FEBS Letters 580 (2006) 5003–5008

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

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Received 12 June 2006; revised 9 August 2006; accepted 9 August 2006

Available online 17 August 2006

Edited by Richard Marais

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

0014-5793/$32.00
doi:10.1016/j.febslet.2006.08.015

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).

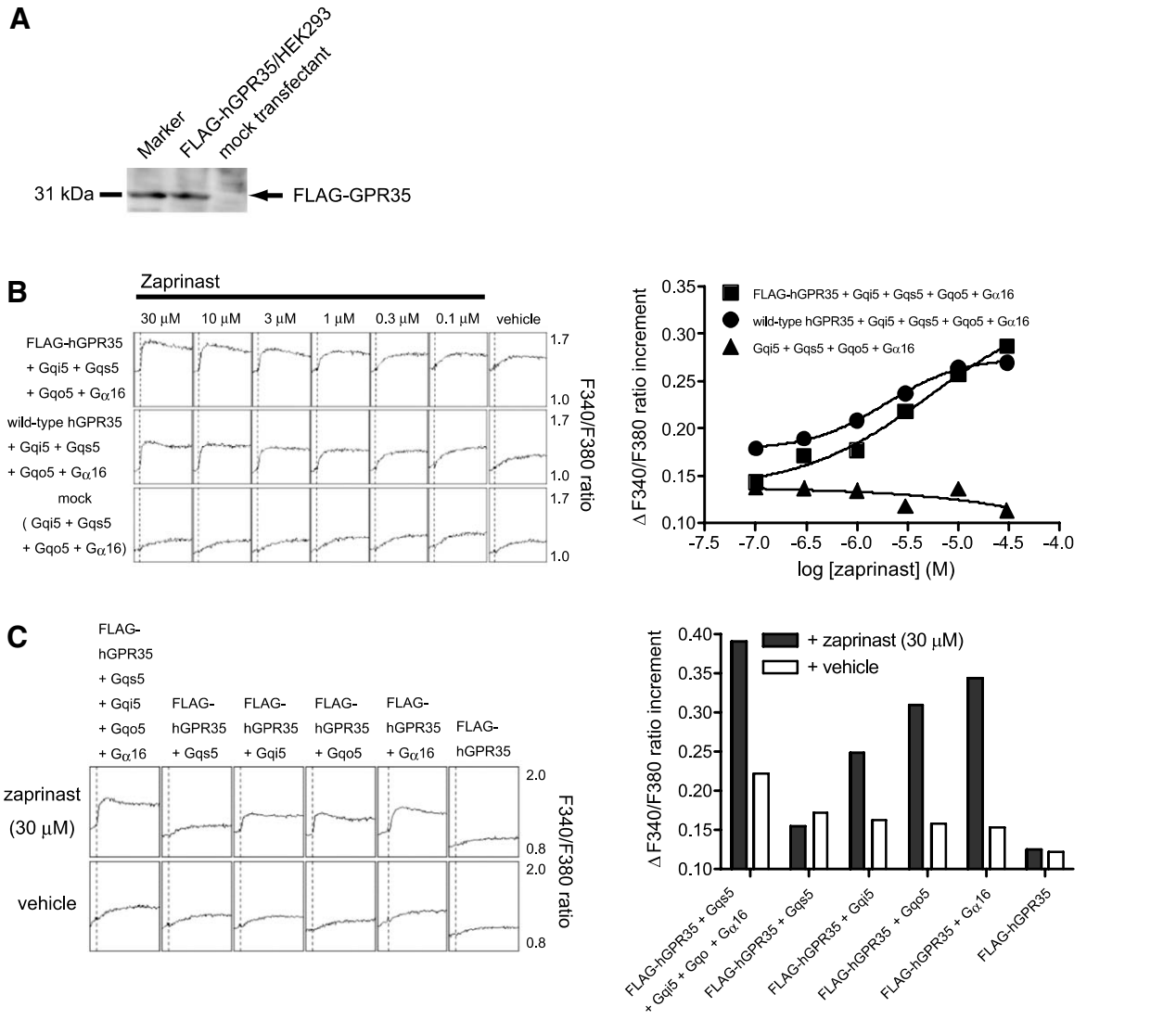

Fig. 1. Discovery of zaprinast as an agonist for hGPR35. (A) Expression of FLAG-hGPR35 in HEK293 cells confirmed by Western blotting. (B) HEK293 cells transiently coexpressing the receptor (FLAG-hGPR35 or wild-type hGPR35) and/or $G\alpha$ proteins (G_{qs5}, G_{qi5}, G_{qo5}, and $G\alpha_{16}$) were 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).

