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**A missense mutation of Leu74Pro of OGR1 found in familial
amelogenesis imperfecta actually causes the loss of the pH-sensing
mechanism**

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Abbreviations:

[Ca²⁺]_i; intracellular Ca²⁺ concentration; DAPI, 4,6-diamidino-2-phenylindole; GAPDH,

glyceraldehyde-3-phosphate dehydrogenase; GPCR, G protein-coupled receptor; GPR4,

G protein-coupled receptor 4; HA, hemagglutinin; OGR1, ovarian cancer G protein-

coupled receptor 1; and TDAG8, T cell death-associated gene 8-

Abstract

Ovarian cancer G protein-coupled receptor 1 (OGR1), also known as GPR68, is a proton-sensing G protein-coupled receptor (GPCR) coupling to $G_{q/11}$ /phospholipase C/ Ca^{2+} signaling pathways. The specific histidine residues at the extracellular surface of OGR1 are suggested to be involved in the proton sensing. Later, some metal ions, including nickel ion (Ni^{2+}), are also indicated to be OGR1 ligands. OGR1 polymorphic variants have recently been found in three families with amelogenesis imperfecta, which suggested that OGR1 is required for the process of dental enamel formation. One of these families possesses a missense mutation from leucine to proline at 74 (L74P) of OGR1. In the present study, we characterized HEK293 cells with L74P OGR1 (L74P-OGR1) and hemagglutinin (HA)-tag, as compared with cells with wild-type OGR1 (WT-OGR1) and HA-tag. We found that either acidic pH or $NiCl_2$ induced intracellular Ca^{2+} mobilization and morphological change in WT-OGR1-transfected cells; however, the extracellular stimulus-induced actions were severely damaged in L74P-OGR1-transfected cells. We further confirmed that either WT-OGR1 or L74P-OGR1 is localized mainly in the surface of the cells, but only WT-OGR1 is internalized in response to acidification or $NiCl_2$.

Thus, the L74P-OGR1 protein may be distributed in the plasma membranes but severely damaged in the receptor functions. We speculate that L74P in the second transmembrane domain in OGR1 may result in conformational changes in the receptor, thereby disturbing the sensing extracellular signals, i.e., protons or metal ions, and/or transducing them to the intracellular signaling machinery through G proteins.

Keywords: extracellular acidification, G protein-coupled receptor, intracellular Ca²⁺, missense variant, OGR1

1. Introduction

Proton-sensing OGR1-family GPCRs include ovarian cancer G protein-coupled receptor 1 (OGR1 or GPR68), G protein-coupled receptor 4 (GPR4), and T-cell death-associated gene 8 (TDAG8 or GPR65), which sense an acidic pH of more than 6.4 [1]. Ludwig et al. reported that OGR1 and GPR4 sense extracellular pH, resulting in the activation of the phospholipase C/ Ca^{2+} and adenylyl cyclase/cAMP signaling pathways through $\text{G}_{q/11}$ and G_s proteins, respectively [2, 3]. Later, proton sensitivity was also reported for TDAG8 [3, 4]. Protonation of histidine residues on the extracellular domains of proton-sensing OGR1-family receptors has been suggested to cause conformational changes in the receptors, thereby facilitating coupling with G proteins [2, 5, 6]. OGR1 has recently been reported to be activated by metal ions including nickel, iron, zinc, cobalt, and manganese; in this case again, extracellular histidine residues may be involved in activation of the receptor [7]. Thus, extracellular protons and divalent metal ions may activate OGR1 through certain extracellular histidine residues [8, 9].

