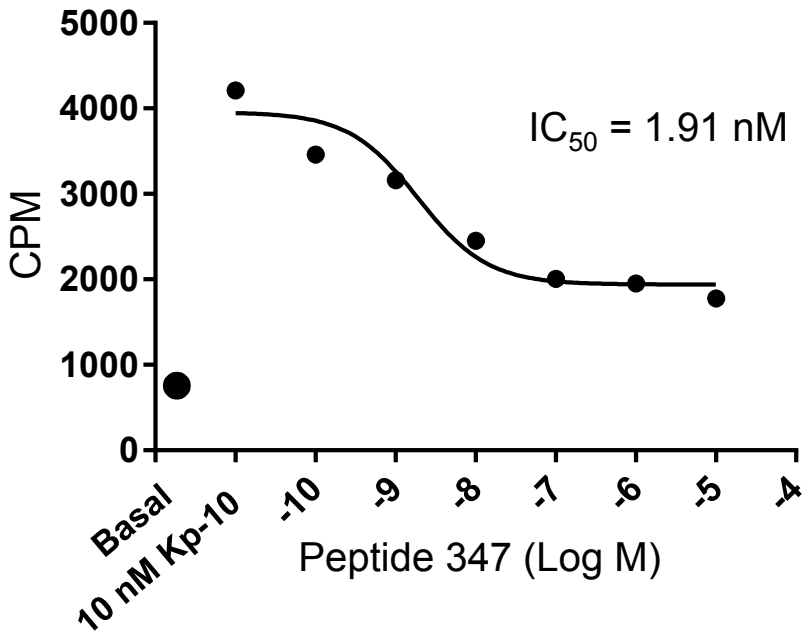
Supplemental Figure 1



Supplemental Figure 1. Peptide 347 inhibition of Kp-10 induced inositol phosphate production in CHO cells. Peptide 347 inhibition (IC_{50}) of Kp-10 (10 nM) stimulation of inositol phosphate in CHO cells transfected with the human Kiss1R. Radioactive inositol phosphate production was assessed using ^{3}H-myoinositol. Detailed information about the methods is presented below.

Methods for Supplemental Figure 1

Materials

Human Kp-10 and Peptide antagonist 347 were custom synthesized by EZBiolabs. The purity was >80% by HPLC analysis. The authenticity of peptides was confirmed by mass spectrometry. The source of all other reagents was Sigma-Aldrich.

Cell Culture

Chinese hamster ovary (CHO) cells stably-expressing the human GPR54 receptor (CHO/GPR54) were obtained from Prof. G. Vassart, Univ. Brussels. The cells were maintained in F12 Ham's nutrient mixture (Gibco) supplemented with 10% fetal calf serum, 2% glutamine and 1% penicillin (10,000 units/ml)/streptomycin (10,000 mg/ml) at 37ºC in a humidified 5% CO_2 atmosphere. COS-7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco)) supplemented with 10% fetal calf serum, 2% glutamine and 1% penicillin (10,000 units/ml)/streptomycin (10,000 mg/ml) at 37ºC in a humidified 5% CO_2 atmosphere.

GPR54 Transfection

COS-7 cells were trypsinized and $1x10^6$ cells/ml place in DMEM and mixed with a 30 μls of the human GPR54-plasmid DNA. Then 300 μl were placed in each cuvette and then pulsed at 250 V and 960 uF using a Gene Pulser (Biorad) and incubated at room temperature for 15 minutes. Cells were then suspended in DMEM and plated into 12-well plates at $1x10^5$ cells/well.

Inositol Phosphate (IP) Stimulation Assay

Assays were performed as previously described (1,2). Prior to stimulation CHO/GPR54 cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS; without calcium or magnesium) then incubated overnight with ^{3}H-myoinositol, labeled HEPES-modified DMEM with 1% penicillin/streptomycin at 37º C. HEPES-modified DMEM supplemented with 1% penicillin/streptomycin and 1% lithium chloride (0.5 ml) was added to cells for 30 min at 37º C to block IP hydrolysis. Cells were then stimulated with 0.5 ml KP-10 (10nM) diluted at 1:100 in the above media for 1 h at 37º C, then with 10 mM formic acid at 4º C for 1 h to lyse cells. Lysates were transferred to plastic tubes containing 0.5 ml Dowex resin to bind the radioactive IP and the resin was then washed with 1 ml water. The resin was next washed with 60 mM ammonium formate/5 mM sodium tetraborate followed by 1 M ammonium formate/0.1 M formic acid to release the bound radiation. Then 800 µl of the radioactive solution were transferred to scintillation vials containing 2.5 ml scintillation fluid and radioactivity counted on a Beta counter for 60 sec. Experiments were repeated 3-5 times. IP production was plotted as mean values ± SEM and analyzed by using a two-way ANOVA followed by Bonferroni post hoc test ($p \geq 0.05$).

Inositol Phosphate (IP) Antagonism Assay

CHO/GPR54 cell monolayers were stimulated with 0.25 ml kisspeptin (10 nM) alone or in combination with 0.25 ml Peptide 347 (100 pM–1 µM), to investigate the inhibition of kisspeptin stimulation of IP production. Experiments were repeated 3 times. IP production was plotted as mean values ± SEM and analyzed using a two-way ANOVA followed by Bonferroni post hoc test ($p \geq 0.05$).

1. Coetsee M, Millar RP, Flanagan CA, Lu ZL: Identification of Tyr(290(6.58)) of the human gonadotropin-releasing hormone (GnRH) receptor as a contact residue for both GnRH I and GnRH II: Importance for high-affinity binding and receptor activation. Biochemistry 2008;47:10305-10313.

2. Lu ZL, Coetsee M, White CD, Millar RP: Structural determinants for ligand-receptor conformational selection in a peptide G-protein coupled receptor. J Biol Chem 2007;282:17921-17929.