Title: Delineation of the GPRC6A receptor signaling pathways using a mammalian cell line stably expressing the receptor

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Abstract

The GPRC6A receptor is a recently deorphanized class C G protein-coupled receptor. We and others have shown that the receptor is co-activated by basic L-α-amino acids and divalent cations whereas other groups have also suggested osteocalcin and testosterone to be agonists. Likewise, the GPRC6A receptor has been suggested to couple to multiple G protein classes albeit via indirect methods. Thus, the exact ligand preferences and signaling pathways are yet to be elucidated. In the present study, we have generated a Chinese Hamster Ovary (CHO) cell line that stably expresses mouse GPRC6A. In an effort to fully establish the signaling properties of the receptor, we test representatives of four previously reported GPRC6A agonist classes for activity in the Gq, Gs, Gi and extracellular-signal regulated kinase signaling pathways. Our results confirm that GPRC6A is activated by basic L-α-amino acids and divalent cations, and for the first time we conclusively show that these responses are mediated through the Gq pathway. We were not able to confirm previously published data demonstrating Gi and Gs-mediated signaling. Neither could we detect agonistic activity of testosterone and osteocalcin. Generation of the stable CHO cell line with robust receptor responsiveness and optimization of the highly sensitive Homogenous Time Resolved Fluorescence technology allows fast assessment of Gq activation without previous manipulations like co-transfection of mutated G proteins. This cell-based assay system for GPRC6A is thus useful in high-throughput screening for novel pharmacological tool compounds, which are necessary to unravel the physiological function of the receptor.
Introduction

The GPRC6A receptor is a recently identified class C G protein-coupled receptor (GPCR) (Wellendorph and Brauner-Osborne, 2004). A promiscuous range of non-selective ligands and a broad but low level expression profile have complicated elucidation of the physiological function of this receptor. Based on studies in GPRC6A knockout mice, the receptor is now suggested to be involved in inflammatory, metabolic and endocrine regulation, however the specific role of GPRC6A is still unknown (Clemmensen et al., 2013a, 2013b; Pi et al., 2008; Pi et al., 2012; Pi et al., 2011; Rossol et al., 2012; Smajilovic et al., 2012; Wellendorph et al., 2009).

In 2004-05 we were the first to clone and deorphanzize GPRC6A, which proved to be activated by L-\(\alpha\)-amino acids and in particular basic amino acids (Wellendorph and Brauner-Osborne, 2004; Wellendorph et al., 2005). Divalent cations such as calcium have also been shown to activate the receptor directly (Pi et al., 2005; Pi and Quarles, 2012) or to serve as co-agonists, which positively modulate the L-\(\alpha\)-amino acid response (Christiansen et al., 2007; Kuang et al., 2005; Wellendorph et al., 2007). Furthermore, others have demonstrated receptor activation upon stimulation with testosterone and osteocalcin in the presence of extracellular calcium (Oury et al., 2011; Pi et al., 2010; Pi and Quarles, 2012; Pi et al., 2011). The closest related GPCR is the odorant goldfish 5.24 receptor, which signals through the G\(_\text{q}\) pathway (Christiansen et al., 2006; Speca et al., 1999). Likewise, we and the group of Hampson have used the oocyte expression system to show that mouse GPRC6A (mGPRC6A) activates a calcium-sensitive chloride channel upon stimulation with L-\(\alpha\)-amino acids and cations indicative of G\(_\text{q}\) coupling (Christiansen et al., 2007; Kuang et al., 2005; Wellendorph et al., 2005). Using mGPRC6A-transfected HEK293 cells and pathway selective inhibitors, the Quarles group has shown that downstream serum-response element (SRE) and/or extracellular signal-regulated kinase...
(ERK) are activated by divalent cations (G_q and G_i pathways (Pi et al., 2005; Pi et al., 2010; Pi and Quarles, 2012)), L-arginine (pathway not investigated, (Pi et al., 2011)), the steroid testosterone (G_i pathway, G_q not investigated (Pi et al., 2010)) and the peptide osteocalcin (G_q pathway, G_i not investigated (Pi et al., 2011)). In addition, the group has shown that all four agonist classes lead to cAMP accumulation in the GPRC6A-HEK293 cell line and thus is likely to also be G_s coupled (Dreaden et al., 2012; Pi et al., 2012). Finally, the Karsenty group has shown that osteocalcin leads to a bell-shaped concentration-dependent increase in cAMP, indicating G_s coupling, but no osteocalcin-mediated activation of the G_q or ERK pathways in TM3 Leydig cells. These osteocalcin responses were, however, not shown specifically to be mediated by GPCR6A (Oury et al., 2011). Thus, conflicting findings regarding GPRC6A signaling have been reported, and physiological relevant ligands and signaling pathways have yet to be identified.

Albeit GPCRs are known to be activated by a very broad range of ligands (Pierce et al., 2002) it is unprecedented that the same receptor subtype should have evolved to be endogenously activated by four highly different structural classes of ligands; amino acids, cations, a steroid and a 49 amino acid peptide (Fig. 1). From a phylogenetic point of view, class C GPCRs are perceived as nutrient receptors derived from amino acid and nutrient transporters from bacteria (Conklin and Bourne, 1994; Kuang et al., 2006). Thus, all other human class C GPCRs with a "Venus-flytrap domain" like GPRC6A sense amino acids, cations and/or sugars and no other subtype in class C has previously been suggested to be activated by steroids or peptides of the size of osteocalcin (Brauner-Osborne et al., 2007). The reports of GPCR6A being activated by testosterone and/or osteocalcin have thus generated considerable curiosity.

A major limitation when testing the GPRC6A receptor is the presence of L-α-amino acids and divalent cations in the cell culture media. These ligands might be
responsible for activation and subsequent desensitization of the receptor, hence it has been
difficult to obtain robust responses when characterizing mGPRC6A recombinantly
expressed in mammalian cell lines (Wellendorph et al., 2005). Accordingly, previous
studies of mGPRC6A signaling have suffered from low-throughput techniques,
manipulations such as co-transfection with chimeric/mutated G proteins biasing the
signaling pathway, and/or measurement of downstream effects such as SRE or ERK
activation, which is difficult to assign to specific signaling pathways.