Homozygous variants in OGR1 have recently been found in three families (termed AI-5, AI-178, and TKTO) with amelogenesis imperfecta, suggesting that OGR1 is

required for the process of dental enamel formation [10]. Two of the homozygous variants, i.e., mutation of family AI-5 (p.Phe129_Asn278del) and that of family AI-178 (p.Lys223Glyfs*113), were predicted to cause a large in-frame deletion of four of the seven transmembrane helices and a frameshift deletion of two of the encoded protein's transmembrane helices, respectively [10]. Thus, any protein produced from these homozygous mutations is likely to lack the physiological function of the wild-type protein. On the other hand, another variant of family TKTO is a homozygous missense mutation from leucine to proline at 74 (p.Leu74Pro), which is located in the second transmembrane helix of OGR1. Leu 74 is conserved in all proton-sensing GPCRs including OGR1, GPR4, and TDAG8, and the replacement of a highly conserved leucine residue with a proline immediately adjacent to another proline residue Pro75 is considered to destabilize the secondary structure of the second transmembrane helix of OGR1 and severely alter the functioning of the protein [10], but the functional evidence of this speculation is lacking.

In the present study, we characterized the OGR receptor with L74P substitution (L74P-OGR1) as compared with the wild-type OGR1 receptor (WT-OGR1) in HEK293 cells expressing these receptors. We found that cellular activities to induce receptor

internalization, intracellular Ca^{2+} mobilization, and cell morphological change in response to extracellular protons and NiCl_2 were severely damaged by the L74P mutation, although both L74P-OGR1 and WT-OGR1 receptors seem to be localized in plasma membranes under unstimulated conditions. Our results suggest that L74P in the second transmembrane domain in OGR1 may cause receptor conformational changes and, thereby, the uncoupling of extracellular signals to intracellular signaling pathways through G proteins.

2. Materials and Methods

2.1. Materials

Lysophosphatidic acid (LPA, 1-oleoyl-*sn*-glycero-3-phosphate) was purchased from Sigma-Aldrich (St Louis, MO); anti-HA (#3724) antibody was from Cell Signaling Technology (Beverly, MA); anti-rabbit Alexa Fluor 488 conjugated secondary antibody and Lipofectamine 2000 Reagent were from Thermo Fisher Scientific (Waltham, MA); fatty acid-free bovine serum albumin (BSA) was from Calbiochem (San Diego, CA);

Fura-2/acetoxymethylester (Fura-2/AM) and 4', 6-diamidino-2-phenylindole (DAPI) were from Dojindo (Tokyo, Japan); NiCl₂ (019-19741) was from Wako Chemicals (Osaka, Japan). YM-254890 was a gift from Dr. M. Taniguchi of Astellas (Tsukuba, Japan). The sources of all other reagents were the same as described previously [4, 6, 11].

2.2. Preparation of receptor cDNA plasmids

The entire coding region of human OGR1 (WT-OGR1, 1128 bp, NM_003485) was cloned from a human cDNA library by reverse transcription polymerase chain reaction (RT-PCR) as described previously [6]. The OGR1 fragment (WT-OGR1) was amplified by PCR with the 5'-primer: GPR68-1 and the 3'-primer: GPR68-2, and the OGR1 fragment (WT-OGR1-HA) containing hemagglutinin (HA)-tag at the C terminus was amplified by PCR with the 5'-primer: GPR68-1 and the 3'-primer: GPR68-2HA (Supplementary Table 1). The OGR1 fragments were subcloned into Hind III/Eco RI sites of pcDNA3.1 (Thermo Fisher Scientific, Waltham, MA). The L74P-OGR1 mutant, an OGR1 missense mutant found in a family with amelogenesis imperfecta [10], was generated by PCR-based mutagenesis. The amplified OGR1 fragments with L74P (L74P-

OGR1 and L74P-OGR1-HA) were subcloned into Hind III/Eco RI sites of pcDNA3.1. Each DNA sequence was confirmed. The HEK293 cells transiently transfected with the OGR1 construct (1128 bp, NM_003485) showed proton concentration-dependent increases in the serum response element-driven transcriptional activity consistent with the previous results with OGR1 (1098 bp, NM_003485), as described in [6]. The HA sequence at the C terminus of OGR1 did not affect the proton concentration-induced $[Ca^{2+}]_i$ change in HEK293 permanent cell lines (data not shown).

