Author correction

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We apologise for an error in the second paragraph of the introduction, which read influx of K^+ rather than efflux of K^+. The correct sentence should read: The increase in intracellular Ca^{2+} then triggers efflux of K^+ through a Ca^{2+}-activated K^+ channel, leading to hyperpolarisation, which is an absolute requirement for entry into mitosis (Kayser et al., 1998).

The error appeared in both the print and online versions of this article.

The neuropeptide head activator is a high-affinity ligand for the orphan G-protein-coupled receptor GPR37

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Summary

The neuropeptide head activator (HA) is a mitogen for mammalian cell lines of neuronal or neuroendocrine origin. HA signalling is mediated by a G-protein-coupled receptor (GPCR). Orphan GPCRs with homology to peptide receptors were screened for HA interaction. Electrophysiological recordings in frog oocytes and in mammalian cell lines as well as Ca^{2+} mobilisation assays revealed nanomolar affinities of HA to GPR37. HA signal transduction through GPR37 was mediated by an inhibitory G protein and required Ca^{2+} influx through a channel of the transient receptor potential (TRP) family. It also required activation of Ca^{2+}-dependent calmodulin kinase and phosphoinositide 3-kinase. Respective inhibitors blocked HA signalling and HA-induced mitosis in GPR37-expressing cells. HA treatment resulted in internalisation of GPR37. Overexpression of GPR37 led to aggregate formation, retention of the receptor in the cytoplasm and low survival rates of transfected cells, confirming the notion that misfolded GPR37 contributes to cell death, as observed in Parkinson's disease.

Key words: G-protein-coupled receptor, GPR37, Head activator, Pael receptor, Parkinson, Signal transduction

Introduction

The undecapeptide head activator (HA) was originally isolated and characterised from hydra, where it mediates head-specific growth and differentiation processes, hence its name. In hydra, HA is produced by nerve cells and is stored in neurosecretory granules, from which it is released to initiate head regeneration and budding, and to maintain the normal head-to-foot morphology of hydra. At the cellular level, HA promotes proliferation of all cell types of hydra by acting as mitogen in the G2-mitosis transition; as for early mammalian development, this transition is the most important checkpoint to control cell-cycle progression. At higher concentrations, HA acts on the determination of stem cells to head-specific fates (Schaller et al., 1996).

HA was isolated with identical sequence from mammalian brain and intestine (Bodenmuller and Schaller, 1981). In adult mammals, HA enhances neurite outgrowth and is neuroprotective. HA is present during early mammalian development and is expressed in cells of the nervous and neuroendocrine system. Like in hydra, HA stimulates entry into mitosis and proliferation of cell lines derived from such origins. The signalling cascade from HA to mitosis includes activation of an inhibitory G protein and requires Ca^{2+} influx, downregulation of adenylyl cyclase and hyperpolarisation of the membrane potential (Kayser et al., 1998; Niemann and Schaller, 1996; Ulrich et al., 1996). For Ca^{2+} influx, a transient receptor potential (TRP)-like channel is responsible, which can be regulated by growth factors, such as insulin growth factor I (IGF-I) and platelet-derived growth factor (PDGF) (Kanzaki et al., 1999), and by HA (Boels et al., 2001). The increase in intracellular Ca^{2+} then triggers influx of K^+ through a Ca^{2+}-activated K^+ channel, leading to hyperpolarisation, which is an absolute requirement for entry into mitosis (Kayser et al., 1998).

In the search for receptors mediating the action of HA on stimulating mitosis in mammalian cells, we concentrated on orphan G-protein-coupled receptors (GPCRs) reacting with small peptides as ligands. GPCRs are the largest family of cell-surface receptors that mediate transduction of signals from the extracellular environment to intracellular effectors. They contain seven transmembrane domains and are activated by ligands of extremely different molecular origins and sizes including light, ions, metabolic intermediates, amino acids, nucleotides, lipids, peptides and proteins. These ligands primarily interact with the extracellular domains, but in part also with transmembrane regions of GPCRs. The classification of GPCRs into subfamilies is primarily based on their homology within the heptahelical structure (Frederiksson et al., 2003), but also on extracellular domains, and has been used to predict ligands for orphan receptors (Boels and Schaller, 2003; Ignatov et al., 2003a; Ignatov et al., 2003b). To find a receptor for HA, we concentrated on GPCR subfamilies reacting with small peptides as ligands.

Several orphan receptors failed to show interactions with HA, including GPR6 and GPR12, for which we found lysophospholipids as cognate ligands (Ignatov et al., 2003a;

Ignatov et al., 2003b). HA had no effect on GPR99, GPR100, GalRL, GPR1, GPR7, GPR8, GPR19, GPR75 and SALPR, just to name a few. Of special interest was a sub-branch of GPCRs that regulate cellular proliferation, namely the endothelin, bombesin and neuromedin receptors. Two orphan receptors are part of this group: GPR37 and GPR37L1 (Marazziti et al., 2001). We focused our interest on GPR37 because of its prominent expression in neurons of the brain compared with a more glial location of GPR37L1 (Marazziti et al., 1997; Zeng et al., 1997). GPR37 has also been isolated and characterised as a substrate for the ubiquitin ligase parkin, hence its alternative name – parkin-associated endothelin-like receptor (Pael R) (Imai et al., 2001). GPR37 was shown to fold improperly in the absence of parkin, and its aggregation to insoluble complexes results in endoplasmic reticulum stress (Imai et al., 2001; Imai et al., 2003). This leads to preferential loss of dopaminergic neurons in the substantia nigra and contributes to neurodegeneration in Parkinson's disease (Yang et al., 2003). Accumulation of GPR37 in Lewy bodies in the brain of patients with Parkinson's disease supports this notion (Murakami et al., 2004).

To study a possible interaction of HA with GPR37, various assay systems were used that allow detection, directly or indirectly, of ligand-receptor interactions. In this paper, we present evidence that HA is a high-affinity ligand for GPR37.

