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Extracellular acidification activates ovarian cancer G-protein-coupled receptor 1 and GPR4 homologs of zebra fish

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ABSTRACT

Mammalian ovarian G-protein-coupled receptor 1 (OGR1) and GPR4 are identified as a proton-sensing G-protein-coupled receptor coupling to multiple intracellular signaling pathways. In the present study, we examined whether zebra fish OGR1 and GPR4 homologs (zOGR1 and zGPR4) could sense protons and activate the multiple intracellular signaling pathways and, if so, whether the similar positions of histidine residue, which is critical for sensing protons in mammalian OGR and GPR4, also play a role to sense protons and activate the multiple signaling pathways in the zebra fish receptors. We found that extracellular acidic pH stimulated CRE-, SRE-, and NFAT-promoter activities in zOGR1 overexpressed cells and stimulated CRE- and SRE- but not NFAT-promoter activities in zGPR4 overexpressed cells. The substitution of histidine residues at the 12th, 15th, 162th, and 264th positions from the N-terminal of zOGR1 with phenylalanine attenuated the proton-induced SRE-promoter activities. The mutation of the histidine residue at the 78th but not the 84th position from the N-terminal of zGPR4 to phenylalanine attenuated the proton-induced SRE-promoter activities. These results suggest that zOGR1 and zGPR4 are also proton-sensing G-protein-coupled receptors, and the receptor activation mechanisms may be similar to those of the mammalian receptors.

1. Introduction

Mammalian ovarian cancer G-protein-coupled receptor 1 (OGR1) and GPR4 were originally reported to be activated by lysolipids, such as sphingosylphosphorylcholine (SPC) and lysophosphatidylcholine (LPC), which act as ligands [1]; however, the direct binding of the lipids to the receptors has not been proven. On the other hand, Ludwig reported that the human receptors sense extracellular protons and activate intracellular signaling pathways through trimeric G proteins [2]. Thus, human OGR1 stimulation causes phospholipase C (PLC) activation and subsequent intracellular Ca^{2+} ($[Ca^{2+}]_i$) mobilization through $G_{q/11}$ proteins, and human GPR4 stimulates adenylyl cyclase activation through G_s proteins, in response to the extracellular acidification. Later, we showed that human OGR1 is also coupled to G_s/cAMP and G_{13}/Rho signaling pathways [3,4], and GPR4 is coupled to G_{13}/Rho and $G_{q/11}$/PLC signaling pathways [4,5], when the receptors were overexpressed in HEK293T cells. Site mutagenesis studies show that the specific histidine residues at the extracellular surface of the receptors are responsible for proton sensing [2,5].

OGR1 and GPR4 expressions are widely detected in many tissues [1]. The receptor expressions are also reported in vascular endothelial and smooth muscle cells [6–19]. Under an acidic pH condition, OGR1 mediates COX-2, MKP-1, IL-6, CTGF, VCAM-1, and ICAM-1 expressions and PGI_2 production in human vascular smooth muscle and airway smooth muscle cells [15–19]. GPR4 mediates VCAM-1, ICAM-1, COX-2, and a number of inflammatory gene expressions [9,10]. The physiological and pathophysiological

Abbreviations: [1]z, zebra fish (*Danio rerio*); OGR1, ovarian cancer G-protein-coupled receptor 1; GPCR, G-protein-coupled receptor.

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roles of OGR1 and GPR4 are examined using OGR1-and GPR4-deficient mice. However, the molecular mechanism by which the receptors are concerned with the phenotype is largely unknown.

Zebra fish can provide a useful vertebrate model system to elucidate the molecular mechanism of the receptor functions in vivo. The embryo is transparent, and development takes place outside the maternal body. These characteristics make them suitable to use for in vivo imaging. Indeed, zebra fish have been used in especially studies of blood vessel formation [20] and cancer invasion [21].

We found zebra fish OGR1 and GPR4 homologs (zOGR1 and zGPR4) in the genome database; however, their characterizations have not yet been reported. In this study, we characterized the functions of the homologs and focused on their ligand specificity and signaling pathways by expressing them in HEK293T cells. We found that these receptors sense protons like the mammalian receptors and activate multiple signaling pathways.

2. Materials and methods

2.1. Materials

A dual luciferase kit was purchased from Promega (Tokyo, Japan); Fura2 AM from Dojindo (Tokyo, Japan); fatty acid-free BSA from Calbiochem-Novabiochem Co. (San Diego, CA); and Lipofectamine 2000 Reagent from Life Technologies (Tokyo, Japan). The sources of all other reagents were the same as described previously [4,5].