To circumvent these limitations and for the first time enable direct measurement of
GPRC6A signaling, we have generated a Chinese Hamster Ovary (CHO) cell line that
stably expresses mGPRC6A (mGPRC6A-CHO) using the Flp-In™ system (Invitrogen,
Paisley, UK). In order to fully elucidate the signaling of GPRC6A, pharmacological
characterization has been carried out using this cell line by testing representatives of the
four previously reported GPRC6A agonist classes using the highly sensitive high-
throughput Homogenous Time Resolved Fluorescence (HTRF®) technology for detection
of the G_q, G_s and G_i pathways, and western blotting for detection of ERK activation.
Material & Methods

Materials

Ham’s F-12 GlutaMAX medium, DMEM GlutaMAX medium, dialyzed fetal bovine serum (dFBS), penicillin (10⁴ U/ml)-streptomycin (10⁴ µg/ml) mixture, hygromycin B (50 mg/ml), Zeocin (100 mg/ml), Dulbecco’s Phosphate-Buffered Saline (DPBS), 0.05% trypsin-EDTA, Hanks’ Balanced Salt Solution (HBSS), the Fluo-4 NW Calcium Assay Kit, TAE buffer (10x), NuPAGE® LDS Sample Buffer (4X), NuPAGE® Sample Reducing Agent (10X), NuPAGE® Novex® 4-12% Bis-Tris Gels (1.5-mm thick, 15-well), NuPAGE® MOPS SDS Running Buffer (20X), NuPAGE® Antioxidant, XCell SureLock® Mini-Cell, NuPAGE® Transfer buffer (20X), PVDF/Filter Paper Sandwiches and XCell II™ Blot Module, c-myc Mouse Monoclonal Antibody and Goat Anti-Mouse IgG HRP Conjugate were all purchased from Invitrogen (Paisley, UK). L-Ornithine (L-Orn), CaCl₂, MgCl₂, LiCl, ATP, paraformaldehyde, Trizma hydrochloride solution pH 7.4 and 7.6, dimethyl sulfoxide (DMSO), probenecid, 3-isobutyl-1-methylxanthine (IBMX), forskolin, poly-d-lysine, HEPES, bovine serum albumin (BSA), Cell Dissociation Solution Non-enzymatic, Tween-20, sodium azide, skim milk powder, RIPA buffer, Tris buffered saline (TBS), U73122 and testosterone were all purchased from Sigma Aldrich (St Louis, MO, USA). Testosterone was dissolved in DMSO to 100 mM. UBO-QIC was purchased from Prof. Evi Kostenis, University of Bonn, Germany and dissolved to 10 mM in DMSO. SuperSignal® ELISA Femto Stable Peroxidase Solution and SuperSignal® ELISA Femto Luminol Enhancer Solution were purchased from Thermo Fischer Scientific (Slangerup, Denmark). Prestained Protein Mw Marker, Donkey Anti-rabbit IGG HRP-link whole Antibody, Mouse IGG HRP-link whole Antibody and Amersham™ ECL™ Prime Western blotting detection reagents were purchased from VWR (Radnor, PA, USA). Uncarboxylated mouse osteocalcin was purchased from Bachem (Bubendorf,
Switzerland). Osteocalcin was dissolved in HBSS buffer containing 20 mM HEPES, 1 mM Ca\(^{2+}\), 1 mM Mg\(^{2+}\) (pH 7.4) to 100 µM. Myo-[2-\(^3\)H]-inositol and polylysine YSi SPA beads were purchased from PerkinElmer (Waltham, MA, USA). Green GoTaq Reaction Buffer (5x), Magnesium Chloride solution (25 mM), GoTaq DNA Polymerase (5 U/µl) and a set of dATP, dCTP, dGTP, dTTP (100 mM each) were all purchased from Promega (Madison, WI, USA) and primers were purchased from TAG Copenhagen A/S (Copenhagen, Denmark). The IP-One kit and the cAMP dynamic 2 kit were purchased from Cisbio (Codolet, France). Complete protease inhibitor cocktail tablets were purchased from Roche (Basel, Switzerland) and Phospho-p44/42 MAPK (ERK1/2) rabbit Antibody and p44/42 MAPK (ERK1/2) mouse Antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). Compound 1 was synthesized by Henrik Johansson and Daniel S. Pedersen (University of Copenhagen, Denmark) as previously published (Gloriam et al., 2011).

Generation of CHO and HEK293 cell lines stably expressing mouse and human GPRC6A

mGPRC6A has previously been tagged in the N-terminus with the signal peptide of mGluR5 to promote cell surface expression and c-myc to enable detection of cell surface expression by enzyme-linked immunosorbent assay (ELISA) (Wellendorph et al., 2005). This construct was transferred from the previously published pEGFP-N1 vector (Wellendorph et al., 2005) to the pcDNA5/FRT/V5-His-Topo vector (Invitrogen, Paisley, UK) by PCR using the primers m6A_cloning_forward, 5’-ACCATGGTCCCTCTGTTGATC-3’ and m6A_cloning_reverse, 5’-TCATATACTTGAACTTTTCTG-3’). Likewise, c-myc-tagged human GPRC6A (hGPRC6A) was transferred from the pEGFPN1 vector to the pcDNA5/FRT/V5-His-Topo vector by using the primers hC6A_cloning_forward, 5’-
AGTGCCACCACATGGCTCTCTGT-3' and hC6A_cloning_reverse, 5'-GCCGCCATCTCCTAAGGCTTATCAT-3'. The absence of mutations in all constructs was verified by DNA sequencing (Eurofins MWG Operon, Ebersberg, Germany). Flp-In-CHO and Flp-In-HEK293 cells were maintained in Ham’s F12 media and DMEM media, respectively, supplemented with 10 % (v/v) dFBS, 2 mM L-glutamine, and 1 % P/S mixture in a humidified atmosphere (95 % air and 5 % CO2). To generate Flp-In-CHO or Flp-In-HEK293 cells stably expressing mGPRC6A (mGPRC6A-CHO or mGPRC6A-HEK293), cells were transfected with a 1:9 ratio mixture of pcDNA5/FRT/V5-His/Topo construct and pOG44 using Polyfect (Qiagen, West Sussex, UK) accordingly to the manufacturer’s instructions. 24 h after the transfection fresh medium was applied. 48 h after the transfection the medium was changed again to fresh medium containing 600 μg/ml or 200 μg/ml hygromycin B to initiate selection of stably expressing mGPRC6A-CHO or mGPRC6A-HEK293 cells, respectively. Likewise, Flp-In-CHO and Flp-In-HEK293 cells stably expressing hGPRC6A were generated.

**RT-PCR**

RNA was extracted from mGPRC6A-CHO and Flp-In-CHO cells using the RNaseq kit (Qiagen, Hilden, Germany), and subsequently 1 μg of RNA was transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). mGPRC6A cDNA was amplified by using intron-spanning primer sequences previously reported (Wellendorph et al., 2009): m6A_RT-PCR_forward 5'-GCCCTGGTCAAATGAAGAAA-3' and m6A_RT-PCR_reverse 5'-TGATGTAGCCACCAGCATGGTA-3'. Amplification of the ubiquitously expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as control, in which the following primers were used: GAPDH_forward 5'-
TGAAGGTCGGTGTGAACGGATTTGG-3’ and GAPDH_reverse 5’CATGTAGGCCATGAGGTCCACCAC-3’. 5 U/µl GoTaq DNA Polymerase were used for the PCR reaction, which was run as follows: 95 °C 5 min, 95 °C 30 sec, 55 °C (for mGPRC6A primers) and 64 °C (for GAPDH primers) 30 sec for annealing, 72 °C 2 min and 72 °C 10 min for 30 cycles. Amplified PCR products were verified on a 1 % agarose gel containing ethidiumbromide, and cDNA bands were detected using the PhotoDoc-It™ Imaging system (UVP, Upland, CA, USA).

Transient expression in tsA201 cells

tsA201 cells were transfected using Polyfect according to the manufacturer’s protocol (Qiagen, Hilden, Germany). Cells were co-transfected either with constructs encoding mGPRC6A and empty vector (transfection ratio 1:1) or constructs encoding mGPRC6A and Gq(G66D) (transfection ratio 1:1) as previously described (Christiansen et al., 2007). tsA201 cells transfected with empty vector were used as negative control.

