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Obestatin does not activate orphan G protein-coupled receptor GPR39 ☆

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Abstract

Recently, the ligand of the orphan G protein-coupled receptor GPR39 has been identified as obestatin, a 23-amino acid peptide derived from the ghrelin precursor protein. We used two methods to study the possible activation of GPR39 by obestatin: cAMP measurements based on a luminescent reporter gene and a fluorometric Ca^{2+} flux method. The former was similar to that reported in the original publication of Zhang et al. [J.V. Zhang, P.G. Ren, O. Avsian-Kretchmer, C.W. Luo, R. Rauch, C. Klein, Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake, Science 310 (2005) 996–999]. The latter method used promiscuous as well as chimaeric G-proteins commonly used to couple orphan G protein-coupled receptors to the phospholipase C pathway, that leads to intracellular Ca^{2+} rise. We could, however, not demonstrate activation of the GPR39 receptor by obestatin via any of these signal transduction pathways. We could activate GPR39 by high concentrations of Zn^{2+}, demonstrating cell surface expression of a functional receptor that could elicit a Ca^{2+} response. The Zn^{2+} response was not affected by obestatin. The identity of the native ligand for GPR39 remains to be elucidated.

Keywords: Obestatin; GPR39; GPCR

Obestatin is a recently described 23-amino acid peptide derived from the same precursor as ghrelin. It has been reported to exhibit effects opposite to those of ghrelin and to reverse ghrelin effects. Furthermore, obestatin was identified as a ligand for the orphan G protein-coupled receptor GPR39 [1].

GPR39 was cloned in 1997 and is related to the growth hormone secretagogue receptor and the neurotensin receptor; it is widely expressed throughout the body [2]. GPR39 is also expressed in the central nervous system including the amygdala, the hippocampus, the auditory cortex, and several other brain regions [3].

When trying to reproduce the findings of Zhang et al. [1], we were unable to elicit a cAMP response from GPR39 stimulated by obestatin. We, therefore, tried to demonstrate GPR39 activation by coupling the receptor to a Ca^{2+} response via promiscuous or chimaeric G-proteins.

Materials and methods

Reagents. Human obestatin was purchased from Peptides International (PGH-3890-PI) and mouse obestatin was purchased from California Peptides (Cat No. 475-97). Short Neuropeptide F was custom synthesized (Invitrogen). The sequence of all peptides was verified by mass spectrometry (results not shown). Forskolin and carbachol were purchased from Sigma, Belgium. Peptides were stored in 70% acetonitrile (HPLC grade, Rathburn) at 4 °C.

Cell culture and transfection. HEK293T cells were a kind gift of Prof. V. Baekelandt (K.U. Leuven). The cells were cultured in DMEM (Sigma, Belgium) supplemented with 50 U/ml penicillin, and 50 μg/ml

☆ *Abbreviations:* cAMP, adenosine 3′,5′-cyclic monophosphate; DMEM, Dulbecco's modified Eagle's medium; HBSS, Hanks' balanced salt solution; Hepes, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; Gα16, G-protein α16; sNPF, short neuropeptide F.

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streptomycin (Invitrogen, The Netherlands) and 10% heat-inactivated fetal calf serum (Invitrogen, The Netherlands). Cells were grown as monolayer in tissue culture flasks (TPP, USA) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

Transfection was carried out in serum-free medium with the Fugene 6 transfection reagent (Roche, USA). Cells were transfected at ± 60% confluency according to the manufacturer's protocol.

Fluorometric Ca^{2+} flux assay. The HEK293T cells were detached 24 h after transfection with trypsin/EDTA (Sigma, Belgium). Cells were subsequently plated out in 96-well plates at 120,000 cells per well. Forty-eight hours after transfection, the cells were loaded with the fluorophore Fluo-4-AM (Molecular probes, The Netherlands) for 1 h at a concentration of 5 μM, after which excess fluorophore was washed away with HBSS buffer (Sigma, Belgium), supplemented with 5 mM $CaCl_2$ and 10 mM Hepes.