2.5. Western blotting

To confirm the expression of FLAG-hGPR35, 1.5×10^6 cells were dissolved in 150 μL of Tris–SDS–BME sample loading buffer (Owl) and analyzed by Western blotting. An aliquot (20 μL) of each sample was separated by SDS–PAGE and blotted onto polyvinylidene difluoride membranes. After blocking with TBS buffer containing 1.2% bovine serum albumin, the blot was probed with anti-FLAG M2 monoclonal antibody (Sigma) and the anti-mouse secondary antibody (Immunotech). The signal was visualized by using ECL Western blotting detection reagents (Amersham). The size of FLAG-hGPR35 observed in this study was about 31 kDa (Fig. 1A).

2.6. Tissue distribution of rGPR35

After euthanasia, DRG and spinal cord tissues were removed from a 10-week-old pregnant female Sprague–Dawley rat, and treated with ISOGEN (Toyobo) to purify total RNAs (all animal experiments performed in this study were approved by the Animal Ethics Committee of the Nagoya Laboratories of Pfizer Japan, based on the internal guidelines for animal experiments and adherence to Pfizer policy). Total RNAs from other tissues were purchased from BD Clontech or Unitech. To generate cDNA templates for RT-PCR, total RNA (5 μg) was reverse-transcribed by using SUPERSCRIPT Preamplification Systems. To assess possible contamination by genomic DNA, a series of samples without reverse transcriptase (−RT) were prepared in parallel as negative controls. Then 1 μL of each reverse-transcribed sample was used as a template for subsequent PCR. PCR was performed to amplify rGPR35 using two primers (5′-AAATTGTAGCATCCTCCCGTGGCC-3′ and 5′-TATCTTGGCTCTTGTGGGGTGTGC-3′) under the following condition: 94 °C for 4 min followed by 35 cycles of 94 °C for 20 s, 58 °C for 30 s, and 72 °C for 1 min, and finally 72 °C for 4 min. As a positive control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also amplified by the same PCR procedure (except that 30 cycles were performed) using the following primers: 5′-GTCTTCACCACCATGGAGAAGGCT-3′ and 5′-GTGATGGCATGGACTGTGGTCATGA-3′. After PCR,

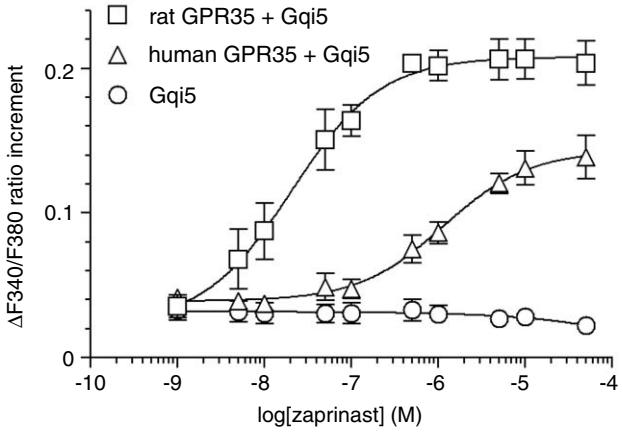

Fig. 3. Effect of zaprinast on rat GPR35. Fura-2-loaded HEK293 cells transiently coexpressing rat or human GPR35 and G_{qi5} were exposed to zaprinast and intracellular calcium mobilization was measured. Concentration–response curves are from three independents experiments with each point determined in quadruplicate.