ELISA

One day before the assay, 10^5 cells/well were cultured in a poly-D-lysine treated white with clear bottom CulturPlate-96 (PerkinElmer, Waltham, MA, USA) at 37 °C and 5 % CO_2 for 24 h. On the day of the assay, cells were fixed to the plate using 50 µl/well of fixing solution (DPBS + 4 % paraformaldehyde) for 5 minutes. The 96-well plate was washed twice with DPBS containing 1 mM Ca^{2+} (DPBS-Ca) followed by addition of 100 µl/well of blocking solution (0.3 g drymilk, 10 µl 1 M CaCl_2, 250 µl Trizma hydrochloride solution pH 7.4, 250 µl Trizma hydrochloride solution pH 7.6, qsp 10 ml dH_2O). The plate was incubated at room temperature (RT) for at least 30 min. After
blocking, the plate was incubated with 75 µl/well of primary antibody (c-myc Mouse Monoclonal Antibody diluted 1:1000 in blocking solution) at RT for 45 min. Subsequently, the plate was washed with 100 µl/well blocking solution and twice with 100 µl/well DPBS-Ca. 75 µl/well of secondary antibody (Goat Anti-Mouse IgG HRP Conjugate diluted 1:1500 in blocking solution) was added and the plate was incubated at RT for 45 min. The plate was then washed four times with 100 µl/well blocking solution and four times with 100 µl/well of DPBS-Ca. 60 µl/well of DPBS-Ca was added and the detection solution was prepared (SuperSignal® ELISA Femto Stable Peroxidase Solution and SuperSignal® ELISA Femto Luminol Enhancer Solution (Thermo Fischer Scientific, Waltham, MA, USA), 1:1). 20 µl/well detection solution was added to the plate, and chemiluminescence was measured immediately on an EnSpire reader (PerkinElmer, Waltham, MA, USA).

**IP-One Assay**

Ligands were prepared in 2x final concentration in ligand buffer (HBSS buffer, 40 mM LiCl, 1 mM Ca²⁺, 1 mM Mg²⁺), and 5 µl/well of ligand solution in triplicates was added to a 384-well OptiPlate (PerkinElmer, Waltham, MA, USA). Subconfluent cells were detached from the cell culture dish by using 37 °C Cell Dissociation Solution. The cells were centrifuged at 1100 rpm for 5-7 min, after which the cell pellet was resuspended in the appropriate volume of 37 °C assay buffer (HBSS buffer, 20 mM HEPES, 1 mM Ca²⁺, 1 mM Mg²⁺ pH 7.4) to achieve a concentration of 10⁷ cells/ml (for the stably expressing mGPRC6A-CHO cells and the control Flp-In-CHO cells) and 6 x 10⁶ cells/ml (for the transiently expressing tsA201 cells). 5 µl/well of cell suspension was added to the plate, which was then sealed and incubated at 37 °C for 1 h, followed by 15 min incubation at RT. The detection solution was prepared as follows: IP-One Conjugate.
& Lysis buffer + 2.5 % anti-IP₁ cryptate Tb conjugate + 2.5 % IP₁-d2 conjugate. 10 µl/well of detection solution was added to the plate, which was then incubated away from light for 1 h at RT. The plate was read on EnVision multilabel reader (PerkinElmer, Waltham, MA, USA); excitation at 340 nm and measurements of emission at 615 nm and 665 nm. The FRET ratios (665 nm/615 nm) were converted to IP₁ concentrations by interpolating values from an IP₁ standard curve generated from an IP₁ calibrator, provided by the manufacturer (Cisbio, Codolet, France).

To test for G₉ pathway signaling, cells were pretreated for one hour before the assay in normal media containing 1 µM UBO-QIC. Ligands to be tested were prepared in ligand buffer containing the same amount of UBO-QIC. For inhibition using U73122, the cell suspensions were made in assay buffer supplemented with 20 µM U73122 and incubated for 10 minutes before addition of the cells.

**IP turnover assay**

The assay was carried out as previously described (Christiansen et al., 2007). Transiently expressing tsA201 cells were stimulated with an EC₂₅ concentration of L-Orn (25 µM) for 30 min at 37 °C in the absence or presence of 0.1 and 1 µM concentrations of osteocalcin and in the presence of 1 mM Ca²⁺ and Mg²⁺. A concentration of 1 mM L-Orn was used to assess the maximum response. Three different forms of osteocalcin were tested. Osteocalcin form 1: human mature carboxylated osteocalcin (Sigma O5761); form 2: mouse mature uncarboxylated osteocalcin (Bachem H-6552), form 3: mouse mature carboxylated osteocalcin (Bachem 4063515). Stock solutions were 1 mM prepared in either DPBS (forms 1 and 2) or 0.1 % trifluoracetic acid in H₂O (form 3).
**Fluo-4 Calcium Assay**

One day before the assay, 10^5 cells/well were cultured in a 96-well black with clear-bottom plate at 37 °C and 5 % CO₂. On the day of the assay, the plate was washed with 100 µl/well of DPBS followed by addition of 50 µl/well of dye loading solution. The plate was incubated for 1 h at 37 °C and 5 % CO₂. The dye loading solution had been prepared by adding 10 ml probenecid-buffer (HBSS buffer, 20 mM HEPES, 1 mM Ca^{2+}, 1 mM Mg^{2+}, pH 7.4, supplemented with 2.5 mM probenecid) to Component A in the Fluo-4 NW Calcium Assay Kit (Invitrogen, Paisley, UK). Ligands were prepared in 4x concentrations and distributed in a clear 96-well ligand plate. After incubation with the dye loading solution, the plate was washed once with 100 µl/well of probenecid-buffer followed by addition of 100 µl/well of probenecid-buffer. The plate was read using a FlexStation Benchtop Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) with an excitation filter of 485 nm and emission at 525 nm. 33 µl/well of ligand was added to the plate.

**cAMP dynamic 2 Assay**

Ligands were prepared in 2x final concentration in ligand buffer (HBSS buffer, 20 mM HEPES, 100 µM IBMX, 1 mM Ca^{2+}, 1 mM Mg^{2+}, pH 7.4). 40 µM forskolin was added to the ligand buffer when measuring G_i signaling. 5 µl/well of ligand solution in triplicates was added to a small volume 384-well white Greiner plate (Greiner Bio-One, Frickenhausen, Germany). Cell suspensions were prepared as described for the IP-One assay in order to achieve a concentration of 2 x 10^6 cells/ml or 1.2 x 10^6 cells/ml for measurements of G_s and G_i signaling, respectively. 5 µl/well of cell suspension was added to the plate, which was then covered with a lid and incubated at RT on a plate shaker at 450 rpm for 30 min. Two detection solutions were prepared as follows; cAMP Conjugate
& Lysis buffer + 5 % anti-cAMP cryptate Tb conjugate and cAMP Conjugate & Lysis buffer + 5 % cAMP d2 conjugate. 5 µl/well of each conjugate solution was added to the plate, which was then incubated away from light for 1 h at RT. The plate was read on EnVision multilabel reader (PerkinElmer, Waltham, MA, USA); excitation at 340 nm and measurements of emission at 615 nm and 665 nm. The FRET ratio (665 nm/615 nm) was converted to cAMP concentrations by interpolating values from a cAMP standard curve generated from a cAMP calibrator provided by the manufacturer (Cisbio, Codolet, France).