A 50-μl aliquot of various concentrations of obestatin, diluted in HBSS buffer, was transferred from the compound plate to the 96-well plate containing the HEK293T cells. The fluorophore was excited at 488 nm. The calcium response was measured for 2 min at 525 nm using a FLEX-Station (Molecular Devices) and data were analyzed using the Softmax Pro software (Molecular Devices).

cAMP measurement. The cellular cAMP levels were measured by a luminescence reporter gene assay. Twenty-four hours after transfection, cells were plated in low glucose DMEM supplemented with L-glutamine, Na-pyruvate, and 200 μM 3-isobutyl-1-methylxanthine (IBMX, Sigma, Belgium). The cells were subsequently exposed to different concentrations of obestatin for 4 or 16 h. The luciferase activity was quantified using a LucLite Kit (Perkin-Elmer Life Sciences) and the luminescence measured on a Microlumat Plus LB96V microplate luminometer (EG&G Berthold, Bad Wildbad, Germany).

Immunocytochemical staining. Cells were fixed by a 10-min incubation with 4% paraformaldehyde (Fluka, ⩾95.0% pure). After fixation, cells were washed and treated with NH_4Cl to block the remaining free aldehyde groups. The cells were then permeabilized by a 5-min incubation with 1% (v/v) Triton X-100. The cells were subsequently incubated with an appropriate dilution of the affinity-purified primary antiserum (Rabbit anti-GPR39, IMGENIX) in PBS supplemented with 1% (w/v) BSA (Sigma, Belgium) and 0.1% (v/v) Triton X-100 for 2 h. After washing the cells, the secondary antiserum was added (Goat anti-rabbit, 1/500 in PBS + 0.1% Triton X-100) for 1 h (DAKO, Glostrup, Denmark). The cells were subsequently washed in PBS + 0.1% Triton X-100 and incubated with the streptavidin–biotin–horseradish peroxidase complex (ABC Kit, Vector Laboratories) for another hour and the detection was subsequently performed with 3,3-diaminobenzidine (DAB) as a chromogen. Images were captured with a Leica DC camera and analyzed by the Leica IM500 Image Manager software.

Statistical analysis. All values shown are means ± SE. Significance was accepted at the 95% confidence level.

Results and discussion

Molecular cloning and expression of GPR39

A cDNA containing the coding region of human GPR39 was a kind gift of Dr. Arjan Buist (Janssen Pharmaceutica). The integrity of the entire cloned insert was verified using automated DNA sequencing of both strands. The insert was amplified by PCR and subcloned into the pcDNA3 mammalian expression vector (Invitrogen). After subcloning the insert sequence was again verified in its entirety by sequencing.

The transfection efficiency of HEK293T cells was estimated using an expression plasmid carrying a fluorescent marker, ZsGreen (pZsGreen1-N1, BD Biosciences). Two days after transfection, up to 100% of the cells demonstrated ZsGreen expression (Fig. 1a and b). This results was confirmed by FACS analysis of the transfected cells.

To determine the expression of GPR39 in HEK293T cells, we performed an immunocytochemical staining on the transfected cells (Fig. 2a) and compared it to pcDNA3 vector-transfected control cells (Fig. 2b). Fig. 2a clearly shows a majority of cells staining with varying intensity

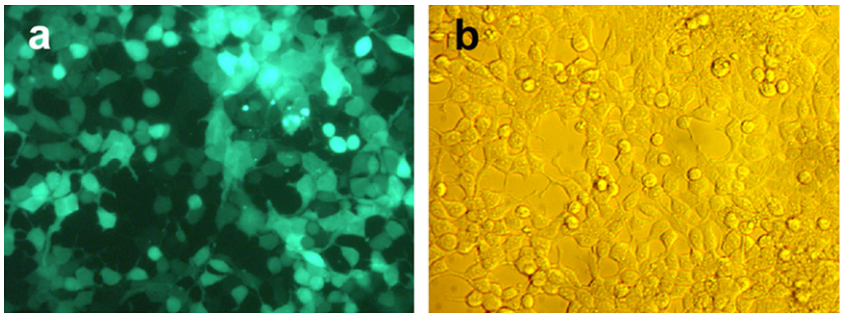

Fig. 1. (a) The transfected cells visualized by fluorescence (b) a phase contrast view of the same monolayer of cells.

