performed two independent times. (C) Representative blot of the hHCA₃ receptor. Lane 1: MMY23(G_{ai1})₃-p426GPD, Lane 2: MMY23(G_{ai1})₃-p426GPD-hHCA₃, Lane 3: MMY24(G_{ai3})₃-p426GPD-hHCA₃. The hHCA₃ receptor specific bands (upper panel) are located around 29kD and 55kD; α -tubulin (lower panel) is located at approx. 130kDa, which was used as loading control/reference (it also appeared in MMY23 carrying p426GPD without receptor). (D) Representative blot of the hHCA₃ receptor in all strains MMY12, MMY14, MMY19, MMY20, MMY21, MMY23, MMY24, and MMY28.

Brown et al (2000) reported that humanized yeast G_{α} proteins only containing a short (5 amino acids) C-terminal fragment of mammalian G_{α} fused to the remainder of the yeast's own Gpa1p G protein represent a significant improvement over other long (approx. 150 amino acids) Gpa1p/ G_{α} chimeras. They chose 9 representative GPCRs to demonstrate this system is very suitable for the analysis of receptor and G protein function and their interplay, and showed some receptors to be more promiscuous than others, such as the adenosine A_{2B} receptor^[30]. In the current study we applied 10 MMY strains, each with a specific humanized or WT yeast G_{α} protein. Corresponding to the replaced last five C-terminal amino acid residues of the mammalian G_{α} subunit, the strains can be classified into five families: $G_{\alpha_{WT}}$ (MMY12), G_{α_s} (MMY28), G_{α_i} (MMY23, MMY24 and MMY25), $G_{\alpha_{12}}$ (MMY19 and MMY20), and G_{α_q} (MMY14, MMY16 and MMY21)^[18, 19, 26] (Table 1). It turned out that the combination of empty plasmid p426GPD with two different G proteins (MMY20 ($G_{\alpha_{13}}$) and MMY28 (G_{α_s})) led to very high constitutive activity of the WT HCA_2 and HCA_3 receptor in the absence of agonist, and they were not examined further. Interestingly, standard commercial medium for yeast growth contains nicotinic acid, hence we used a special medium without nicotinic acid, as its presence would invariably activate all HCA_2 receptors. For comparison we applied the same medium for our studies on the HCA_3 receptor and its mutant, although nicotinic acid does not activate the HCA_3 receptor (see Fig. 1C).

Wise et al. (2003) reported EC_{50} values of nicotinic acid and acifran for the HCA_2 receptor of 904 nM and 3,000 nM, respectively, using a yeast growth assay through the *FUS1-LacZ* reporter gene in the MMY16 ($G_{\alpha_{16}}$) strain. They also studied the effect of acifran on the HCA_2 (EC_{50} value of 2,100 nM) and HCA_3 receptor (EC_{50} value of 20,000 nM) in a [³⁵S]GTP γ S binding assay^[31]. We came to comparable, although not similar, potencies for the agonists. For example, in the better-coupled MMY24 ($G_{\alpha_{13}}$) strain the EC_{50} value of nicotinic acid at the $hHCA_2$ varied between 35 nM (Table 2) and 240 nM (Table 3) in different experimental conditions. The EC_{50} value of acifran for the $hHCA_2$ receptor was 56 nM in the same MMY24 strain. Nicotinic acid is without effect at the $hHCA_3$ receptor, while the EC_{50} value of acifran for this receptor subtype varied from 14 μ M

(Table 2) to 88 μM (Table 3). It is somewhat unfortunate that no antagonists have been identified yet for the two receptors, which would allow a more detailed pharmacological analysis.

The different G protein coupling profiles of the hHCA₂ receptor and hHCA₃ receptor

Although the hHCA₂ and hHCA₃ receptor share 95% sequence identity, their G protein coupling profiles are quite different. In the presence of nicotinic acid the hHCA₂ receptor activates almost all G protein pathways except G _{α q} (MMY14) (Fig. 2A and Table 3). In fact, the hHCA₂ receptor activated G _{α q} as well, when the agonist nicotinic acid was present for a longer time (48 h, data not shown). In contrast, the hHCA₃ receptor activated only two G protein pathways, MMY23 (G _{α ii1}) and MMY24 (G _{α ii3}), with no further activation of other G protein pathways after prolonged presence (48 h) of the agonist acifran (data not shown). In the model yeast system the hHCA₂ receptor thus activates G _{α} in the following order: G _{α ii1} > G _{α ii3} > G _{α az} > G _{α 14} > Gpa1(G _{α WT}) > G _{α 16} > G _{α q}. However, the G _{α} protein coupling profile of the hHCA₃ receptor is less promiscuous: G _{α ii1} > G _{α ii3} >>> all other pathways. Expression levels of the hHCA₂ receptor in most strains were quite comparable except for MMY16, MMY25 and MMY28 (Fig. 5A and B). Apparently, there was no clear connection between the activation profile and the expression of the receptor. For instance in the MMY14 strain there is good receptor expression, but receptor activation only happened after prolonged incubation of the agonist (see above). Expression of the hHCA₃ receptor was observed in all strains examined, with relatively high levels in MMY19, MMY23 and MMY24 strains (Fig. 5C and D).

