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Zaprinast, a well-known cyclic guanosine monophosphate-specific phosphodiesterase inhibitor, is an agonist for GPR35

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Abstract **We found that zaprinast, a well-known cyclic guanosine monophosphate-specific phosphodiesterase inhibitor, acted as an agonist for a G protein-coupled receptor, GPR35. In our intracellular calcium mobilization assay, zaprinast activated rat GPR35 strongly (geometric mean EC_{50} value of 16 nM), whereas it activated human GPR35 moderately (geometric mean EC_{50} value of 840 nM). We also demonstrated that GPR35 acted as a $G\alpha_{i/o}$- and $G\alpha_{16}$-coupled receptor for zaprinast when heterologously expressed in human embryonic kidney 293 (HEK 293) cells. These findings will facilitate the research on GPR35 and the drug discovery of the GPR35 modulators.**

Keywords: GPR35; Zaprinast; Orphan G-protein-coupled receptor; Calcium mobilization assay

1. Introduction

The G protein-coupled receptors (GPCRs) are a large family of cell surface receptors that account for over 30% of current drug targets [1]. Sequencing of the human genome has led to the discovery of novel GPCRs, and many of them are orphan receptors for which the natural ligands have not yet been identified. To determine the biological functions of these orphan GPCRs, identification of their natural ligands is the first step. However, despite extensive attempts at receptor–ligand pairing, a number of GPCRs are still orphan receptors. GPR35 [2] is one of these orphan GPCRs. It shares homology with some of the purinergic receptors [2], GPR23/P2Y9 (the receptor for lysophosphatidic acid) [3], and HM74 (the receptor for nicotinic acid) [4]. Although chromosomal mapping and the expression of GPR35 in a number of human tissues have been investigated in previous studies [2,5,6], little is known about this receptor.

Here we report that zaprinast [7], a well-known cGMP specific-phosphodiesterases (cGMP-PDEs) inhibitor [8], acts as an agonist for GPR35. The cyclic nucleotide phosphodiesterases are a large group of structurally-related enzymes [9,10]. Among them, PDE4, PDE7, and PDE8 are specific enzymes for cyclic adenosine monophosphate (cAMP), whereas PDE5, PDE6, PDE9 are specific enzymes for cyclic guanosine monophosphate [9,10]. Other PDEs have dual activities [9,10]. They differ in their mode of action, intracellular distribution, tissue distribution, relative activities, and K_m values [9,10]. Various stimuli induce cellular responses by increasing the intracellular levels of cAMP and cGMP, and PDEs account for degradation of these intracellular second messengers to terminate the signals and the cellular responses [9,10]. Therefore, regulation of PDEs activities is important to control the intracellular second messenger levels and physiological responses, and specific PDE inhibitors are utilized as both research tools and remedies [10]. For example, sildenafil (Viagra™) is a potent selective PDE5 inhibitor and an orally active drug for erectile dysfunction [7]. Zaprinast, a lead compound for sildenafil, is known as a moderate inhibitor for cGMP-PDEs, especially PDE5 and PDE6 (IC_{50} values for PDE5, PDE6, PDE9 are 0.5–076, 0.15, and 35 μM) [7,11]. Zaprinast also inhibits PDE10, and PDE11 weakly (IC_{50} values are 22 and 11–33 μM, respectively) [11]. By using an intracellular calcium mobilization assay, we show that zaprinast activates GPR35-G protein pathways and this activity of zaprinast in this assay is not attributable to inhibition of PDEs. We have also found that GPR35 acts as a $G\alpha_{i/o}$- and $G_{\alpha 16}$-coupled receptor for zaprinast when heterologously expressed in human embryonic kidney 293 (HEK293) cells.

2. Materials and methods

2.1. Chemicals

Zaprinast, 8-bromoguanosine 3′,5′-cyclic monophosphate (8Bromo-cGMP), T-0156 and T-1032 were purchased from Sigma.