Results

HA stimulates internalisation of GPR37 in COS-7 cells

We tried to express GPR37 heterologously in Chinese hamster ovary (CHO-K1) cells, in human embryonic kidney (HEK-293) cells and in green monkey kidney (COS-7) cells. Transient transfection efficiencies in HEK-293 and CHO-K1 cells were far below 5%, and cells expressing GPR37 looked sick and decreased in number at 48 hours compared with 24 hours after transfection. Transfection efficiencies in COS-7 cells were better and reached levels in the range of 15-30% (Fig. 1A). COS-7 cells were therefore suitable for experiments with individual, transfected cells. There was no difference in expression levels between GPR37 with (Fig. 1B) and without (Fig. 1C) FLAG tag at the C-terminus. This indicated that the tag did not interfere with GPR37 protein biosynthesis and localisation. GPR37 immunoreactivity was visible in the cytoplasm of transfected COS-7 cells, but also extended to cell protrusions, hinting at cell-surface expression (Fig. 1B,C). Cell-surface expression was confirmed by treating living cells before fixation with a monoclonal antibody against GPR37 (Fig. 1D) that reacts with extracellular epitopes of GPR37 (Imai et al., 2001).

HA treatment of COS-7 cells transiently transfected with GPR37-FLAG led to internalisation of the receptor. This was visible as disappearance of the GPR37-FLAG immunoreactivity from the protrusions after 10 minutes (compare Fig. 1E and F), and as translocation into the cytoplasm after 20 minutes (Fig. 1G). Protrusions started to show FLAG staining again after 30-60 minutes (Fig. 1H,I).

GPR37 aggregation is prevented by stable inducible expression in HEK-293 cells

Transient expression of GPR37 led in all cell lines assayed to aggregation of complexes with apparent molecular masses of $\geqslant$250 kDa (Fig. 2A). Surface biotinylation showed that only the monomeric receptor appeared at the outer cell membrane (Fig. 2B), indicating that most of the overproduced GPR37 was not properly folded, stayed in the cytoplasm and was probably

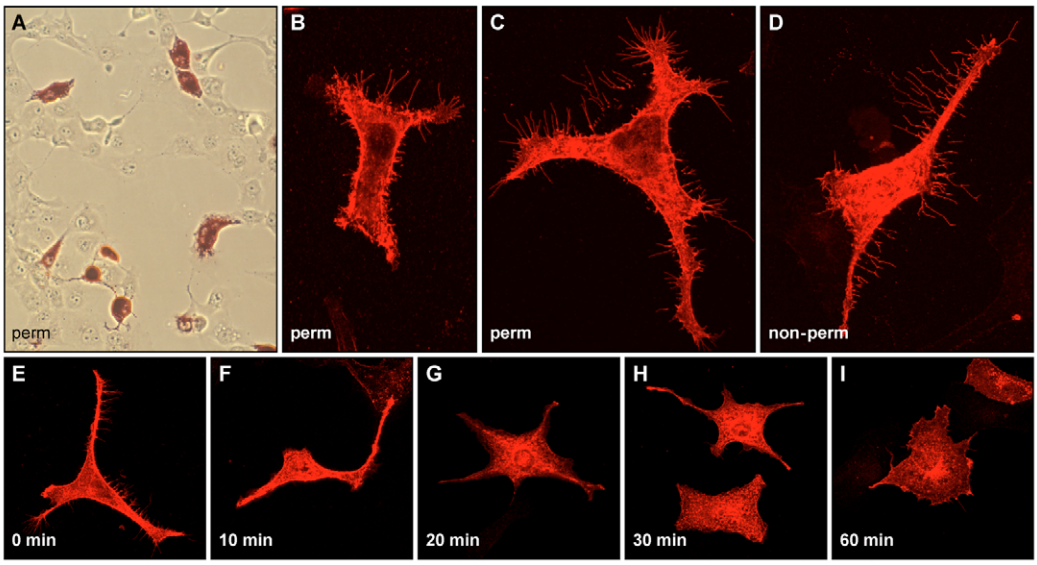

Fig. 1. GPR37 is expressed at the cell surface of COS-7 cells and internalises after HA treatment. (A-I) COS-7 cells were transfected with GPR37 with (B,D-I) or without (A,C) FLAG tag, immunostained with anti-GPR37 antibody (A,C,D) or with anti-FLAG antibody (B,E-I) and visualised with alkaline phosphatase-coupled secondary antibodies for light microscopy (A) or with Cy3-coupled antibodies for confocal analysis (B-I). Cells were permeabilised (perm) by fixation with 1% acetic acid in ethanol and by washing with Triton X-100, except in D, where living cells were incubated with the primary antibody before fixation (non-perm) to show surface staining. (E-I) COS-7 cells 48 hours after transfection with GPR37-FLAG were treated at 37°C with 2 nM HA for 0, 10, 20, 30 and 60 minutes, respectively, and immunostained with anti-FLAG antibody.

degraded (Imai et al., 2001). To prevent aggregation and subsequent degradation, we integrated GPR37 stably into HEK-T-REx cells with a construct that allowed induction by tetracycline (HEK-T-REx–GPR37). Incubation of cells with the tetracycline derivative doxycycline for 24 hours resulted in production predominantly of the monomeric form of GPR37 (Fig. 2C, first lane). Without doxycycline induction, GPR37 was not detectable (Fig. 2C, second lane). Confocal image analysis revealed that, after induction with doxycycline, GPR37 localised mainly to the outer cell membrane, both in permeabilised (Fig. 2D) and non-permeabilised cells (Fig. 2F). The non-induced cells showed no GPR37 immunoreactivity (Fig. 2E,G). To study internalisation, HEK-T-REx–GPR37 cells were incubated in defined medium for 24 hours with doxycycline to induce GPR37 expression. Subsequent treatment with HA for 10 minutes led to rapid internalisation of GPR37 (Fig. 2H,I). This internalisation was much faster in HEK than in COS-7 cells, probably as a result of differences in β-arrestin levels (Ménard et al., 1997).