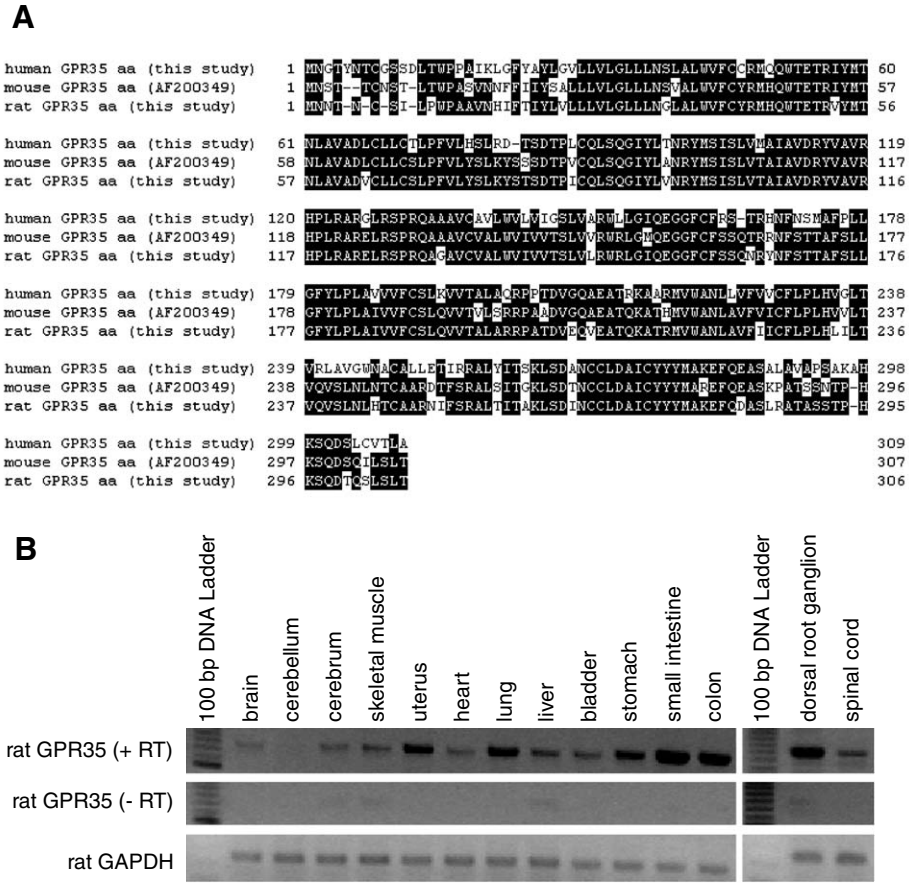

Fig. 2. Cloning and tissue distribution of rGPR35. (A) Alignment of the deduced amino acid sequences of human, mouse, and rat GPR35. (B) Tissue distribution of rGPR35 (expected size; 887 bp) determined by semi-quantitative RT-PCR (+RT). As a negative control (−RT), RT-PCR without the reverse transcriptase was performed in parallel. GAPDH (expected size; 245 bp) was also amplified in parallel as a positive control (rat GAPDH).

the samples were separated by electrophoresis and DNA was visualized by ethidium bromide staining. The PCR products were confirmed by sequencing.

3. Results

3.1. Identification of zaprinast as an agonist for hGPR35

To discover ligands for GPR35, we prepared HEK293 cells transiently coexpressing FLAG-hGPR35, three chimeric $G\alpha$ proteins (G_{qs5}, G_{qi5}, and G_{qo5}) [12], and promiscuous $G\alpha_{16}$ [12], because the downstream signaling molecules of GPR35 were unknown at that time. The chimeric $G\alpha$ proteins and $G\alpha_{16}$ are used to redirect $G\alpha_s$- and/or $G\alpha_{i/o}$-mediated signals to the $G\alpha_q$ signaling pathway, thus allowing evaluation of the activity of most GPCRs by a calcium mobilization assay using the above-mentioned G proteins [12]. After cells were loaded with Fura-2, about 1000 compounds were assayed by using the FDSS6000 to monitor intracellular calcium mobilization. Among them, zaprinast (a well-known PDEs inhibitor) induced intracellular calcium mobilization in HEK293 cells coexpressing FLAG-hGPR35 and the four exogenous G proteins (Fig. 1B), while there was little change of calcium in the control transfectant only expressing the chimeric $G\alpha$ proteins and $G\alpha_{16}$ (Fig. 1B). Further analysis showed that zaprinast induced intracellular calcium mobilization in the transfectant coexpressing FLAG-hGPR35 and the four exogenous $G\alpha$ proteins in a concentration-dependent manner (Fig. 1B). A similar result was obtained in an experiment using wild-type hGPR35 (Fig. 1B). These results suggest that zaprinast is an agonist for hGPR35.