2.2. Cloning of rat GPR35 (rGPR35)

To generate cDNA templates for RT-PCR, 5 μg of total RNA from rat colon (Clontech) was reverse-transcribed by using SUPERSCRIPT Preamplification Systems (Invitrogen), and an aliquot (1 μL) of the products was subjected to PCR. To amplify rGPR35, two primers (5′-TCCGTCAGATGAGCCCTAGGACC-3′ and 5′-CACAGGTTC CTCTGGCCCTTGGCATG-3′) were designed on the basis of the nucleotide sequence of mouse GPR35 (GenBank Accession No. BC027429), and PCR was performed under the following conditions: 94°C for 4 min followed by 35 cycles of 94 °C for 20 s, 50 °C for

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Abbreviations: GPCR, G protein-coupled receptor; PDE, phosphodiesterase; HEK293, human embryonic kidney 293; hGPR35, human GPR35; FLAG-hGPR35, FLAG-tagged human GPR35; rGPR35, rat GPR35; DRG, dorsal root ganglion; cGMP, cyclic guanosine monophosphate

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30 s, and 72 °C for 1 min, and finally 72 °C for 4 min. Subsequently, the PCR products were separated by electrophoresis. Although some non-specific amplification was observed, a product of the expected size (900–1000 bp) was obtained. The fragment was gel-purified and TA-cloned using TOPO TA Cloning Kit (Invitrogen). Then several of the clones obtained were sequenced. Three independent experiments were performed to determine the sequence of rGPR35 gene without PCR errors (GenBank Accession No. AB240684).

2.3. Expression vectors

The original cDNA for human GPR35 (hGPR35) was isolated from human dorsal root ganglion (DRG) cDNA library (Life Technologies) using GENETRAPPER III cDNA Positive Selection System (Life Technologies) and an oligonucleotide probe (5′-ATG GTNYAYA TGCCNGGNGAYG-3′). Compared with the published sequence of hGPR35 (GenBank Accession No. AF027957), the hGPR35 obtained in this study had three non-synonymous single nucleotide polymorphisms (T108M, R174A, and R294S). Among them, T108M and R294S have been reported previously [5], while R174A was registered in the NCBI human genome database. The fragment for FLAG-tagged human GPR35 (FLAG-hGPR35) was generated by using the following pair of primers: 5′-CCGGAATTCGCCACCATGGATTAC-AAGGATGACGACGATAAGAATGGCACCTACAACACCTG-3′ and 5′-TCGTCTAGAATTAGGCGAGGGTCACGCACA-3′. All of cDNAs used in this study were subcloned into pcDNA3.1 (Invitrogen).

2.4. Cell transfection and calcium mobilization assay

Transfection of HEK293 cells was performed by using FuGENE 6 Transfection Reagent (Roche) according to the manufacturer's instructions. In brief, 400 000 cells were transfected with 40 μg of the expression vectors using 60 μL of FuGENE 6 Transfection Reagent. Two days after transfection, the cells were washed twice with Hanks' balanced salt solution (HBSS) without $CaCl_2$ or $MgCl_2$ (Gibco) and loaded with 5 μM Fura 2-AM (Dojindo) in HBSS containing 0.05% Pluronic F-127 (Sigma) for 1 h at 37 °C. After the incubation, the cells were harvested by centrifugation and diluted to $1–3 \times 10^5$ cells/mL in HBSS containing $CaCl_2$, $MgCl_2$, and $MgSO_4$ (Gibco). Then aliquots of the cell suspension (90 μL) were dispensed into 96-well plates (Coster). Addition of 10 μL of each ligand solution and measurement of intracellular calcium mobilization were performed with FDSS6000 (Hamamatsu Photonics). Concentration–response curves were determined using GraphPad Prism 3 (GraphPad Software).