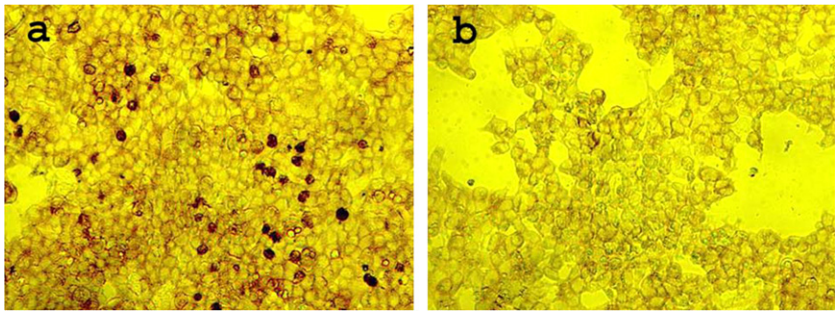

Fig. 2. Immunocytochemical staining for GPR39 in GPR39-transfected cells (a) and control cells (b).

whereas no significant GPR39 immunoreactivity was detected in control HEK293T cells (Fig. 2b).

Measurement of cellular cAMP after obestatin incubation of GPR39-transfected cells

It has been shown by Zhang et al. that incubation of GPR39-transfected cells with obestatin results in an increase in cAMP measured by an ELISA [1]. Therefore, we added different concentrations of obestatin to GPR39-transfected HEK293T cells and measured cellular cAMP by a Luciferase-based system (LucLite, Perkin-Elmer) (Fig. 3).

No significant increase in cAMP levels was observed after incubation of the cells with obestatin in concentrations ranging from 0.3 to 100 nM. In contrast, forskolin could elicit a significant increase in cAMP.

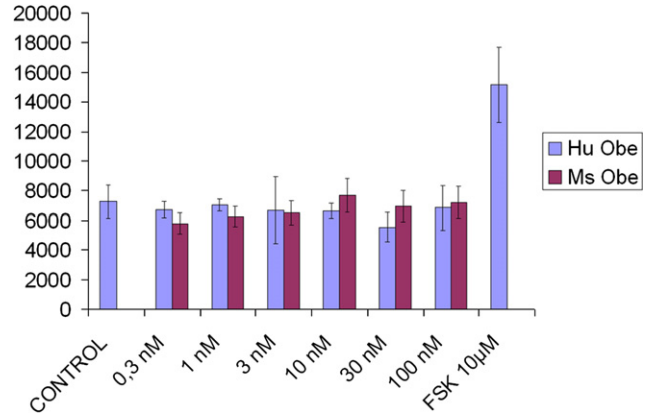

Fig. 3. Luminescence-based cAMP measurement in GPR39-transfected cells stimulated with forskolin (FSK) or various concentrations of human (Hu Obe) or mouse (Ms Obe) obestatin. All compounds were tested in triplicate at each concentration. Results are shown as means ± SEM in arbitrary light units.