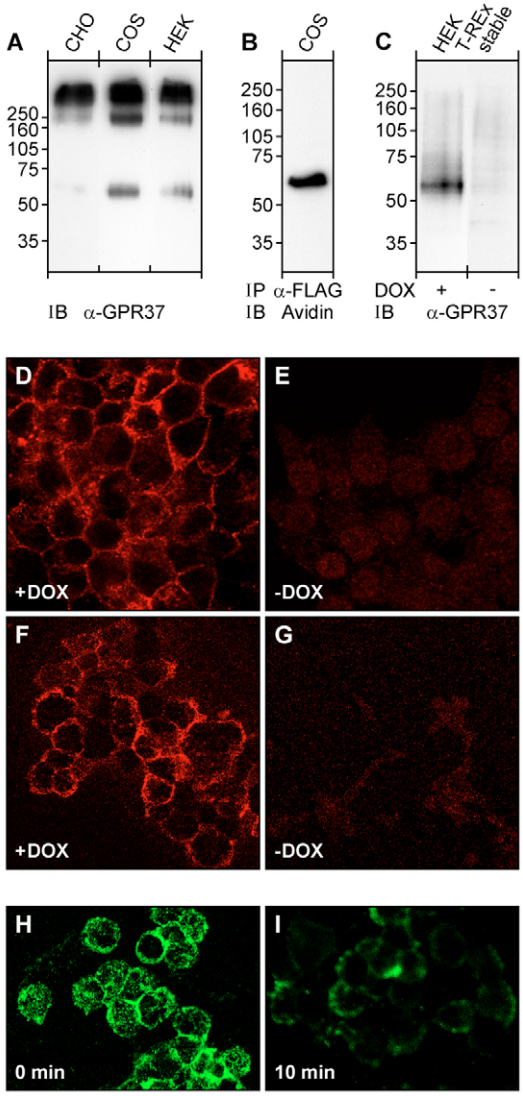

Fig. 2. Inducible, stable expression of GPR37 prevents aggregate formation. (A) CHO-K1, COS-7 and HEK-293 cells were transiently transfected with GPR37, and membrane fractions were assayed by immunoblotting (IB) with anti-GPR37 antibody (α-GPR37). (B) COS-7 cells transiently transfected with GPR37-FLAG were cell-surface biotinylated, and the solubilised membrane fraction was immunoprecipated (IP) with anti-FLAG antibody (α-FLAG) and visualised after immunoblotting with avidin. (C) GPR37 was introduced stably into the flip-in cell line HEK-T-REx, where GPR37 expression is inducible by doxycycline (DOX). Membrane fractions were subjected to western blotting with anti-GPR37 antibody (α-GPR37) with (first lane) and without (second lane) induction for 24 hours with doxycycline. (D-I) HEK-T-REx–GPR37 cells with (D,F,H,I) and without (E,G) doxycycline induction for 24 hours were immunostained with anti-GPR37(R2) antibody after permeabilisation (D,E) and with anti-GPR37 antibody without permeabilisation (F-I). (H,I) HEK-T-REx–GPR37 cells were treated with 2 nM HA for 0 and 10 minutes at 37°C, respectively, fixed with 2% formaldehyde for 10 minutes and subsequently immunostained with anti-GPR37 antibody.

HA binds to GPR37

To show direct interaction of HA with GPR37, COS-7 cells were analysed after incubation with 2 nM HA by fluorescence resonance energy transfer (FRET). Localisation of HA was detected with a HA-specific polyclonal antiserum and was visualised with a Cy2-coupled secondary antibody (green). To detect GPR37, monoclonal antibodies directed against the extracellular domain of human GPR37 were used in combination with a Cy3-coupled secondary antibody (red). Fig. 3A-D shows a typical example of FRET between HA and ectodomains of GPR37. After bleaching a discrete area in a GPR37-positive cell (Fig. 3A,B), an increase in HA fluorescence was observed (Fig. 3C,D). The difference in staining pattern is due to the fact that COS-7 cells, in addition to GPR37, express endogenous HA receptor(s) (Boels et al., 2001). The experiment was repeated several times on different days yielding similar results. On average, the calculated energy-transfer efficiencies were in the range of 19.4±4.5%, indicating the close association of GPR37 and HA. Non-transfected cells were negative, and no transfer of signal was obtained if an antibody against the FLAG tag at the C-terminus of GPR37 was used (data not shown).

For visualisation of HA binding to GPR37, a fluorescent derivative of HA was produced. For this purpose, the fluorophore Cy3B was coupled to the ϵ-amino group of Lys7 of HA. The neuroblastoma cell line NH15-CA2, which reacts with HA (Ulrich et al., 1996) and endogenously expresses GPR37 (Fig. 3E), was used as positive control. Binding of Cy3B-labelled HA to NH15-CA2 cells was observed starting from a concentration of 50 nM, with optimal binding at 150 nM, achieved after incubation for 10 minutes at 37°C (Fig. 3F). Pre-incubation with unlabelled HA for 50 minutes prevented Cy3B-HA binding (Fig. 3G). Cy3B-labelled HA did not bind to HEK-T-REx–GPR37 cells without induction of GPR37 expression by doxycycline (Fig. 3H), but reacted after induction for 24 hours with doxycycline (Fig. 3I). Pre-incubation with unlabelled HA inhibited binding (Fig. 3J), demonstrating that the two ligands compete for the same receptor and that the receptor is either occupied or, more likely, internalised after interaction with HA.

HA induces an increase in Ca^{2+} mobilisation in cells expressing GPR37

To confirm the interaction of HA with GPR37, Ca^{2+} mobilisation was measured in CHO-K1 cells stably

transfected with apoaequorin as Ca^{2+} sensor and with the promiscuous G-protein subunit Gα16 for signal enhancement. After reconstitution with the aequorin cofactor coelenterazine, agonist action was monitored as increase in bioluminescence (Stables et al., 1997). Since GPR37 was not sufficiently expressed in this cell line (CHO) by transient transfection, a stable cell line was established that, in addition to Gα16 and apoaequorin, also expressed GPR37 (CHO-GPR37). Treatment of these cells with HA dose dependently led to an increase in Ca^{2+} mobilisation with an EC_{50} value of 3.3 nM (Fig. 4A). To our surprise, an endogenous response was also observed (Fig. 4A). Northern blots were negative, but immunocytochemistry (Fig. 4B) and western blots (Fig. 4C) probed with an antiserum against the very conserved intracellular C-tail confirmed presence of GPR37 in CHO cells. The hippocampal mouse cell line HT22, expressing GPR37 endogenously, was used as a positive control (Fig. 4C). The active monomeric form of GPR37 was predominantly present both in CHO-GPR37 and HT22 cells.