2.3. Cell culture

HEK293 cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) as described previously [6]. The cells were cultured in a humidified air/CO₂ (19:1) atmosphere at 37 °C. The cells were plated on 6 cm dishes for the extraction of total RNA for RNA analysis, on 12-well glass slides for the study with a confocal laser-scanning microscope, and on 10 cm dishes for the evaluation of $[Ca^{2+}]_i$ change. When cells had become 80-90% confluent, the culture

medium was changed to fresh medium containing 0.1% BSA to make them quiescent overnight.

2.4. Transfection of OGR1 receptors

For the transfection of OGR1 receptors in HEK293 cells, the cells were plated at 10^5 cells on a 6-well plate 1 day prior to transfection. Transfections were carried out by the lipofection technique with Lipofectamine 2000 Reagent (Thermo Fisher Scientific, Waltham, MA) as described previously [4, 6]. The HEK293 cells were transfected with the pcDNA3.1 empty vector alone or the vector containing WT-OGR1, L74P-OGR1, WT-OGR1-HA, and L74P-OGR1-HA. Permanent cell lines were selected with neomycin (G418 sulfate at 0.5 mg/ml).

2.5. Measurement of $[Ca^{2+}]_i$

The cells on a 10 cm dish were gently harvested with PBS containing 0.05% trypsin-EDTA. After a 20 min incubation of the cells with 1 μ M Fura-2/AM at 37 °C in Ham's F-10 medium containing 0.1% BSA, the cells were washed two times with ice-cold

HEPES-buffered medium composed of 20 mM HEPES, pH7.8, 134 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 2 mM CaCl₂, 2.5 mM NaHCO₃, 5 mM glucose, and 0.1% BSA and finally suspended in the same medium. The Fura 2-loaded cells were warmed for 3 min at 37 °C, and then the [Ca²⁺]_i change was monitored in the intensities of 540 nm fluorescence obtained by the two excitations (340 nm and 380 nm), which were monitored by CAF-110 fluorometer (JASCO, Tokyo, Japan). Other experimental conditions were the same as those previously described [11, 12].

2.6. RNA analysis

For analysis of the mRNA for OGR1-family receptors, total RNA was prepared from HEK293 cells in accordance with the manufacturer's instructions for RNAiso Plus (TAKARA BIO INC, Otsu, Japan). RT-PCR was performed with specific primers, as shown in Supplementary Table 1. The cDNAs were synthesized from the DNase I-treated RNA samples with or without reverse transcriptase and subjected to RT-PCR. The thermal cycling conditions were as follows: 2 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 50-62 °C, 1 min at 72 °C, and 2 min at 72 °C. The annealing conditions used were:

the vector multicloning site (MCS) containing OGR1 with pcDNA3-T7 and pcDNA3-BGH at 50 °C, GAPDH with GAPDH-1 and GAPDH-2 at 55 °C, OGR1 with GPR68-1 and GPR68-2, and OGR1-HA with GPR68-1 and GPR68-2HA at 62 °C. The amplified fragments were subjected to 1% agarose gel electrophoresis. The human mRNA sequence (822-1061, BC_025925) could be detected by the mouse GAPDH primers, as described previously [13].

2.7. Analysis of receptor internalization and morphological cell changes

Permanent cell lines expressing WT-OGR1-HA and L74P-OGR1-HA were grown in 12-well glass slides in DMEM supplemented with 10% FBS. The cells were serum starved for 8 h in fresh HEPES-buffered RPMI-1640 containing 0.1% BSA. The cells were then stimulated for 0.5 h with 1N HCL (final pH 6.8) or 100 μ M NiCl₂ in the same medium at pH 7.4. The cells were then washed with PBS and fixed for 10 min with 4% formaldehyde in PBS at room temperature. The fixed cells were used for immunostaining for the localization of OGR1-HA receptors with anti-HA antibody (1:800 dilution). The positive signals were detected by Alexa Fluor 488 conjugated secondary antibody (1:200

dilution). The fixed cells were also stained with DAPI in PBS for 5 min and mounted on a glass slide. Analysis was performed at 405 nm and 488 nm laser lines to excite the DAPI and Alexa Fluor 488, respectively, using a confocal laser-scanning microscope (FV1000-D, Olympus, Tokyo, Japan).