**ERK Assay**

3 x 10^5 cells/well (for mGPRC6A-CHO) and 4.5 x 10^5 cells/well (for Flp-In-CHO) were cultured in a 6-well clear culture plate 48 h before the assay. On the day of the assay, cells were washed with 2 ml/well of washing buffer (HBSS, 20 mM HEPES, 1 mg/ml BSA, 1 mM Ca^{2+}, 1 mM Mg^{2+}, pH 7.4, although without Ca^{2+} and Mg^{2+} when testing Ca^{2+} as a ligand) for 4 h at 37 °C. Ligands were prepared in final concentrations in ligand buffer (HBSS, 20 mM HEPES, 1 mM Ca^{2+}, 1 mM Mg^{2+}, pH 7.4, although without Ca^{2+} and Mg^{2+} when testing Ca^{2+} as a ligand), and 900 µl/well was added. The plates were incubated for 20 minutes at 37 °C, as pilot studies had shown maximum ERK activation 20 minutes after addition of L-Orn or Ca^{2+}. Subsequently the cells were washed twice with ice-cold DPBS. 70 µl/well of ice-cold lysis buffer (7 ml RIPA buffer + 1 protease inhibitor cocktail tablet) was added and the plates were incubated on ice for 10-15 min. The protein concentration in each sample was determined by using the Bio-Rad protein determination kit (Bio-Rad Laboratories, Hercules, CA, USA). SDS-PAGE was run by using the XCell SureLock™ Mini-Cell kit and samples were loaded on a NuPAGE® 4-12 % Bis Tris Gel. The gel was run at 175 V and 125 mA for 1 h, and the proteins were
subsequently transferred from the gel to a PVDF membrane by using the XCell
SureLock™ Mini-Cell kit and the XCell II™ Blot Module. At first, the membranes were
prepared by 2 min incubation in 96 % ethanol, and the transfer was then run at 30 V and
110-170 mM for 1 h. Subsequently, the membranes were incubated in blocking solution (5
% dry milk in TBST (TBS (1x) with 0.2 % Tween-20) for 1 h at RT on a tilting table
followed by over-night incubation at 4 °C. Membranes were subjected to 1 h incubation in
primary antibody (rabbit anti-P-ERK1/2 antibody diluted 1:1000 in 1 % dry milk in TBST
with 0.02 % sodium azide) followed by 1 hour incubation in secondary antibody (anti-
rabbit antibody HRP conjugate diluted 1:5000 in 1 % dry milk in TBST) at RT on a tilting
table. The Amersham™ ECL™ Prime Western blotting-detection reagents were mixed
1:1 and 1 ml of the detection mix was then added to the protein-side of the membranes.
Imaging of the chemiluminescence was carried out by using the FluorChem® HD2 system
from Alpha Innotech (San Leandro, CA, USA). Then the membranes were stripped of the
first antibodies by 0.2 M NaOH followed by incubation with total ERK (T-ERK)
antibodies (mouse anti-T-ERK1/2 antibody and anti-mouse antibody HRP conjugate) and
imaging as described above. Relative quantification of the western blots was carried out
by measuring the intensity of the bands using the publicly available ImageJ program
(http://rsbweb.nih.gov/ij/download.html) and by normalizing the P-ERK response to the
corresponding T-ERK response.

Data analysis

All data analysis has been carried out using Prism GraphPad version 5.0a for Mac
OS X (GraphPad Software, San Diego, CA, USA). Concentration-response curves have
been fitted by non-linear regression using the equation for sigmoidal concentration-
response function with variable slope:
\[ R = R_{\text{min}} + \frac{(R_{\text{max}} - R_{\text{min}})}{1 + 10^{\log EC_{50} - X)} \cdot n_H \]

in which \( X \) is the logarithm of the concentration, \( R \) is the response, \( R_{\text{max}} \) is the maximal response, \( R_{\text{min}} \) is the minimal response, \( EC_{50} \) is the concentration giving half maximum response and \( n_H \) is the Hill coefficient, which describes the steepness of the curve. Statistical analysis (Unpaired Student’s t-test or One-way ANOVA followed by Dunnett’s test) has been performed where appropriate and as indicated in the figure captions. Statistical significance has been determined at the following levels: * \( P<0.05 \), ** \( P<0.01 \) and *** \( P<0.001 \).
Results

Expression of mouse GPRC6A in the CHO cell line

A stable mGPRC6A-CHO cell line was generated and mRNA expression of the receptor was verified by RT-PCR (Fig. 2A). Furthermore, the cell surface expression of the receptor was determined by ELISA, as surface expression of GPCRs is a prerequisite for functional activity. Prior to insertion into the Flp-In-CHO genome, the receptor was tagged with a c-myc epitope at the N-terminus, thereby allowing receptor expression to be detected using anti-c-myc antibody. The surface expression of the receptor in mGPRC6A-CHO was found to be around seven fold higher than the non-specific background level detected in Flp-In-CHO (Fig. 2B) thus confirming that the receptor is present in the cell membrane when stably expressed in the CHO cell line.

In parallel, a CHO cell line stably expressing c-myc-tagged human GPRC6A (hGPRC6A) was generated. mRNA expression was verified by RT-PCR (data not shown), however no surface expression of the receptor was detected when using anti-c-myc antibody in ELISA (Fig. 2B). Accordingly, L-Orn was inactive in the IP-One and P-ERK assays in this cell line (data not shown). This is consistent with previously published data (Wellendorph and Bräuner-Osborne, 2004; Wellendorph et al., 2005), in which no surface expression or functional responses of hGPRC6A was obtained in any of the applied expression systems.

Activation of the Gq signaling pathway

The Gq protein directly activates the phosphatidylinositol-specific phospholipase C (PI-PLC), which is responsible for hydrolysis of phosphatidylinositol 4,5 bisphosphate into the second messenger molecules diacylglycerol and inositol 1,4,5 trisphosphate (IP3). IP3 is degraded into D-myo-inositol bisphosphate and D-myo-inositol monophosphate.
(IP₁) (Luttrell, 2008; Trinquet et al., 2011). A further degradation of IP₁ is inhibited by lithium chloride (Parthasarathy et al., 1994), which can be added to the ligand buffer, and thus allows assessment of G₉ activity by measuring IP₁ accumulation (Trinquet et al., 2006).

Four ligands (L-Orn, Ca²⁺, testosterone and osteocalcin) were tested for their ability to activate the G₉ pathway in the mGPRC6A-CHO and Flp-In-CHO cell lines using the HTRF® IP-One assay. L-Orn was tested at 500 µM due to pilot studies showing a maximum IP-One response in mGPRC6A-CHO at this concentration, and Ca²⁺ was tested in 5 mM concentrations due to assay interference above this concentration (data not shown). Testosterone and osteocalcin have previously been reported to induce maximum response in the ERK pathway at 80 nM and 60 ng/ml respectively (Pi and Quarles, 2012), thus the ligands were tested in corresponding concentrations in the IP-One assay. Also, they were tested in 10 fold higher and 10 fold lower concentrations.