HA stimulates a current increase in frog oocytes expressing GPR37

In our hands, the frog oocyte system has proven to be very reliable and robust for studying the interaction of ligands with orphan GPCRs (Ignatov et al., 2003a; Ignatov et al., 2003b). Since HA signal transduction for mitotic stimulation is coupled to an inhibitory G protein (Kayser et al., 1998; Ulrich et al., 1996), frog oocytes were injected not only with complementary RNAs (cRNAs) coding for human GPR37, but also with cRNAs coding for the G-protein-coupled inwardly rectifying K^+ channel GIRK, which is activated by βγ subunits of inhibitory G proteins (Kofuji et al., 1995). The concatemer between GIRK1 and GIRK2 (GIRK1/2) was chosen to enhance the current increase and improve the signal to noise ratio (Wischmeyer et al., 1997). Treatment with HA led to an additional increase in the basal inward current induced by changing the external bath medium to high K^+ in oocytes expressing GPR37 together with GIRK1/2 (Fig. 5A). A minute, negligible response was also obtained with medium alone (Fig. 5A). The effect of HA was concentration dependent, and a dose-response curve yielded an EC_{50} value of 5.6 nM (Fig. 5B). Since HA was diluted about twofold by addition to the oocyte bath medium, this EC_{50} value is in agreement with that obtained in the Ca^{2+}-mobilisation assay in CHO cells. Oocytes expressing GIRK1/2 without GPR37 were unresponsive to HA (Fig. 5B). Comparable dose-response curves were obtained from 30 oocytes.

HA signal transduction

One of the most prominent effects of HA is that it stimulates cells to enter mitosis (Hampe et al., 2000; Kayser et al., 1998; Ulrich et al., 1996). At the G2-mitosis transition, histone H3 is phosphorylated and is, therefore, an excellent marker for mitotic events. HEK-T-REx–GPR37 cells were incubated with and without doxycycline for 24 hours, before HA was added for 1.7 hours. Cells induced with doxycycline to express GPR37 showed an increase over uninduced cells in the percentage of mitotic cells, as visualised with the antibody against phosphorylated histone H3 (Fig. 6A). This suggested a direct role for GPR37 in mediating the action of HA as a mitogen. To monitor HA signalling mediated by GPR37, transiently transfected COS-7 cells and HEK-T-REx–GPR37 cells were subjected to electrophysiological analysis by patch clamping. Treatment of cells with HA led

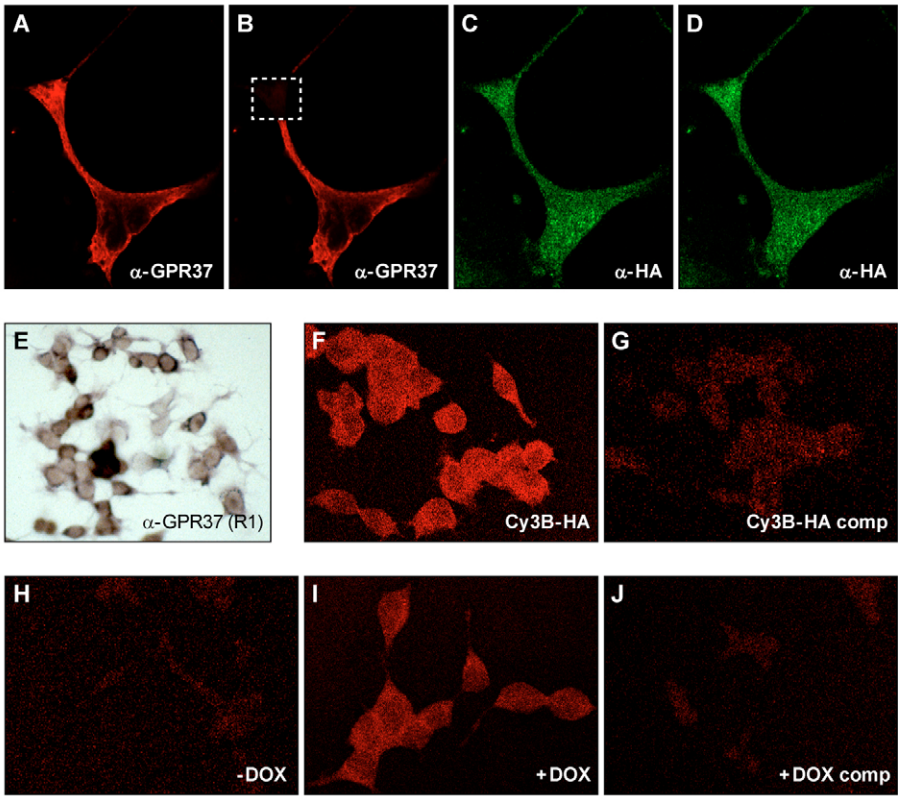

Fig. 3. HA colocalises with and binds to GPR37. (A-D) Interaction of HA and GPR37 analysed by FRET. Shown is a typical example of FRET between HA and an extracellular epitope of GPR37. COS-7 cells transiently transfected with GPR37 were treated with 2 nM HA for 20 minutes on ice to prevent internalisation, followed by incubation for 20 minute on ice with the antiserum against HA (α-HA). After fixation with 4% formaldehyde in PBS, cells were immunostained with anti-GPR37 antibody (α-GPR37). GPR37 immunoreactivity was visualised with Cy3 (A,B) and that of HA with Alexa Fluor 488 (C,D). (A) The Cy3 signal (GPR37) is shown after excitation at 568 nm. (B) A discrete area was photobleached using intense 568 nm laser. The Alexa Fluor 488 signal (HA) after excitation at 488 nm is shown before (C) and after (D) photobleaching. In this example, the Alexa Fluor 488 signal was increased by 18%. (E-G) The neuroblastoma cell line NH15-CA2 was used as a positive control to show specific Cy3B-HA binding to endogenous HA receptors. (E) NH15-CA2 cells endogenously express GPR37, as visualised with anti-GPR37(R1) antibody [α-GPR37(R1)]. (F,G) Binding is optimal at 150 nM of Cy3B-HA after incubation for 10 minutes at 37°C (Cy3B-HA) and is inhibited by pretreatment for 50 minutes at 37°C with 100 nM unlabelled, monomerised HA (Cy3B-HA comp). (H-J) HEK-T-REx–GPR37 cells bound Cy3B-HA only after GPR37 induction with doxycycline (±DOX), and binding was competed with unlabelled HA (+DOX comp).