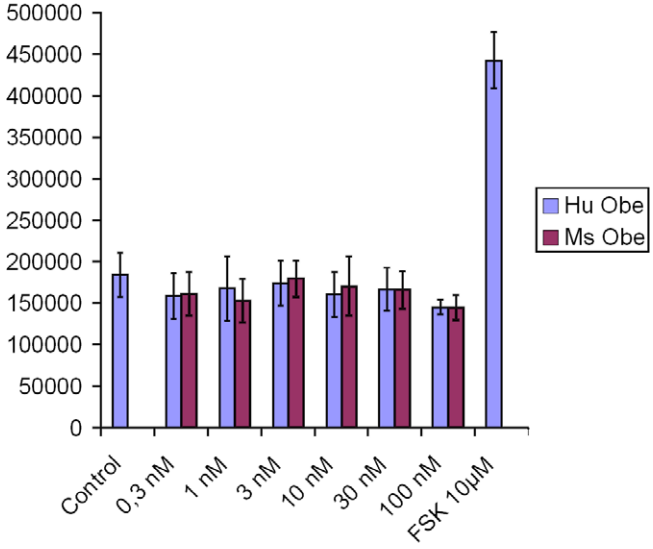

Fig. 4. Luminescence-based measurements of cellular cAMP levels in GPR39-transfected HEK293T cells after 16-h incubation with different concentrations of human or mouse obestatin or with forskolin (FSK). Abbreviations see Fig. 3.

Because Zhang et al. used an incubation of 16 h, we repeated our experiment with human and mouse obestatin using a 16 h incubation period but could not detect an increase in cAMP under those circumstances either (Fig. 4).

Measurement of Ca^{2+} response after obestatin incubation of GPR39-transfected cells

To detect obestatin-induced GPR39 activation by another method, we also co-transfected HEK293T cells with GPR39 and a promiscuous G protein: Gα16. Activation of the G protein-coupled receptor should result via Gα16 in a Ca^{2+} flux, which can be detected by the fluorophore Fluo-4-AM using a fluorescence reader (Flexstation, Molecular Devices).

We could not detect any activation of GPR39 in HEK293T cells by mouse obestatin at concentrations ranging from 1 nM to 1 μM (Fig. 5a). In contrast, 10 μM carbachol resulted in a large Ca^{2+} flux by activation of the endogenous muscarinic receptor on HEK293T cells (Fig. 5a).

To rule out that our negative results were due to the inability of GPR39 to couple to Gα16, we cotransfected the HEK293T cells with GPR39 and chimaeric G-proteins, Gqi5 and Gqs5. These chimaeric G proteins link Gi- and Gs-coupled receptors, respectively, to Gq. We could, however, not detect any activation of GPR39 by obestatin with either of these G-proteins (data not shown). We have repeated both experiments at least twice. We also repeated the experiment with human obestatin at the same concentrations from 1 nM to 1 μM with similar (negative) results (data not shown).

To test our signal transduction system, we transfected HEK293T cells with the GC7395 receptor from *Drosophila melanogaster* which is activated by short neuropeptide F (sNPF) [4]. sNPF, in the same concentrations as used for obestatin, elicited a large Ca^{2+} response in CG7395-transfected HEK293Tcells, cotransfected with Gα16-protein (Fig. 5b). These data demonstrate the sensitivity of our receptor activation assay.

To test the presence of functional GPR39 on the surface of the transfected cells, we incubated the cells with 1 mM Zn^{2+}. It has been shown before that Zn^{2+} can activate GPR39 [5]. In the presence of 1 mM Zn^{2+} we could demonstrate a clear Ca^{2+} flux in the GPR39-transfected cells whereas no flux was observed in pcDNA-transfected control cells (Fig. 6). The activation of GPR39 by Zn^{2+} showed a clear dose–response effect between 0.1 and 1 mM, as described [5]. Addition of obestatin did not affect this Zn^{2+} effect.