A significant increase in IP₁ concentration was seen in mGPRC6A-CHO when testing both L-Orn and Ca²⁺, and Ca²⁺ also triggered a minor, but statistically significant response in Flp-In-CHO cells. Neither testosterone nor osteocalcin induced IP₁ accumulation in any of the concentrations tested (Fig. 3A). Given that two other groups have previously reported osteocalcin to be a GPRC6A agonist, we also tested this ligand in our previously published assay where mGPRC6A is co-transfected with the mutated G₉(G66D) protein and receptor activation is measured as generation of IP₁-3 by using scintillation proximity assay beads (Christiansen et al., 2007). In addition, osteocalcin was tested in the presence of ~EC₂₀ of L-Orn which would allow detection of both agonism and positive allosteric modulation. However, osteocalcin was also inactive in these settings (Fig. 3B).
The mGPRC6A-CHO cell line was shown to retain a stable, functional L-Orn response in the IP-One assay for at least 30 passages. In parallel, we also generated a stably expressing mGPRC6A-HEK293 cell line using the Flp-In system. However, this cell line was proven to lose the L-Orn-mediated IP-One response after only a few passages, and thus was not characterized further (data not shown).

Concentration-response curves of L-Orn established a potency in mGPRC6A-CHO (Fig. 4A) of EC₅₀ = 41.0 µM (pEC₅₀ ± SEM = 4.40 ± 0.02, n = 32). This potency is comparable with a previously published EC₅₀ value of L-Orn at mGPRC6A transiently co-expressed with G_q(G66D) in tsA201 cells; EC₅₀ = 63.6 µM (Christiansen et al., 2007). The lack of L-Orn response in the host cell line Flp-In-CHO strongly indicates that the response in mGPRC6A-CHO is in fact mediated by the GPRC6A receptor. This is further confirmed by the complete inhibition of L-Orn -induced IP₁ accumulation by a GPRC6A-selective antagonist, compound 1 (Gloriam et al., 2011) (Fig. 4A). This antagonist has proven to be selective towards GPRC6A when tested against a panel of receptors, including the homologous calcium-sensing receptor (Gloriam et al., 2011).

Ca²⁺-mediated IP₁ accumulation was also measured with and without the antagonist present (Fig. 4D), which showed that compound 1 completely inhibited the Ca²⁺-induced IP₁ production in mGPRC6A-CHO, whereas the minor Ca²⁺ response in Flp-In-CHO was not significantly changed when compound 1 was added, thereby demonstrating that the Ca²⁺ response in mGPRC6A-CHO is mediated by GPRC6A.

Additionally, we used the specific G_q inhibitor UBO-QIC (Fujioka et al., 1988) to demonstrate that the observed IP₁ response of L-Orn was indeed mediated by G_q activation (Fig. 4B). However it is noted that the basal level of IP₁ was decreased upon incubation with UBO-QIC. A similar decrease of basal IP₁ was also observed in the Flp-In-CHO cell line upon stimulation with UBO-QIC (Fig. 4E), thus indicating constitutive activity of the
Gq pathway in CHO cells. The Ca\(^{2+}\)-mediated IP\(_1\) production in mGPRC6A-CHO cells was likewise inhibited by UBO-QIC (data not shown). To further demonstrate the involvement of the Gq signaling pathway, we used the specific PI-PLC inhibitor U73122 (Smallridge et al., 1992), which indeed inhibited the L-Orn-induced IP\(_1\) response (Fig. 4C).

The stably expressing mGPRC6A-CHO cell line was tested in the Fluo-4 NW Calcium Assay, which measures release of intracellular calcium in response to ligand stimulation. Concentrations of L-Orn ranging from 30 µM to 5 mM were tested for their ability to increase the level of intracellular calcium, however no responses were seen at any of the concentrations tested. 1 mM ATP was included as a positive control (Fig. 2C). These data indicate that the Fluo-4 Calcium assay is not a sufficiently sensitive method for detection of mGPRC6A signaling in the mGPRC6A-CHO cell line. Thus, the IP-One assay seems to be more sensitive than previously employed methods, as it enables measurements of robust responses by direct Gq coupling.

To further elucidate the sensitivity of the IP-One assay, we tested our previously published transient expression system, in which tsA201 cells were co-transfected with mGPRC6A and a mutated Gq\((G66D)\) protein. First, the receptor surface expression was determined in the different cellular expression systems by ELISA. Comparable levels of mGPRC6A surface expression were seen between the stable CHO expression system and tsA201 cells transiently expressing mGPRC6A alone. However, when co-expressing Gq\((G66D)\), the surface expression of mGPRC6A was decreased approximately 40 % (Fig. 2B). In parallel, the functional response of mGPRC6A was tested in the stable and transient expression systems using the IP-One assay. 1 mM L-Orn induced a 1.9 fold increase in IP\(_1\) levels in the stably expressing mGPRC6A-CHO cell line. Despite a comparable level of surface expression in mGPRC6A-tsA201, 1 mM L-Orn induced only a minor increase in IP\(_1\). However, with Gq\((G66D)\) co-expressed it was possible to achieve a
1.5 fold increase in IP₁ upon stimulation with L-Orn (Fig. 2D). Thus, the signal-enhancing $G_q^{(G66D)}$ protein seems necessary in order to achieve a markedly functional response in the IP-One assay when using a transient expression system. The functional response is lower than in the stable mGPRC6A-CHO cell line, but the level of receptor surface expression is accordingly lower in the transiently expressing tsA201 cells. Collectively, the results indicate that the IP-One assay is not sufficiently sensitive to enable direct measurements of functional responses in the transiently expression system without having $G_q^{(G66D)}$ present.

$G_s$ and $G_i$ signaling

Both $G_s$ and $G_i$ proteins affect the enzymatic activity of adenylate cyclase, which is responsible for the conversion of ATP into the second messenger cAMP. $G_s$ proteins stimulate adenylate cyclase whereas the $G_i$ protein inhibits the enzyme resulting in increased and decreased levels of cAMP, respectively (Kristiansen, 2004; Luttrell, 2008). In the HTRF® cAMP dynamic 2 assay, activation of the $G_s$ and $G_i$ pathways are assessed by measuring the level of cAMP (Degorce et al., 2009). The four ligands (L-Orn, Ca²⁺, testosterone and osteocalcin) were tested in the same concentration as in the IP-One assay, except that Ca²⁺ was tested at both 5 mM and 10 mM concentrations.

Activation of the $G_s$ signaling pathway was accounted for by measuring whether any of the applied ligands induced cAMP production. Forskolin and ATP were used as positive controls, as they triggered significant increases in cAMP in both mGPRC6A-CHO and Flp-In-CHO. None of the other ligands induced any cAMP production, indicating no $G_s$ coupling of GPRC6A when stably expressed in CHO cells (Fig. 5A).