For morphological changes, the photographed cells were analyzed by Fit Ellipse using ImageJ software. The results were expressed as a distribution of observed frequencies of the ratio of the major axis to the minor axis of the cells (fold).

2.8. Data presentation

All experiments were performed in duplicate or triplicate. The results of multiple observations are presented as the mean \pm SEM or as representative results from more than two different batches of cells unless otherwise stated. Statistical significance was assessed by the Student's *t*-test; values were considered significant at $*p < 0.05$ and $**p < 0.01$.

3. Results

3.1. Alteration of the predicted secondary structure for the human OGR1 receptor due to the missense mutation of L74P

The histidine residues (H17, H20, H84, H169, and H269) in human OGR1 have been shown to be involved in proton sensing [2, 5]. We have provisionally speculated that the L74P mutation immediately adjacent to another proline residue was considered likely to destabilize the secondary structure of the second transmembrane helix of OGR1 and severely alter the functioning of the protein [10]. In the present study, we first employed a secondary structure prediction for human OGR1 by PSIPRED V4.0 (Supplementary Fig. 1). The replacement of L74P in the OGR1 receptors seems to cause an alteration of the helix structure in the second transmembrane domain (Supplementary Fig. 2). Thus, we speculate again that the change in the secondary structure of the second transmembrane in the L74P mutant might cause the loss of OGR1 receptor function.

3.2. Reduction of OGR1 receptor activity coupling to Ca²⁺ signaling pathways due to the missense mutation of L74P

We examined the expression level of mutated (L74P) OGR1 tagged with HA (L74P-OGR1-HA) in wild-type OGR1 tagged with HA (WT-OGR1-HA) in the cells. Unfortunately, we failed to compare the protein expression level by Western blotting; therefore, the expression level was confirmed by RT-PCR, which showed a comparable expression level of L74P-OGR1-HA with WT-OGR1-HA (Fig. 1). Using receptor-transfected cells, we examined Ca^{2+} mobilization, a typical OGR1 receptor signaling pathway, in response to acidic pH and NiCl_2 . In Fig. 2, a typical trace pattern is shown in A-C, and summarized results are shown in D. Either acidic pH of 6.7 or 100 μM NiCl_2 clearly increased $[\text{Ca}^{2+}]_i$ in WT-OGR1-HA-transfected cells (Fig. 2B). In contrast, the L74P-OGR1-HA-transfected cells (Fig. 2C) failed to significantly increase in $[\text{Ca}^{2+}]_i$ to acidic pH and NiCl_2 , as was the case of the vector-transfected control cells (Fig. 2A). On the other hand, a lipid mediator LPA-induced Ca^{2+} mobilization was comparable in all the cell types employed, suggesting that at least the Ca^{2+} signaling machinery is not affected by the transfection of OGR1-HA, regardless of whether the receptor is wild type or mutant (Fig. 2D).

The dose-dependent effect of acidic pH (Fig. 3A) and NiCl₂ (Fig. 3B) on the increase of [Ca²⁺]_i is shown. In WT-OGR1-HA cells, a significant [Ca²⁺]_i increase was detected at pH 7.2 (60 nM proton) and almost maximal at pH 7.0 (100 nM). In L74P-OGR1-HA cells as well as vector cells, however, even a more acidic pH to 6.4 (400 nM) failed to induce a significant increase in [Ca²⁺]_i (Fig. 3A). The situation was similar to that of NiCl₂; a significant increase was observed at 3 μM and a maximal dose was 100 μM in WT-OGR1-HA cells. However, a significant Ca²⁺ response was not detected in L74P-OGR1-HA cells at any dose of NiCl₂ examined (Fig. 3B). Thus, NiCl₂ in addition to extracellular protons may interact with WT-OGR1-HA to induce the Ca²⁺ response but fail to activate the L74P-OGR1-HA.