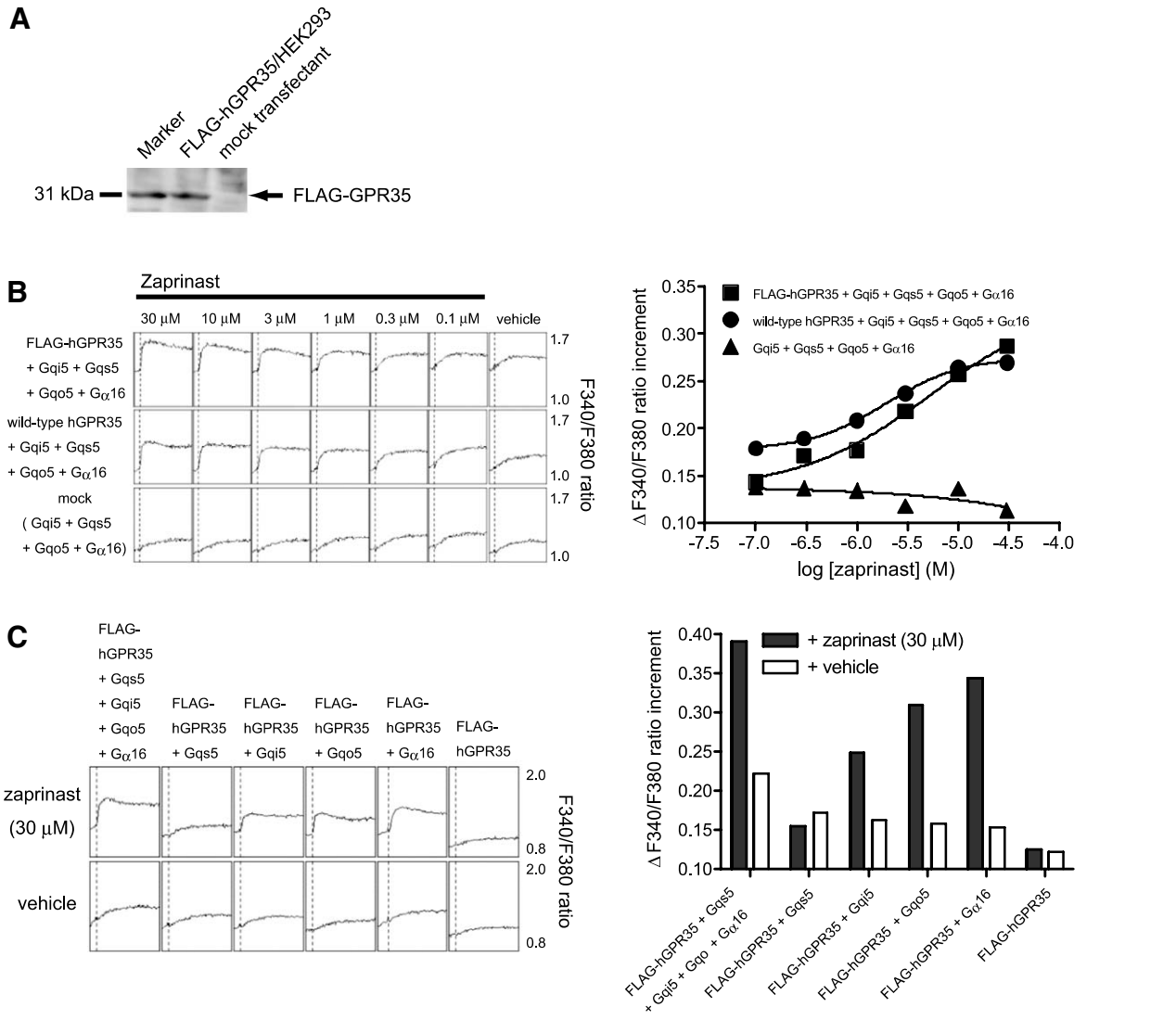

Fig. 1. Discovery of zaprinast as an agonist for hGPR35. (A) Expression of FLAG-hGPR35 in HEK293 cells confirmed by Western blotting. (B) HEK293 cells transiently coexpressing the receptor (FLAG-hGPR35 or wild-type hGPR35) and/or $G\alpha$ proteins (G_{qs5}, G_{qi5}, G_{qo5}, and $G\alpha_{16}$) were loaded with Fura-2, and then were exposed to zaprinast (broken line; addition of zaprinast). Dose–response curves are shown in parallel. EC_{50} values for FLAG-hGPR35 and wild-type hGPR35 were 5.2 and 1.9 μM, respectively. (C) GPR35 acted as a $G\alpha_{i/o}$- and $G\alpha_{16}$-coupled receptor for zaprinast in HEK293 cells. The Fura-2-loaded HEK293 cells transiently coexpressing FLAG-hGPR35 and a $G\alpha$ protein (G_{qs5}, G_{qi5}, G_{qo5}, or $G\alpha_{16}$) were exposed to zaprinast (broken line; addition of zaprinast).