When testing for $G_i$ activity adenylate cyclase is preactivated with forskolin to generate basal levels of cAMP which allows inhibition to be measured upon receptor
activation (Degorce et al., 2009). 5 mM and 10 mM Ca$^{2+}$ significantly inhibited the cAMP level in both cell lines (Fig. 5B), and other Ca$^{2+}$ concentrations also triggered equal responses in both mGPRC6A-CHO and Flp-In-CHO (data not shown). Thus the Ca$^{2+}$-induced inhibition of cAMP is a non-specific effect. None of the other ligands triggered decreased levels of cAMP, thereby indicating that GPRC6A is not a Gi coupled receptor in the CHO cell line.

As L-Orn induces a significant concentration-dependent response in the G$_{q}$ pathway, different concentrations ranging from 100 µM to 10 mM have been tested in the cAMP assay. However, none of the tested L-Orn concentrations induced any response in the G$_{s}$ or in the G$_{i}$ signaling pathway (data not shown).

Activation of the ERK pathway

Many GPCRs are also capable of initiating the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway, which is involved in regulation of cell proliferation through modulation of gene transcription. Two of the most important kinases are ERK1 and ERK2, which are activated upon phosphorylation of specific serine and threonine residues (P-ERK1/2). Both G protein-dependent and -independent pathways such as the β-arrestin pathway can trigger ERK activation (Luttrell, 2008). The four ligands (L-Orn, Ca$^{2+}$, testosterone and osteocalcin) were tested for their ability to activate ERK in the mGPRC6A-CHO and Flp-In-CHO cell lines by using the western blot technique.

10 mM L-Orn was shown to induce increased levels of P-ERK in the mGPRC6A-CHO cells but not in the Flp-In-CHO cells. The level of T-ERK was similar in all samples. Quantification of the western blots proved the L-Orn response to be significant only in mGPRC6A-CHO and not in Flp-In-CHO (Fig. 6A). At 1 mM L-Orn, no activation
of ERK was seen in mGPRC6A-CHO (Fig. 6B). Collectively, these results show that L-Orn triggers GPRC6A-mediated ERK activation, which is consistent with the Gq activation seen in mGPRC6A-CHO upon L-Orn stimulation, although higher concentrations of L-Orn seem to be needed in order to achieve a detectable response in the ERK pathway.

10 mM Ca2+ triggered increased P-ERK levels in both mGPRC6A-CHO and Flp-In-CHO cells, and quantification of the responses showed significant activation of ERK in both cell lines (Fig. 6C). 3 mM and 5 mM Ca2+ also induced equal ERK activation in mGPRC6A-CHO and Flp-In-CHO cells (data not shown), thus the Ca2+-mediated ERK phosphorylation at these three concentrations is unrelated to GPRC6A. Different concentrations of both testosterone and osteocalcin were also tested for their ability to phosphorylate ERK, however neither of the concentrations triggered any increase in the level of P-ERK in mGPRC6A-CHO. 10 mM Ca2+ was included as a positive control. Quantification of the 80 nM testosterone and 0.01 µM osteocalcin bands further confirmed that no increase in P-ERK was seen (Fig 6D and 6E). Thus testosterone and osteocalcin do not activate the ERK pathway in the mGPRC6A-CHO cell line.
Discussion

Conflicting data regarding the signaling of GPRC6A have been reported, thus the physiological relevant ligands and signaling pathways of the receptor are still elusive. In the present study we have generated a CHO cell line that stably expresses the mouse GPRC6A receptor. This cell line was used for systematically testing of the different intracellular signaling pathways upon stimulation with previously reported GPRC6A-agonists (L-Orn, Ca^{2+}, testosterone and osteocalcin) in order to delineate the signaling properties of the GPRC6A receptor.

L-Orn was shown to trigger a GPRC6A-mediated response in mGPRC6A-CHO cells when testing the G_q signaling pathway as evidenced by IP_1 generation. This is in accordance with previously published data showing that GPRC6A is a promiscuous L-α-amino acid-sensing receptor with a preference for basic amino acids activating an endogenous calcium-sensitive chloride channel in *Xenopus* oocytes (Kuang et al., 2005; Wellendorph et al., 2005). Previously, a 2 x 2 hours washing protocol and a signal-enhancing mutated G_q(G66D) protein construct have been a requisite for achieving responses through the G_q pathway in mammalian cell lines (Christiansen et al., 2007; Wellendorph et al., 2007), however this is not a necessity in the current setup. To our knowledge, this is the first time that robust G_q protein activation has been demonstrated for the GPRC6A receptor without using techniques for improving signaling or assays that does not measure G protein pathways directly.

When testing the mGPRC6A-CHO cell line in the Fluo-4 NW Calcium assay, no functional response could be detected, contrary to the IP-One assay, which therefore seems to be a more sensitive method. In our previously published GPRC6A setup it was only possible to measure responses when co-expressing the signal-enhancing G_q(G66D) protein. When applying this expression system in our current IP-One setup, only a minor
response could be detected when expressing mGPRC6A alone. Thus, despite using the more sensitive IP-One assay, this method is still not sufficiently effective to enable robust functional measurements in transient expression systems without using the signal-enhancing Gq(G66D) protein. Conclusively, our ability to characterize mGPRC6A signaling through coupling to endogenous G proteins arises from a combination of using the highly sensitive HTRF® technology and our stably expressing CHO cell line.

Divalent cations have been claimed to work as either direct agonists at the GPRC6A receptor (Pi et al., 2005) or co-agonists with amino acids (Christiansen et al., 2007). In the present study, Ca\(^{2+}\) behaves as an agonist at mGPRC6A when measuring activation of the Gq pathway. In the HTRF® IP-One assay, cells in high suspension density are subjected to ligand stimulation for 1 hour during which the level of IP\(_1\) accumulates. It is well known that L-amino acids can be released from cells during such an incubation, which can lead to indirect activation of amino acid receptors (Desai et al., 1995; Thomsen et al., 1994). It is thus plausible that the observed Ca\(^{2+}\) response is caused by positive modulation of low-level amino acid levels that build up during the ligand incubation. However, we cannot rule out that Ca\(^{2+}\) is directly activating the mGPRC6A receptor, as it is not possible to confirm the presence/absence of amino acid build-up in the buffer with the present assay setup.

L-Orn cannot necessarily be classified as an agonist either, as the IP-One experiments were conducted in a buffer containing 1 mM Ca\(^{2+}\) and 1 mM Mg\(^{2+}\). It seems possible that both basic amino acids and divalent cations are equally required in order to obtain robust GPRC6A-responses, and in that regard the ligands may instead be classified as co-agonists. This is consistent with the fact that both amino acids and cations will be present in concentrations capable of activating GPRC6A under most physiological situations. Thus, our results confirm previous results that basic L-\(\alpha\)-amino acids and
divalent cations are ligands for the mGPRC6A receptor (Christiansen et al., 2007; Kuang et al., 2005; Pi et al., 2005; Wellendorph et al., 2005).

Through the use of the specific G<sub>q</sub> inhibitor UBO-QIC and the PI-PLC inhibitor U73122 we unequivocally confirm that the L-Orn and Ca<sup>2+</sup>-induced IP<sub>1</sub> production is specifically mediated by the G<sub>q</sub> signaling pathway in this system.