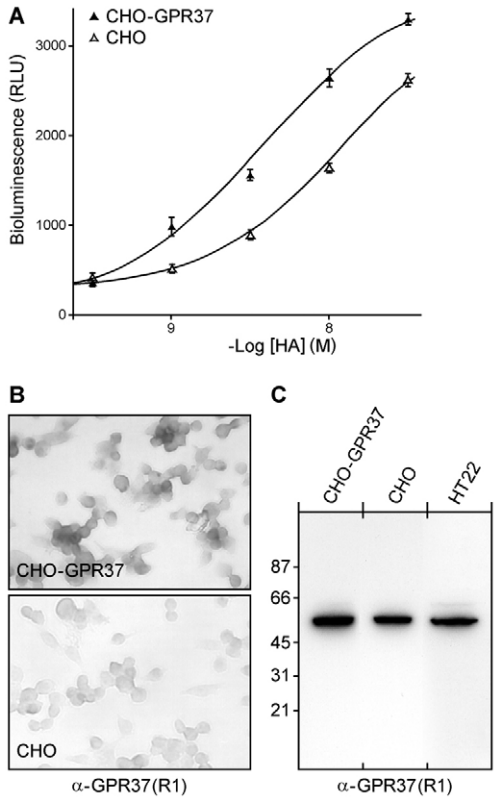

Fig. 4. HA stimulates Ca^{2+} mobilisation in CHO-K1 cells stably transfected with GPR37-FLAG, Gα16 and apoaequorin. (A) The Ca^{2+}-bioluminescence response was measured at 469 nm and is expressed in relative light units (RLU), from which the medium response was subtracted. Values are given as means ± s.d. CHO-Gα16-AEQ cells stably expressing GPR37-FLAG (CHO-GPR37) responded with an EC_{50} value of 3.3 nM; the endogenous response of CHO-Gα16-AEQ cells (CHO) resulted in an EC_{50} value of 11 nM. Data show representative results of three independent experiments. (B) CHO-GPR37 (upper panel) and CHO cells (lower panel) reacted with anti-GPR37(R1) antibody [α-GPR37(R1)], a polyclonal antiserum produced against the conserved intracellular C-tail. (C) Western blot analysis of membrane fractions confirmed an increased expression of GPR37 in transfected cells. The mouse hippocampal cell line HT22, expressing GPR37 endogenously, was used as a positive control.

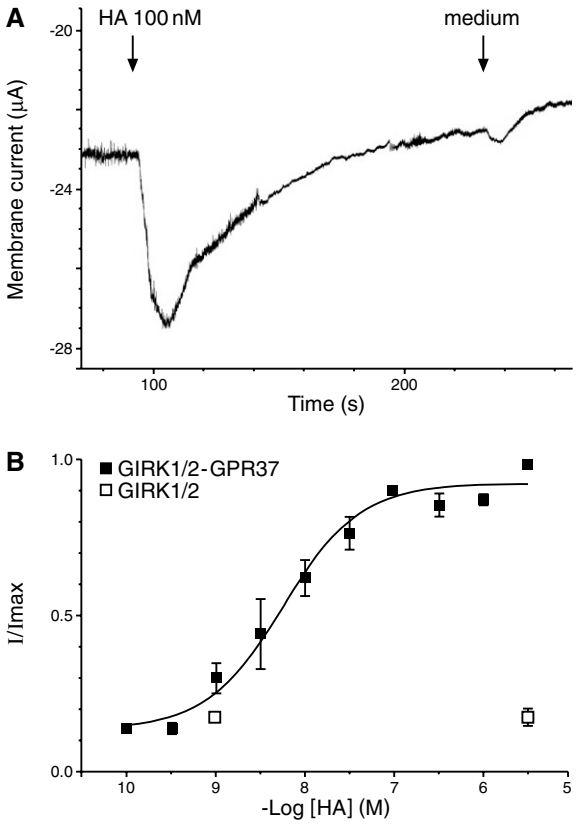

Fig. 5. HA is a high-affinity ligand for GPR37 expressed in frog oocytes. (A) Currents induced by 100 nM HA were recorded from *Xenopus* oocytes injected with cRNAs coding for GPR37 and for GIRK1/2. Stimulation with medium served as control. (B) The current increase was dependent on HA concentration. Dose-response curves for a HA-induced increase in GIRK1/2-mediated inward currents were normalised against maximal currents obtained for each oocyte. Current increases were averaged over four oocytes prepared and injected on the same day. The values represent means ± s.d. Data are representative of several independent experiments.

to an increase in membrane currents (Fig. 6B), which was blocked by La^{3+} and by SKF (Fig. 6B), both of which are known inhibitors of TRP-like Ca^{2+} channels. These Ca^{2+} channels, upon stimulation of receptors with ligands, translocate from an intracellular pool to the plasma membrane, for which activation of phosphoinositide 3-kinase (PI 3-kinase) and Ca^{2+}-dependent calmodulin (CaM) kinase is a prerequisite (Boels et al., 2001). The HA-induced increase in current was prevented by pre-incubating cells with pertussis toxin, wortmannin and KN93 (Fig. 6C), which demonstrates that an inhibitory G protein, PI 3-kinase and CaM-kinase II, respectively, are involved in the HA-GPR37 signalling cascade. A preliminary scheme of HA signalling is shown in Fig. 7.