2.2. Preparation of receptor cDNA plasmids and expression

The entire coding regions of zOGR1 (1032 bp, GenBank accession No. XM_001339552), zGPR4 (1122 bp, GenBank accession No. XM_687123), zOGR1-H4F (the 12th, 15th, 162th, and 264th positions of histidine from the N terminus were substituted with phenylalanine), GPR4-H78F (the 78th position of histidine from the N terminus was substituted with phenylalanine), and GPR4-H84F (the 84th position of histidine from the N terminus was substituted with phenylalanine) were synthesized and cloned into a pBo-CMV vector (Takara, Japan) with a Kozak sequence (CCACC) in front of the 1st methionine codon.

The wild-type or the substituted constructs were transfected into HEK293T cells using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA) and plated onto 12 multiplates, as described previously [4].

2.3. Cell cultures

HEK293T cells were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. The cells were cultured in DMEM containing 10% (v/v) FBS (Life Technologies) in a humidified air/CO_2 (19:1) atmosphere.

2.4. Measurement of intracellular calcium

The change in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured using a fura-2 method as described previously [22,23]. The changes in the intensities of 540 nm fluorescence obtained by 340 nm and 380 nm excitations were monitored by an FP-8200 spectrofluorometer (JASCO, Tokyo, Japan).

2.5. Dual luciferase reporter assay

cAMP response element (CRE)-, serum response element (SRE)-, or nuclear factor of activated T-cells (NFAT)-driven promoter activity was assayed using the PathDetect Signal Transduction Pathway cis-Reporting Systems (Agilent Technologies, Santa Clara, CA) as described in the previous paper [4].

2.6. Reverse transcriptase (RT) polymerase chain reaction (PCR)

Total RNA was extracted from each 10 embryos or baby fishes at 3, 24, 48, 72, and 96 h post fertilization (hpf). RT-PCR was carried out as follows: preheat at 95 °C for 4 min, then proceed to 34 cycles (OGR1 and GPR4) or 30 cycles (β-actin) at 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s. The forward primers were CGGGACTGCAACTTCATTGAG for zOGR1, GAAGTGAGACCATGTGCAAC for zGPR4, and GTGATGGACTCTGGTGATGGTGT for zβ-actin. The reverse primers were AGTGGAGTGTGTGTTGAACCTTC for zOGR1, AGAGGTCTGCTATCGAGAGGTTC for zGPR4, and TGAAGCTGTAGCCTCTCTCGGTC for zβ-actin. The expected size of each product was 204 bp for zOGR1, 201 bp for zGPR4, and 148 bp for zβ-actin.

2.7. Data presentation

All the experiments were performed in duplicate or triplicate. The results of multiple observations are presented as the mean ± SE from more than three different batches of cells unless otherwise stated. Statistical significance was assessed by ANOVA; values were considered significant at $p < 0.05$ (*).

3. Results

3.1. Zebra fish OGR1 and GPR4 homologs sense protons to activate multiple signaling pathways

Transient expression of wild-type zebra fish OGR1 (zOGR1) and GPR4 (zGPR4) homologs in HEK293T cells induced CRE-, SRE-, and NFAT-driven transcriptional activation when the extracellular pH was reduced from 7.6 to 6.3, indicating that these homologs sense protons to activate multiple signaling pathways (Fig. 1A, B). The activated signaling pathways were different between zOGR1 and zGPR4: zOGR1 activates all three (CRE, SRE, and NFAT) pathways. On the other hand, zGPR4 activates CRE and SRE pathways but not the NFAT pathway.

In the previous study [4], we showed that CRE, SRE, and NFAT promoters were activated through the G_s-protein/adenylyl cyclase/cAMP signaling pathway, $G_{12/13}$-protein/Rho signaling pathway, and G_q-protein/phospholipase C–Ca^{2+} signaling pathway in HEK293T cells, respectively. In agreement with this, the $[Ca^{2+}]_i$ was increased, which reflects phospholipase C activation when extracellular pH was reduced from 7.6 to 6.3 in the zOGR1 expressed cells (Fig. 1C). On the other hand, the $[Ca^{2+}]_i$ concentration in the zGPR4 expressed cells was not significantly increased from that in vector-transfected HEK293 cells at pH 6.3 (Fig. 1C). The significant activation of CRE and SRE promoters was observed in the zOGR1 and zGPR4 expressed cells, even at a neutral pH of 7.4 (Fig. 1A, B). Thus, zOGR1 and zGPR4 are stimulated under neutral pH as if the receptors had constitutive activity.

3.2. The similar position of histidine residue in zOGR1 and zGPR4 to that of human receptors is involved in the receptor activation

The histidine residues in the extracellular surface of human OGR1 and GPR4 are crucial for sensing protons [2,5]. The amino acid identity between human and zebra fish OGR1 is 57%, and that between human and zebra fish GPR4 is 73%. The four residues of the five crucial histidine residues (depicted as H in bold type in Fig. 2A) to sense protons in human OGR1 are conserved in zOGR1. To clarify that the histidine residues of zOGR1 play a role to sense protons, we made a construct in which the four histidine residues of the 12th,