GPRC6A has previously been reported to activate the downstream signaling molecules SRE and ERK via G<sub>i</sub> (Pi et al., 2005; Pi et al., 2010) and to increase cAMP levels via G<sub>s</sub> in HEK293 cells (Dreaden et al., 2012). Activation of the G<sub>i</sub> pathway by osteocalcin in Leydig TM3 cells has also been suggested, albeit not directly demonstrated to be mediated by GPRC6A (Oury et al., 2011). In the present study, we show that mGPRC6A neither couples to G<sub>s</sub> nor G<sub>i</sub> when stably expressed in the CHO cell line. CHO cells have been used extensively to study G<sub>s</sub> and G<sub>i</sub> coupled GPCRs for two decades (Horie et al., 1995; Jones et al., 1991; Schroder et al., 2010) and it is thus not due to lack of components of these signaling pathways that we fail to measure G<sub>s</sub> or G<sub>i</sub> mediated responses by any of the four ligand classes.

The Quarles group has previously demonstrated activation of the P-ERK pathway upon stimulation with basic amino acids, divalent cations, testosterone and osteocalcin (Pi et al., 2005; Pi et al., 2010; Pi et al., 2011) albeit the latter ligand activity was not confirmed in Leydig TM3 cells by the Karsenty group (Oury et al., 2011). Here we have shown that L-Orn triggers mGPRC6A-specific ERK phosphorylation. These results were expected, since ERK phosphorylation is a downstream effect of several signaling pathways including the G<sub>q</sub> pathway (Luttrell, 2008), which is activated by L-Orn in mGPRC6A-CHO cells. In analogy, activation of G<sub>q</sub> by the homologous calcium-sensing receptor also leads to ERK1/2 phosphorylation (Kifor et al., 2001; Thomsen et al., 2012). However, it is noteworthy that high L-Orn concentrations were needed to detect responses.
in western blotting compared to the amount of L-Orn needed to give maximum effect in the IP-One assay. Thus the potency of L-Orn seems to be lower in the ERK pathway than in the G_q pathway, and consequently it has not been possible to determine an exact EC_{50} value of L-Orn in the ERK signaling pathway.

Ca^{2+}-induced ligand activation of mGPRC6A has also been shown in the G_q signaling pathway, thus it was expected that Ca^{2+} likewise would activate ERK in mGPRC6A-CHO. However, the response in the ERK pathway turned out to be non-specific. These non-specific effects may be triggered by unknown mechanisms e.g. G protein-independent effects in the CHO cell lines, which potentially mask the G_q-mediated Ca^{2+} response in the ERK pathway.

Contrary to previous reports, neither testosterone nor osteocalcin elicited any response in the ERK pathway or any of the other tested G protein signaling pathways. Thus, the role of testosterone and osteocalcin as GPRC6A agonists, as stated previously by others (Oury et al., 2011; Pi et al., 2005; Pi et al., 2010; Pi et al., 2012; Pi et al., 2011), cannot be supported by the present study. Due to the large interest in osteocalcin as a GPRC6A agonist, we also tested this ligand in a combined agonist/positive allosteric modulator mode in tsA cells (a transformed HEK293 cell line (Chahine et al., 1994)) where we also failed to detect receptor activity.

Our results have not provided us with an explanation for the contradictory data regarding GPRC6A signaling. It is likely that inconsistencies between reports on GPRC6A pharmacology is accounted for by the choice of cell expressing system, cell growth conditions and/or the applied methods. Different cell lines might display different levels of receptor density, G proteins and other signaling molecules, which can lead to cell-type specific agonist signaling profiles (Kenakin, 2011). The results can also be influenced by the messenger molecule being measured, which may limit signal
amplification and sensitivity. Others have measured ERK phosphorylation and SRE activation (Pi et al., 2005), that are both downstream to many signaling pathways and which might consequently give rise to increased interference from non-specific pathways. In comparison more upstream signaling pathway components have been measured in the current work, which, along with the application of a GPRC6A selective antagonist and the specific inhibitors UBO-QIC and U73122, convincingly validate the specificity of the observed signaling.

In conclusion, we provide clear evidence that the mGPRC6A receptor is G_\text{q}\text{-} coupled in response to L-\alpha-amino acids and divalent cations. In addition, we report the successful development of a stable mGPRC6A-CHO cell line and its usefulness for measuring G_\text{q}\text{-}mediated responses in the robust and sensitive HTRF® IP-One assay. Notably, this precludes the previously employed 'tricks' of using chimeric receptors, mutated/chimeric G proteins and/or extensive pre-washing of the cells, which allows direct and unbiased measurement of receptor activation. The assay enables screening for novel and more selective GPRC6A ligands that may serve as pharmacological tool compounds, which are highly required to study the physiological function and therapeutic potential of the receptor. We were unable to confirm previously reported activation of G_\text{i} and G_\text{s} pathways by GPRC6A, and the agonistic activity of testosterone and osteocalcin. Further studies are thus warranted to fully elucidate which ligand classes and G protein pathways are utilized by the GPRC6A receptor.
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Conducted experiments: Jacobsen, Nørskov-Lauritsen, Thomsen, Smajilovic, Wellendorph

Contributed new reagents or analytic tools: Thomsen, Bhatia

Performed data analysis: Jacobsen, Nørskov-Lauritsen, Thomsen, Smajilovic, Wellendorph

Wrote or contributed to the writing of the manuscript: Jacobsen, Nørskov-Lauritsen, Thomsen, Smajilovic, Wellendorph, Larsson, Lehmann, Bhatia, Bräuner-Osborne
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Footnotes:

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1 These authors contributed equally to this work.
Figure legends:

Fig. 1. Structures of previously reported GPRC6A ligands. (A) the amino acid L-ornithine, (B) the steroid testosterone, (C) the peptid osteocalcin (porcine) (adapted from Protein Data Bank ID PDB1Q8H), (D) the GPRC6A-selective antagonist compound 1 and (E) the amino acid sequence of bovine osteocalcin (bOC) and the differences to porcine osteocalcin (pOC).

Fig. 2. Evaluation of cell lines stably and transiently expressing GPRC6A. (A) RT-PCR performed on total RNA extracted from the mGPRC6A-CHO and Flp-In-CHO cell lines. Water was used as a negative control. Bands were detected by using intron-spanning primers specific for (top) mGPRC6A and (bottom) the ubiquitously, endogenously expressed GAPDH. Only the shown bands were detected. (B) ELISA to detect cell-surface expression of c-myc tagged GPRC6A in the mGPRC6A-CHO, hGPRC6A-CHO and Flp-In-CHO cell lines and in tsA201 cells transiently expressing mGPRC6A alone, mGPRC6A and G_q(G66D) or empty vector alone. Surface-expressed receptors were detected by using c-myc mouse monoclonal antibody and goat anti-mouse IgG HRP conjugate. Luminescence was measured after addition of a chemiluminescent HRP substrate. Data are shown as means ± SEM of at least three independent experiments performed in triplicates. (C) Measurements of intracellular Ca^{2+} release. Responses to L-Orn and ATP were measured in mGPRC6A-CHO by using the Fluo-4 NW Calcium Assay Kit. Data are ΔRFU (peak fluorescence units after agonist addition subtracted fluorescence before agonist addition) and are shown as means ± SEM of two independent experiments performed in triplicates. (D) Measurements of IP_1 accumulation in response to 1 mM L-Orn as assessed by the HTRF® IP-One assay. Responses were measured in the mGPRC6A-CHO, hGPRC6A-CHO and Flp-In-CHO cell lines and in tsA201 cells.
transiently expressing mGPRC6A alone, mGPRC6A and G_{q(G66D)} or empty vector alone. Data are means ± SEM of at least three independent experiments performed in triplicates.