Discussion

We present evidence that HA is a high-affinity ligand for GPR37. After heterologous expression in frog oocytes and in mammalian cells, EC_{50} values in the low nanomolar range were obtained. Electrophysiological analysis revealed that GPR37 activation by HA involved the same signalling cascade (Fig. 7) as found earlier for the endogenous HA receptor (Boels et al., 2001; Kayser et al., 1998; Ulrich et al., 1996). Interaction with HA resulted in GPR37 internalisation and stimulated entry into mitosis.

HA is bound to a carrier-like molecule both in hydra and in mammals, which improves the half-life and function of HA (Roberge et al., 1984; Schaller et al., 1996). The HA-binding protein HAB was isolated from hydra using HA-affinity chromatography, and later SorLA was discovered as an orthologue of HAB (Hampe et al., 2000). SorLA is a multi-ligand sorting receptor that, in addition to HA, binds glial-cell-derived neurotrophic factor (GDNF), PDGF and apolipoprotein E (ApoE) (Gliemann et al., 2004; Taira et al., 2001; Westergaard et al., 2004). HAB and SorLA are type I

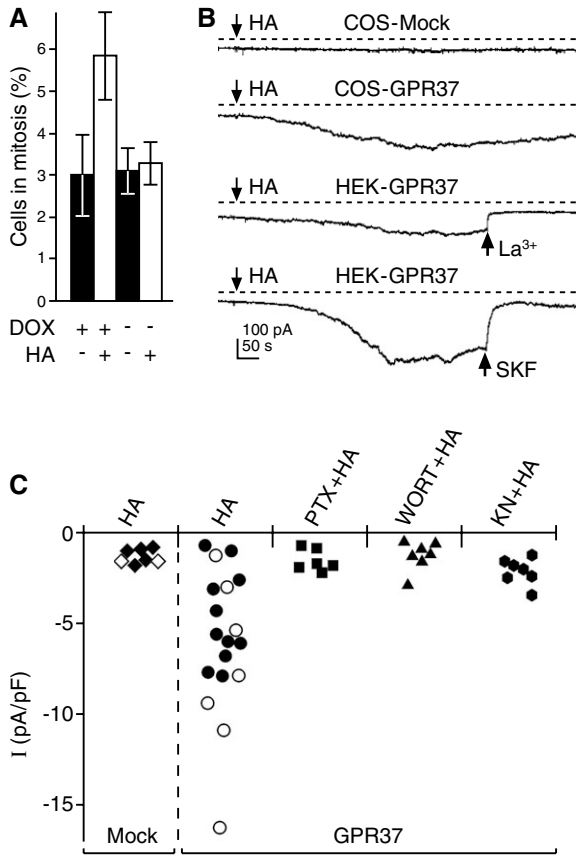

Fig. 6. GPR37 mediates HA signalling to stimulate mitosis. (A) HEK-T-REx–GPR37 cells were treated with and without doxycycline for 24 hours. Incubation with 2 nM HA for 1.7 hours led to an increase of cells in mitosis after induction of GPR37 expression. Immunostaining of cells with anti-phospho-histone H3 (1:1000) was used to determine cells in mitosis. 6×350 cells were counted, and the percentage of stained mitotic cells is given as means ± s.d. (B) Membrane currents were measured in the perforated patch configuration at a holding potential of –80 mV. Treatment with 1 nM HA induced an increase in membrane currents in COS-7 cells transiently expressing GPR37 (COS-GPR37), but not in mock-injected cells (COS-Mock). Membrane currents activated by HA in HEK-T-REx–GPR37 cells were blocked by application of 1 mM La^{3+} or 10 μM SKF. (C) Membrane-current densities were recorded from mock- and GPR37-transfected COS-7 cells. HA signal transduction was inhibited by pretreating cells for 2-3 hours with 200 ng ml^{-1} pertussis toxin (PTX), for 30-60 minutes with 100 nM wortmannin (WORT), or 30 μM KN93 (KN). Each symbol represents one cell measured in the whole-cell (filled symbols) or the perforated patch (open symbols) configuration

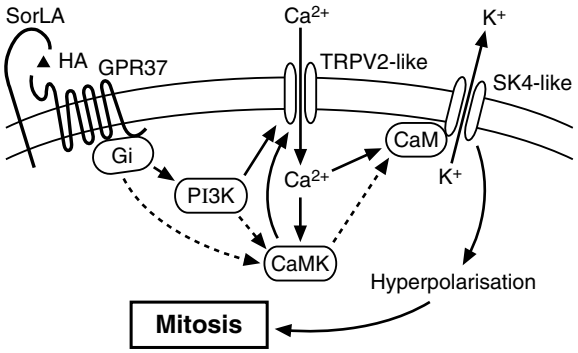

Fig. 7. Scheme of the signalling pathway from HA through GPR37 to stimulate mitosis. After binding of HA to GPR37 with or without help of the coreceptor SorLA, a pertussis-toxin-sensitive inhibitory G protein (Gi) is activated, which interacts through the phosphoinositide 3-kinase (PI3K) and the calcium-calmodulin dependent kinase II (CaMK) with a Ca^{2+} channel of the transient receptor potential family (TRPV2-like). The resulting Ca^{2+} influx activates a Ca^{2+}-dependent K^+ channel of the small and intermediate conductance family (SK4-like), leading to hyperpolarisation, which is a prerequisite for cells to enter mitosis. Dashed lines indicate hypothetical pathways.

transmembrane receptors with a large extracellular domain that can be shed by metalloprotease cleavage (Hampe et al., 2000). This represents an ideal mechanism to regulate the range of action of a morphogen like HA. GPR37 contains a relatively large extracellular domain, which is unusual for a peptide receptor. The notion that SorLA interacts with this domain of GPR37 as co-receptor to enhance HA binding (Hampe et al., 2000) is plausible and outlined in Fig. 7.