The function of the C terminus in G protein activation and selectivity

Fusing the C-terminus of the HCA₃ receptor to the HCA₂ receptor did not change the ligand preference of the HCA₂ receptor, as in both cases nicotinic acid activated the receptor (Fig. 4A and B). This is not surprising, as the ligand binding site resides most likely in the upper part of the transmembrane domain, in the absence of a crystal structure^[1]. The same finding was noticed for the

HCA₃ receptor when its C-terminus was deleted; both proteins were activated by acifran (Fig. 4C and D). Indeed, the typical selectivity profile for the two WT receptors remained intact when the three reference compounds were tested on the constructs (Table 2). Tunaru et al. systematically mutated each of the amino acid residues in the putative binding pocket of hHCA₂ into the corresponding residues of hHCA₃ and found that three amino acids, Asn86, Trp91 and Ser178 of hHCA₂ are required for high-affinity binding of nicotinic acid^[32]. Ahmed et al. reported on hHCA₃-specific amino acid residues, again in the N-terminal half of the receptor, mediating the specific activation of hHCA₃ by 2-OH-octanoic acid^[33]. As the C-terminus may be close to the intracellular G protein binding site we anticipated substantial effects on G protein coupling of either adding (HCA₂+C construct) or deleting (HCA₃-C construct) that part of the receptor. For instance, if the longer C terminus of the HCA₃ receptor would have been a determinant for the quite selective G protein preference of this receptor, addition of that part of the receptor to the HCA₂ receptor might have caused a similar narrow preference. However, this was by no means the case, as the HCA₂+C construct was even less fastidious than the WT receptor (Fig. 4A and B). Removing the C-terminus of the HCA₃ receptor (HCA3-C) made the receptor a bit more promiscuous but not very much (Fig. 4C and D).

Conclusions

The hHCA₂ receptor is more promiscuous in its G protein coupling preference than the hHCA₃ receptor, which may also be the case in physiology. However, these differences are not controlled by their C-terminal tails. This is an important finding as the C-terminus of GPCRs has been implicated in many other relevant physiological processes such as folding of the receptor, maturation and trafficking to the cell membrane, association with other intracellular proteins including scaffold and adapter proteins^[34]. Apparently G protein coupling may not belong to this enumeration, if the findings for the HCA₂ and HCA₃ receptor can be extended to other GPCRs.

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Supplement data

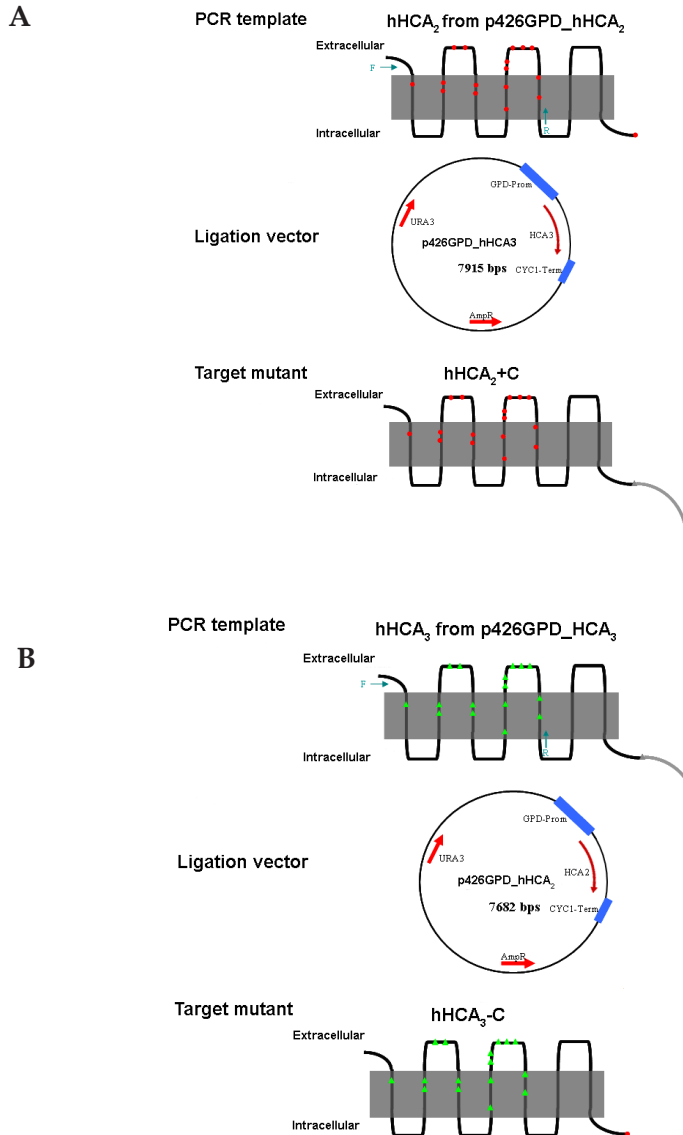


Fig. S1. Schematic drawing of the construction of the hHCA₂+C mutant (A) and the hHCA₃-C mutant (B). There are 16 different amino acids in both receptors preceding the C terminus; they are color-coded in both the hHCA₂/hHCA₂+C receptors (●) and the hHCA₃/hHCA₃-C receptors (▲). The one different amino acid at C-terminus is shown between the hHCA₂/hHCA₂+C receptors (●) and the hHCA₃/hHCA₃-C receptors (▲); the 24-amino acid C-terminal extension is shown as gray lines in the hHCA₃/hHCA₂+C receptors. F: forward primer; R: reverse primer. The size of PCR products is 213 amino acids.