Our results cast considerable doubt on the earlier report by Zhang et al. that obestatin is a ligand for GPR39. The reason for this discrepant result remains to be elucidated. We used the same (sequence-verified) receptor and the same (mass spectrometry-verified) ligand in the same cell line (HEK293T) and assayed the same second messenger

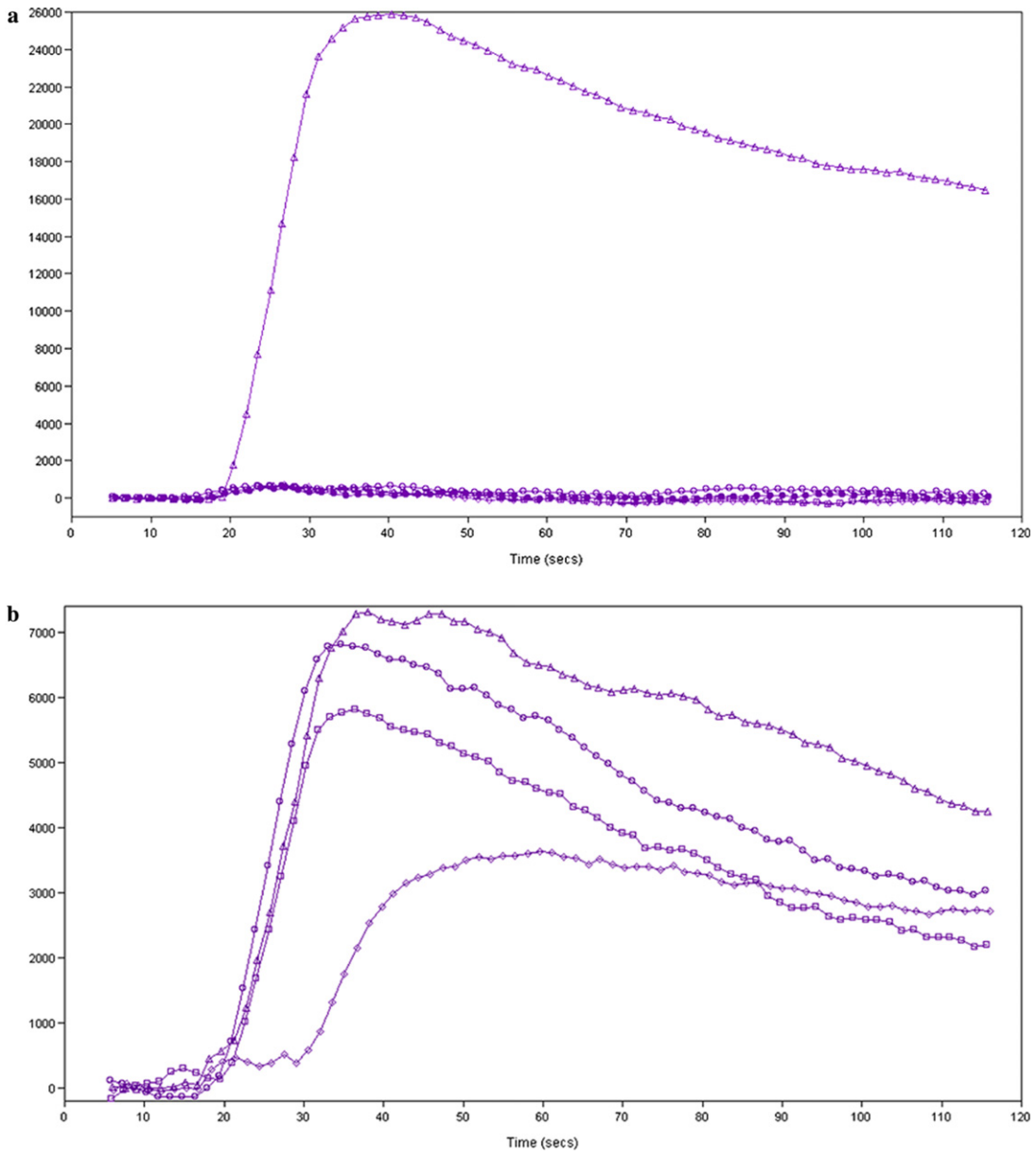

Fig. 5. (a) Ca^{2+}-based fluorescence (arbitrary units) in GPR39-transfected HEK293T cells after addition of 10 μM carbachol (Δ) or 1 nM (□), 10 nM (Δ), 100 nM (◊) or 1 μM (●) obestatin. (b) Ca^{2+}-based fluorescence (arbitrary units) in CG7395-transfected HEK293T cells after addition of 1 nM (◊), 10 nM (□), 100 nM (○) or 1 μM (Δ) sNPF.