GPCRs play key physiological roles, and their dysfunction is implicated in several diseases. This might be reflected by the fact that about half of the current drugs, and certainly more in the future, are targeted to these receptors. GPR37 is of special interest for pharmacology, since it was shown to contribute to Parkinson's disease. GPR37 was characterised as a substrate for the E3 ubiquitin ligase parkin (Imai et al., 2001). Ubiquitylation marks proteins for degradation. Parkin mutations have been shown to be causative for neurodegeneration in Parkinson's disease, where dopaminergic neurons of the substantia nigra are especially affected (Imai et al., 2001; Yang et al., 2003). We found that overexpression of GPR37 resulted in complexes of molecular masses ⩾250 kDa. Aggregated GPR37 did not translocate to the cell surface, as shown by cell-surface biotinylation experiments, and most probably led to preferential cell death of transfected cells. We could express GPR37 successfully in frog oocytes and in mammalian cells after stable integration into the chromosome. Since frog oocytes are cultured at room temperature, more time for proper folding may have been advantageous for GPR37 expression. Similarly, lower levels of GPR37 transcripts by stable expression may have caused less stress for the cells. The fact that insoluble GPR37 was enriched in brains of patients with juvenile Parkinson's disease (Imai et al., 2001) and its presence in Lewy bodies (Murakami et al., 2004) supports the notion that GPR37 misfolding contributes to neuronal cell death (Imai et al., 2003). This might be confirmed by the recent finding that GPR37-knockout mice showed altered dopaminergic signalling and were resistant to the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which preferentially kills dopaminergic neurons (Marazziti et al., 2004). As SorLA was found to be downregulated in the brains of patients with Alzheimer's disease (Scherzer et al., 2004), it is intriguing to speculate that a connection exists between SorLA, GPR37 and HA to improve neuronal cell survival.

Materials and Methods

Monomerisation of HA and synthesis of Cy3B-labelled HA

HA was from Bachem AG. Monomerisation was achieved by heating a 10 μM solution of HA in 0.1 N HCl for 5 minutes to 95°C. After neutralisation with NaOH to pH 7.0, samples were stored frozen at –20°C and used 2-3 times only (Bodenmuller et al., 1986). For labelling Cy3B, 150 nmoles of monomerised HA were lyophilised and dissolved in 100 μl dimethylformamide containing 0.2% *N*-methylmorpholine. Cy3B-mono-*N*-hydroxysuccinimide (NHS) ester (Amersham Biosciences) was dissolved in the same buffer (0.5 mg in 50 μl) and incubated with HA overnight in the dark. The Cy3B-labelled HA was purified by C18 reverse-phase HPLC, yielding approximately 30-40 nmoles of Cy3B-labelled HA.

Molecular biology

Human GPR37 cDNA was inserted into pcDNA 3.1 (+) and into pcDNA3-FLAG-His6C as described earlier (Imai et al., 2001). GPR37 and GPR37-FLAG were subcloned into the dual-function vector pXOON, a kind gift from T. Jespersen, optimised for expression both in frog oocytes and in mammalian cells (Jespersen et al., 2002). GPR37-FLAG was introduced into CHO-K1 cells stably expressing Gα16 and apoaequorin (CHO-Gα16-AEQ) (Stables et al., 1997) with the vector pIRES-P, a kind gift from S. Hobbs (Hobbs et al., 1998). Stable integration was monitored by immunostaining with antibodies against FLAG (Sigma-Aldrich). For inducible expression, GPR37 was transfected into HEK-293 cells using the Flp-In T-REx system of Invitrogen (Karlsruhe, Germany). The concatemeric construct between GIRK1 and GIRK2 (GIRK1/2) was kindly provided by A. Karschin (Wischmeyer et al., 1997). All constructs were confirmed by sequencing.

Expression of GPR37 in *Xenopus laevis* oocytes and electrophysiology

For functional expression in frog oocytes, the GPR37 cRNA was transcribed in vitro with T7 polymerase from the *Xba*I-linearised pXOON-GPR37-FLAG vector and co-injected at a ratio of 5:1 with cRNA of the concatemeric GIRK1/2 construct transcribed from the *Nhe*I-linearised plasmid. For recordings, oocytes were superfused with ND-96 medium (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes, pH 7.5). Two-electrode voltage-clamp recordings were performed with electrodes pulled to a tip resistance of 0.5-2.0 MΩ. A Gene Clamp 500B amplifier (Axon Instruments), pClamp9 (Axon Instruments) and Origin (Microcal Software) served for data acquisition and analysis. Whole cells were clamped at –100 mV. For agonist measurements, the medium was changed to high K^+ (ND-96 with 96 mM KCl, 2 mM NaCl). After the initial inward current had reached a plateau, agonists were applied in high K^+ medium. Agonist treatment was terminated by wash-out with low K^+ to control intactness of the oocyte membrane. All recordings were performed at room temperature.

Cell culture, transfection and immunostaining

NH15-CA2, HT22 and COS-7 cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS), HEK-T-REx-GPR37 cells with 10% newborn calf serum (tetracycline-free) and CHO-K1 cells in DMEM-F12 with 5% FCS. For routine culture, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 10 mM Hepes, pH 7, were added to these media. CHO-Gα16-AEQ cells stably expressing GPR37 required the addition of 750 μg ml^{-1} geneticin, 200 μg ml^{-1} hygromycin and 5 μg ml^{-1} puromycin. HEK-T-REx-GPR37 cells were induced to express GPR37 by incubation in 1 μg ml^{-1} doxycycline. Lipofectamine 2000 (Invitrogen), Fugene 6 (Roche Diagnostics), or electroporation were used for transfection. To assay ligands, cells were transferred overnight into serum-free defined medium consisting of the respective basal media to which 5 μg ml^{-1} insulin, 30 μg ml^{-1} transferrin, 20 μM ethanolamine, 30 nM sodium selenite, 1 μM sodium pyruvate, 1% non-essential amino acids and 2 mM glutamine were added.