Next, we examined which of the $G\alpha$ proteins was involved in mediation of the signaling. When FLAG-hGPR35 was coexpressed in HEK293 cells with G_{qi5}, G_{qo5}, or $G\alpha_{16}$, zaprinast

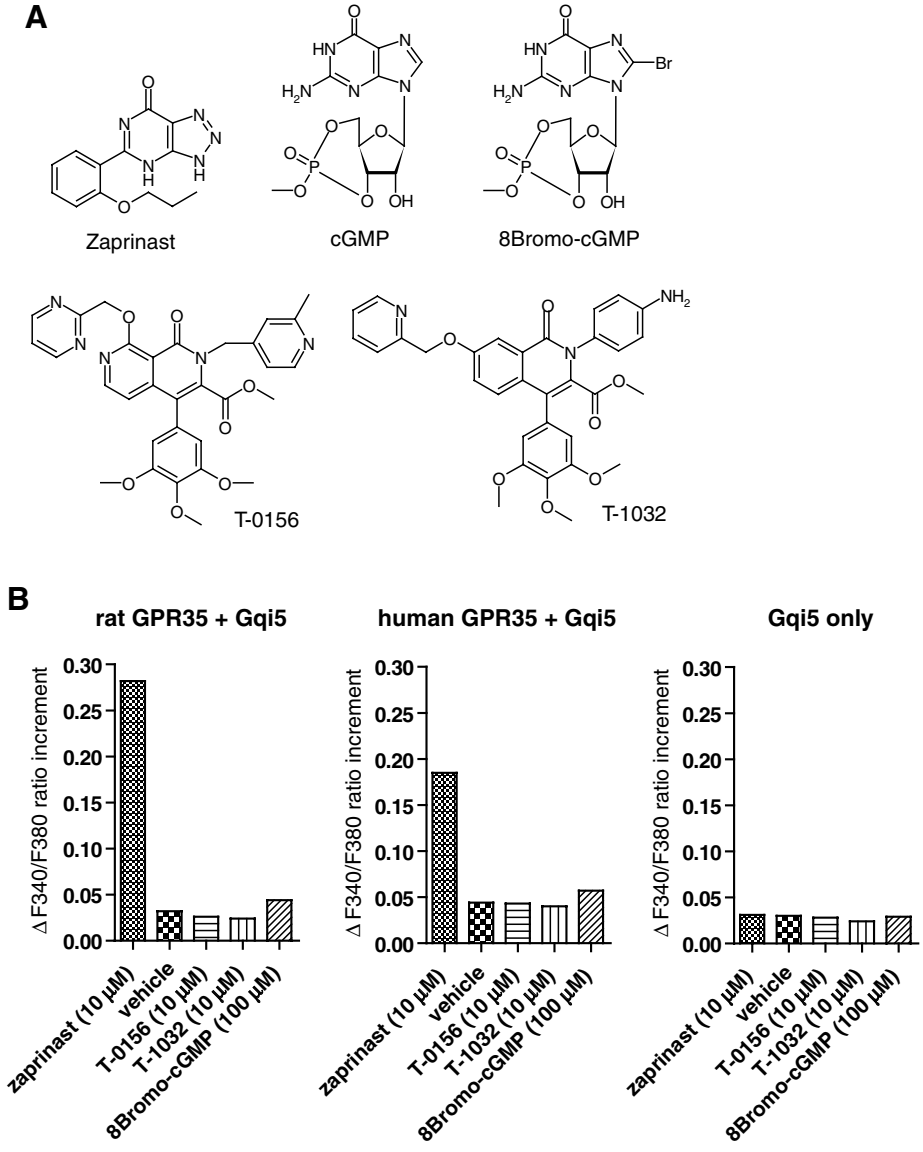

Fig. 4. Effects of selective PDE5/PDE6 inhibitors (T-0156 and T-1032) and 8Bromo-cGMP on the intracellular calcium mobilization in the HEK293 cells expressing GPR35 and G_{qi5}. (A) Structures of assayed compounds. For comparison, structure of cGMP is also shown. (B) Fura-2-loaded HEK293 cells transiently coexpressing rat or human GPR35 and G_{qi5} were exposed to T-0156 (10 μM), T-1032 (10 μM), 8Bromo-cGMP (100 μM) or zaprinast (10 μM).