(cAMP). Zhang et al. measured cellular cAMP levels by an ELISA whereas we used a luciferase detection system for this purpose. This is, however, an unlikely reason for the discrepant results because the LucLite luciferase reporter assay is known for its high sensitivity [6] with a threshold of detection of 10^{-15} g, clearly surpassing that of an ELISA. We excluded low transfection efficiency or low levels of receptor expression as possible reasons for our failure to detect GPR39 activation by obestatin. Furthermore, we have failed to detect GPR39 activation by obestatin in a Ca^{2+} flux-based assay using a variety of (promiscuous and chimaeric) G-proteins under conditions where both an endogenous GPCR and another transfected GPCR could clearly trigger a Ca^{2+} response. Since we could clearly activate the GPR39-transfected cells and not the control cells with Zn^{2+}, we have also ruled out the possibility of the presence of a non-functional GPR39 receptor in our assay, as well as the possibility that our GPR39 could not couple to the transfected G-protein to elicit a Ca^{2+} response.

Finally, we explored the possibility that different Zn^{2+} levels (perhaps from the water used) might account for the differences in GPR39 response. Zn^{2+} is known to potentiate the activation of some GPCRs by their natural ligand [7–9], and has been shown to activate GPR39 by itself at high concentrations. Although Zn^{2+} did activate GPR39 as reported before, obestatin did not potentiate this response.

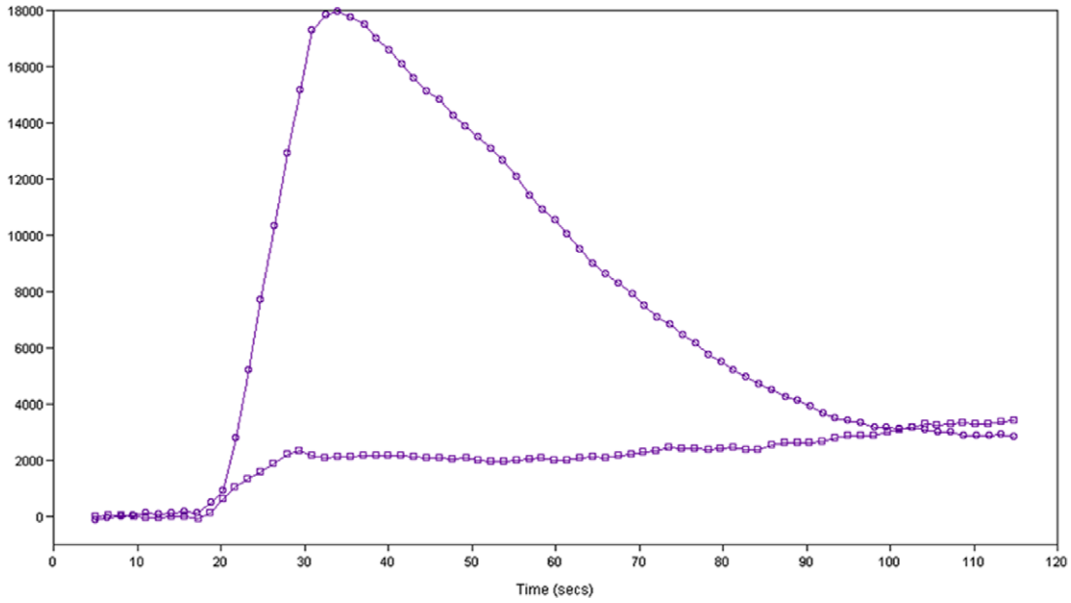

Fig. 6. Ca^{2+}-based fluorescence (arbitrary units) in GPR39-transfected HEK293T cells (○) and control cells (□) after addition of 1 mM Zn^{2+}.

Acknowledgments

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