For immunocytochemistry, cells were fixed either with 4% formaldehyde in PBS for 30 minutes at room temperature or with ice-cold 1% acetic acid in ethanol for 5 minutes. After washing with 0.1% Triton X-100 and pre-absorption with 1% bovine serum albumin, first and second antibodies were applied. For cell-surface staining, living cells were incubated with ligand and/or antisera for 20-30 minutes on ice, washed, fixed and visualised as indicated. No Triton X-100 was added to prevent permeabilisation. For western blotting, cells were harvested by treatment with 2 mM EDTA in PBS for 10 minutes, collected by centrifugation, and ultrasonicated for 20 seconds in Tris-HCl buffer, pH 7.4, containing 2 mM EDTA and a protease-inhibitor cocktail (Roche Diagnostics). After centrifugation at 100,000 ***g***, the membrane pellets were dissolved in sample buffer and separated by SDS-PAGE. The monoclonal mouse anti-GPR37 antibody, recognising an extracellular domain of recombinant human GPR37, was used at a dilution of 1:400, the polyclonal rabbit antisera against the intracellular C-terminal domain of GPR37, anti-GPR37(R1) and anti-GPR37(R2), were diluted 1:1000 and 1:2000, respectively. All GPR37-specific antibodies were produced in the laboratory of Takahashi and have been described previously (Imai et al., 2001). The antibody against FLAG (M2) was from Sigma-Aldrich and that against phospho-histone H3 from Biomol. Cy2 or Cy3 secondary antibodies were used for confocal analysis, and alkaline phosphatase- or peroxidase-conjugated secondary antibodies were used for light microscopy and western blotting. Western blots were visualised by ECL. Biotinylated proteins were detected with an avidin-peroxidase conjugate (Bio-Rad).

FRET analysis

For fluorescence resonance energy transfer (FRET) experiments, HA was reacted with the highly specific HA antiserum 102.8, which binds to HA in the picomolar range and was described earlier (Schaller et al., 1984). It was used at a dilution of 1:3000 and visualised with Alexa Fluor 488 goat anti-rabbit as donor (Invitrogen). GPR37 was detected with anti-GPR37 antibody and visualised with Cy3 anti-mouse antibody (Amersham) as acceptor. The energy transfer was detected as increase in donor fluorescence (Alexa Fluor 488) after complete photobleaching of the acceptor molecule (Cy3). Initial images were recorded after excitation at 488 and 568 nm. A discrete area of the sample was illuminated with intense 568 nm light (laser power 100%) for a few minutes to destroy completely the acceptor fluorescence. The cell was then rescanned using excitation at 488 nm. An increase within the photobleached area was used as a measure for the amount of FRET obtained. The efficiency of energy transfer (E) was expressed as E=1-(D1/D2), where D1 is the donor fluorescence before, and D2 after, photobleaching. Data were collected for 4-5 different fields from a single coverslip; 2-3 coverslips were used for each measurement; the experiment was repeated at least three times.

Biotinylation of surface proteins

COS-7 cells were transiently transfected with GPR37-FLAG using the Fugene 6 reagent (Roche Diagnostics). 48 hours after transfection, cells were washed 2× with PBS and biotinylated for 30 minutes at room temperature with 1 mM S-NHS-biotin (Perbio Science). The reaction was stopped by addition of 0.5 M Tris-HCl, pH 7.5, for 5 minutes at room temperature, and the cells were washed with PBS to remove free biotin. Cell lysates were prepared in a buffer consisting of 5 mM EDTA, 10 mM Tris-HCl, pH 7.4, and protease-inhibitor cocktail. Samples were ultrasonicated for 20 seconds and centrifuged at 100,000 ***g*** for 30 minutes. Pellets were solubilised in buffer containing 1% Triton X-100, 0.5% NP40, 150 mM NaCl, 7 mM EDTA, 1 mM EGTA, 10 mM Tris-HCl, pH 7.4, and protease-inhibitor cocktail for 30 minutes on ice, followed by centrifugation at 16,000 ***g*** for 15 minutes at 4°C. The supernatant was used for immunoprecipitation.

Immunoprecipitation with anti-FLAG M2-agarose

Since high concentrations of NP40 inhibited binding to FLAG-agarose, the supernatant from the NP40-solubilised and biotinylated COS-7 cells was diluted fivefold with TBS (150 mM NaCl, 50 mM Tris-HCl, pH 7.4) to reduce the NP40 concentration to 0.1%. Samples were incubated with 100 μl anti-FLAG M2-agarose (Sigma-Aldrich) overnight at 4°C and then centrifuged at 1500 ***g*** for 5 minutes at 4°C. Pellets were resuspended in 1 ml TBS and centrifuged again at 16,000 ***g*** for 2 minutes at 4°C. After washing with TBS, pellets were dissolved in 50 μl sample buffer and subjected to western blotting.

Electrophysiology with mammalian cells

For electrical recordings, COS-7 cells were microinjected with 50 ng μl^{-1} GPR37-pcDNA3 and 5 ng μl^{-1} EGFP-N1-pcDNA3, the latter being used to facilitate detection of successfully transfected cells. Membrane currents were recorded in the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) or the perforated-patch configuration with nystatin (Horn and Marty, 1988). An EPC9 patch-clamp amplifier was used in conjunction with the PULSE-stimulation and data-acquisition software (HEKA Elektronik). The patch electrodes were made from 1.5 mm diameter borosilicate glass capillaries with resistances of 2.5-4 MΩ. Data were low-pass filtered at 3 kHz and compensated for both fast and slow capacity transients. Series resistance was compensated by 75-90%. All experiments were performed at room temperature (22-25°C). The pipette solution contained 140 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 2.5 mM EGTA, 10 mM HEPES and had a calculated free Ca^{2+} concentration of 66 nM. The pH was adjusted to 7.3 with KOH. The standard external solutions contained 140 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM KCl, 10 mM HEPES and 10 mM glucose, buffered to pH 7.3 with NaOH. Nystatin was dissolved in dimethyl sulfoxide (DMSO). Its final concentration in the standard pipette solution was 0.2 mg ml^{-1}. All chemicals for electrophysiology were purchased from Sigma-Aldrich.

Statistical analysis

The results are expressed as means of 3-6 determinations ± s.d. Curve fittings were performed with the Prism program (GraphPad). Each experiment was repeated at least three times.

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