induced intracellular calcium mobilization (Fig. 1C). However, no response to zaprinast was observed in either cells expressing G_{qs5} and FLAG-hGPR35 or cells expressing FLAG-hGPR35 alone (Fig. 1C). These results show that hGPR35 functions as a $G\alpha_{i/o}$- and $G\alpha_{16}$-coupled receptor for zaprinast in HEK293 cells.

3.2. Cloning and tissue distribution of rGPR35

Next, we have cloned rGPR35 to evaluate the effect of zaprinast on this receptor. To identify and isolate rGPR35 cDNA clone, we designed a pair of primers based on the sequence of mouse GPR35, and the entire ORF of rGPR35 was amplified by RT-PCR using these primers. Then the PCR product of the expected size was TA-cloned and sequenced. The PCR product contained a 921-bp ORF of rGPR35 (data not shown). The amino acid sequence of the cloned rGPR35 showed 72% and 85% identity with human and mouse GPR35, respectively (Fig. 2A). We also investigated tissue distribution of rGPR35 by semi-quantitative RT-PCR (Fig. 2B). Consistent with previous studies preformed in human [5,6], we detected expression of rGPR35 in lung, stomach, small intestine, colon, and skeletal muscle. In addition, a relatively high level of expression in uterus and DRG, as well as moderate expression in brain, cerebrum, heart, liver, bladder, and spinal cord, was shown in this study (Fig. 2B).

3.3. Zaprinast as a potent agonist for rGPR35

To confirm that zaprinast was an agonist for rGPR35, HEK293 cells transiently coexpressing rat or human GPR35 and G_{qi5} were prepared, and the transfectants were exposed to zaprinast (Fig. 3). In this assay, zaprinast caused the moderate activation of hGPR35 (geometric mean EC_{50} value of 840 nM), while zaprinast had a potent effect on the transfectant co-expressing rGPR35 and G_{qi5} (geometric mean EC_{50} value of 16 nM). These results suggest that the zaprinast is able to act as a potent agonist for rGPR35.

3.4. PDE5/PDE6 inhibitors without GPR35 agonist activity

To further support our findings in this study, we evaluated the effect of phosphodiesterase inhibitors of a different chemical class on HEK293 cells transiently coexpressing either rat or human GPR35 and G_{qi5}. T-0156 and T-1032 are known as potent PDE5 inhibitors (IC_{50} values are 0.23 and 1 nM, respectively) [11,13,14] and also inhibit PDE6 (IC_{50} values are 56 and 28 nM, respectively) [11,13,14]. T-0156 and T-1032 are structurally different from zaprinast, a cGMP analog (Fig. 4A) [13,14]. As shown in Fig. 4B, T-0156 (10μM) and T-1032 (10 μM) did not induce substantial intracellular calcium mobilization in HEK293 cells transiently coexpressing either rat or human GPR35 and G_{qi5}, although zaprinast did so. These results indicate that T-0156 and T-1032 do not act as GPR35 agonists, and support our conclusion that the effect of zaprinast on intracellular calcium mobilization is attributable to its agonist activity for GPR35, not to inhibitory activity for PDE5/PDE6. In addition, we also confirmed that 8-bromoguanosine 3′,5′-cyclic monophosphate (8Bromo-cGMP), a membrane-permeable analogue of cGMP, did not induce the intracellular calcium mobilization in these transfectants (Fig. 4B), suggesting that elevation of intracellular cGMP level was unlikely to be responsible for the intracellular calcium mobilization in this assay system.