**Fig. 3.** Activity testing in the G_{q} signaling pathway. (A) Measurements of IP_{1} accumulation as a result of G_{q} activation. Responses to the ligands L-Orn, Ca^{2+}, testosterone and osteocalcin were measured in mGPRC6A-CHO and Flp-In-CHO cells, respectively, by means of the HTRF®, IP-One assay. Data are means ± SEM of four independent experiments performed in triplicates. Significant differences from basal (ligand buffer) were calculated by performing a one-way ANOVA followed by Dunnett’s post-test (* P<0.05, *** P<0.001). (B) Activity testing of three different commercially available recombinant forms of osteocalcin by using the IP turnover assay. Osteocalcin were tested in the presence of EC_{25} of L-Orn in tsA201 cells co-transfected with mGPRC6A and G_{q(G66D)}. Results are shown as CPM and are means ± SD of a single representative experiment performed in duplicates. An additional experiment gave similar results. Significant differences from basal (ligand buffer) were calculated by performing a one-way ANOVA followed by Dunnett’s post-test (not significant, P > 0.05).

**Fig. 4.** Validation of mGPRC6A pharmacology as mediated by the G_{q} signaling pathway. (A), (B) and (C), concentration-response curve of L-Orn in mGPRC6A-CHO cells in the presence and absence of (A) the GPRC6A-selective antagonist compound 1, (B) the specific G_{q} inhibitor UBO-QIC or (C) the specific PI-PLC inhibitor U73122. Responses are shown as IP_{1} accumulation as measured by the HTRF® IP-One assay. Data are means ± SD of a single representative experiment performed in triplicates. Two additional experiments gave similar results. (D) Ca^{2+}-induced IP_{1} production in mGPRC6A-CHO and Flp-In-CHO cells in the presence and absence of compound 1. Data are normalized to
the basal level of IP<sub>1</sub> in ligand buffer and are shown as means ± SEM of three independent experiments performed in triplicates. Statistical comparison was performed within each group (each cell line) by using an unpaired student’s t-test (* P<0.05). (E) the basal level of IP<sub>1</sub> in ligand buffer in the Flp-In-CHO cell line in the presence and absence of 1 µM UBO-QIC. Data are means ± SEM of three independent experiments performed in triplicates. Statistical comparison was performed by using an unpaired student’s t-test (**) P<0.01).

Fig. 5. Measurements of (A) cAMP accumulation as a result of G<sub>s</sub> activation and (B) cAMP inhibition as a result of G<sub>i</sub> activation. Responses to ATP, forskolin, L-Orn, Ca<sup>2+</sup>, testosterone and osteocalcin were measured in mGPRC6A-CHO and Flp-In-CHO cells, respectively, by using the HTRF<sup>®</sup> cAMP assay. (A) Data are means ± SEM of three independent experiments performed in triplicates. Significant differences from basal (ligand buffer) were determined by performing a one-way ANOVA followed by Dunnett’s post-test (*** P<0.001). (B) Data are normalized to the cAMP production in response to 20 µM forskolin and are shown as means ± SEM of five independent experiments performed in triplicates. Significant differences from basal (20 µM forskolin) were determined by performing a one-way ANOVA followed by Dunnett’s post-test (** P<0.01, *** P<0.001).

Fig. 6. Measurements of ERK activation in mGPRC6A-CHO and Flp-In-CHO cells upon stimulation with (A) 10 mM L-Orn, (B) 1 mM L-Orn, (C) 10 mM Ca<sup>2+</sup>, (D) 80 nM testosterone and (E) 0.01 µM osteocalcin. ERK activation was assessed by using antibodies against phosphorylated ERK (P-ERK) and total ERK (T-ERK) after western
blotting. Quantification of the western blots has been performed by using the ImageJ program (http://rsbweb.nih.gov/ij/download.html) and by normalizing the P-ERK response to the corresponding T-ERK response. Furthermore, the data have been normalized to the basal level of P-ERK/T-ERK in ligand buffer and are shown as means ± SEM of (A, B, C) three or (D, E) two independent experiments. Statistical comparison between basal (ligand buffer) and the ligand has been performed within each group (each cell line) by using an unpaired student’s t-test (** P<0.01, *** P<0.001).
Figure 1

A

\[
\text{H}_2\text{N}-\text{CH}_{2}-\text{CH}_{2}-\text{CH}_{2}-\text{CH}_{2}-\text{CO}_{2}\text{H}
\]

B

\[
\text{CH}_3-\text{C}_\text{H}_2-\text{C}_\text{H}_2-\text{C}_\text{H}_2-\text{OH}
\]

C

D

\[
\text{CH}_3-\text{C}_\text{H}_2-\text{C}_\text{H}_2-\text{C}_\text{H}_2-\text{OC}_6\text{H}_5
\]

E

\[
\text{NH}_2-\text{YLDHWLGPAPAPYDPDLPEPKREVCELNPDCDELADHGIFQEAYRFFYGPV-COOH}
\]

pOC: G R A
Figure 2

Panel A: Gel electrophoresis image showing bands for mGPRC6A and GAPDH.

Panel B: Bar graph showing chemiluminescence values for different groups.

Panel C: Graph showing ΔRFU levels with varying L-Orn concentrations.

Panel D: Graph showing ratio of [IP1]/[IP1]_basal for different groups.
Figure 3

A  

mGPRC6A-CHO

B  

mGPRC6A+Gq(G66D)tsA201

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| [P] \_nM               |               |               |              |             |
| 500 μM                 | 1000 μM       | 1500 μM       | ***          |             |

Figure 3 B: mGPRC6A+Gq(G66D)tsA201

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<th>1 μM</th>
<th>25 μM L-Orn</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>+0.1 μM</th>
<th>+1 μM</th>
<th>+0.1 μM</th>
<th>+1 μM</th>
<th></th>
</tr>
</thead>
</table>

| CPM                    |               |               |              |             |
| 500 μM                 | 1000 μM       | 1200 μM       | ***          |             |

Figure 3 B: mGPRC6A+Gq(G66D)tsA201

<table>
<thead>
<tr>
<th>Ligand buffer (500 μM)</th>
<th>L-Orn (500 μM)</th>
<th>Ca^{2+} (5 mM)</th>
<th>Testosterone</th>
<th>Osteocalcin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<thead>
<tr>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
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<th>+0.1 μM</th>
<th>+1 μM</th>
<th>+0.1 μM</th>
<th>+1 μM</th>
<th></th>
</tr>
</thead>
</table>

| CPM                    |               |               |              |             |
| 500 μM                 | 1000 μM       | 1200 μM       | ***          |             |
Figure 6

A

B

C

D

E

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