4. Discussion

In this study, we demonstrated that zaprinast (a cGMP-PDE inhibitor) induced the intracellular calcium mobilization in the cells coexpressing GPR35 and G_{qi5}, G_{qo5}, or $G\alpha_{16}$. Induction of intracellular calcium mobilization by zaprinast in the GPR35-expressing transfectants was due to selective activation of the GPR35-$G\alpha$ (G_{qi5}, G_{qo5}, or $G\alpha_{16}$) signaling pathway, rather than inhibition of phosphodiesterases (PDE5, PDE6, PDE9, PDE10, and PDE11), direct activation of $G\alpha$ proteins, or non-selective stimulation of endogenous GPCR-$G\alpha$ signaling pathway, because expression of both GPR35 and $G\alpha$ proteins (G_{qi5}, G_{qo5}, or $G\alpha_{16}$) was necessary preconditions for this action of zaprinast (Figs. 1B, C, and 3). Thus, these observations strongly suggest that zaprinast acts as an agonist for GPR35. Zaprinast potently induced intracellular calcium mobilization in the transfectant coexpressing rGPR35 and G_{qi5} with an EC_{50} value of 16 nM (Fig. 3), while effects of zaprinast on PDEs are moderate or weak (see Section 1). In addition, the selective PDE5/PDE6 inhibitors T-0156 and T-1032 did not have any effect on the transfectants coexpressing GPR35 and G_{qi5} (Fig. 4B). Furthermore, 8Bromo-cGMP did not induce intracellular calcium mobilization in our assay (Fig. 4B). These facts also support our conclusion.

Several recent reports have suggested that zaprinast may possess pharmacological activities other than PDEs inhibition. For example, Wibberley et al. demonstrated a nitric oxide (NO)-independent role for zaprinast in the regulation of urethral sphincter tone [15]. Yoon et al. reported that intrathecal zaprinast had an antinociceptive effect in the rat formalin test, and that this effect was not related to the NO-cGMP-potassium channel pathway [16]. Because cGMP PDEs are deeply involved in the NO-cGMP signaling pathway (NO activates soluble guanylyl cyclase to increase the intracellular cGMP level, while cGMP PDE terminates NO/cGMP-dependent signals by degrading cGMP) [17], these NO/cGMP-independent effects of zaprinast may be mediated by GPR35 activation. Numerous reports about pharmacological studies of PDEs inhibition by zaprinast have been published [7]. However, it may be necessary to repeat those experiments with different structural classes of selective PDE inhibitors since the GPR35 agonist activity of zaprinast was revealed in this study.

It is important to note that zaprinast is a lead compound for sildenafil (Viagra™) [7], indicating that zaprinast has favorable chemical properties for drug design. It may be feasible to identify potent and selective agonists and/or antagonists for hGPR35 without phosphodiesterase inhibitory activity from among compounds related to zaprinast. In fact, we have already identified a number of GPR35 agonists with different potency and species selectivity among zaprinast derivatives (Taniguchi et al, Patent Application WO2005085867(A2)), suggesting that zaprinast may serve as a lead compound to develop drugs that modulate GPR35 activity.

During the review process of this manuscript, Wang et al. reported that kynurenic acid was a natural ligand for GPR35 [18]. They independently showed that GPR35 acted as a $G\alpha_{i/o}$- and $G\alpha_{16}$-coupled receptor using kynurenic acid, which was consistent with our results obtained with zaprinast. However, affinity of kynurenic acid for GPR35 is relatively low (EC50 values of 7.4–39.2 μM) [18]. Thus, other chemical classes of natural ligand with higher affinity may be expected. Because GPR35 shares homology with GPCRs belonging to

the P2Y family [2] and zaprinast is a xanthine derivative [7], our findings may provide a hint to discover the natural ligand for GPR35.

Acknowledgments: We thank Dr. Tetsuhiko Nakagawa for preparation of rat DRG and spinal cord, Kenji Taki for providing cDNA of hGPR35, Dr. Don Walker for information about PDEs inhibitors, Dr. Tetsujo Sujaku for collaboration, Masahiro Nanjo for patent application, and Drs. Yoichi Kurebayashi and Hiroshi Iwata for valuable advices.

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