The induction of [Ca²⁺]_i by acidic pH or NiCl₂ in WT-OGR1-HA cells was unchanged by the removal of extracellular Ca²⁺ (Fig. 3C). Moreover, the Ca²⁺ response was reduced to a basal level with YM254890, an inhibitor of G_{q/11}-protein (Fig. 3D). Similar results were obtained with 1 μM LPA in WT-OGR1-HA cells (data not shown). Thus, Ca²⁺ is mobilized from an intracellular pool, possibly through G_{q/11}-protein/phospholipase C activation in WT-OGR1-HA cells. These results suggest that the

missense mutation at L74P of OGR1 attenuates receptor activity coupling to the G_{q/11}-protein/phospholipase C/Ca²⁺ signaling pathways.

3.3. The L74P mutation of OGR1 causes a halt of the receptor internalization and loss of morphological change of the cells in response to extracellular stimuli

Ligand activation of GPCRs has been shown to cause the redistribution of receptors from the cell surface to the intracellular space through a process of endocytosis known as internalization [14]. It is interesting to consider whether OGR1 is internalized in response to acidic pH and whether the L74P mutation attenuates the receptor processing. For this purpose, OGR1-HA receptors were monitored by a HA-tag antibody. Using a confocal laser-scanning fluorescence microscope, we acquired fluorescent images of cells that expressed OGR1-HA receptors (Fig 4A). At pH 7.4, WT-OGR1-HA receptors were detected both in the cell surface and the intracellular space. Treatment of the cells with acidic pH 6.8 or 100 μ M NiCl₂ at pH 7.4 for 0.5 h resulted in a change in the receptor distribution; thus, receptors located on the edge or surface of cells (i) are reduced, and most of the receptors are detected in intracellular spaces (ii and iii). The WT-OGR1-HA

detected in the intracellular space even at pH 7.4 might be partly related to the pH level employed, which is a threshold of the receptor activation (Fig. 3A), and might possibly cause partial receptor internalization (Fig. 4A).

WT-OGR1-HA-expressing HEK293 cells exhibit epithelial-like morphology with a slim shape (i). The extracellular stimuli also changed the morphology of the cells to a round shape (ii and iii). Thus, the peak ratio of major/minor axis in WT-OGR1-HA expressing cells was around 2-4, and the ratio changed to 1-2, reflecting a round shape by either acidic pH or NiCl₂ (Fig. 4B).

On the other hand, the L74P-OGR1-HA in cells is distributed in both the plasma membrane and the intracellular space similarly to WT-OGR1-HA-expressing cells; however, the distribution pattern was not altered by acidic pH and NiCl₂ (Fig. 4A). Moreover, these extracellular stimuli did not appreciably affect the cell morphology and distribution pattern of the ratio of the major/minor axis of the cells (Fig. 4B). These results suggest that the missense mutation at L74P of OGR1 causes a reduction in the ability of receptor internalization and signaling activity causing morphological change in cells by extracellular protons or NiCl₂.

4. Discussion

Among three families with amelogenesis imperfecta with variants in OGR1 receptors, one family possesses a missense mutation of Leu74Pro (L74P) in OGR1 [10]; however, where and how the damage to the mutant receptors, i.e., receptor transcription, sorting, interaction with ligands, coupling with G proteins, etc., occurs remain uncharacterized. In the present study, we characterized the Leu74Pro (L74P) mutation of OGR1 in the receptor transfection experiments using HEK293 cells. While the wild-type receptor-transfected cells showed a remarkable increase in intracellular Ca^{2+} mobilization, possibly through G_q and phospholipase C activation, and a clear morphological change from a slim shape to a round shape in response to protons and nickel ions, we hardly detected a significant Ca^{2+} response and cell morphological change in L74P mutant receptor-transfected cells. Importantly, receptor peptides either for WT-OGR1-HA or L74P-OGR1-HA seem to be delivered by a protein-sorting mechanism to plasma membranes, although the delivery might still be in process, or receptor recycling is

already functioning at the proton levels of basal pH 7.4, as evidenced by the presence of the receptor proteins in the intracellular space as well (Fig. 4B). Nevertheless, wild-type receptors, but not mutant receptors, are internalized into the intracellular space in response to protons and nickel ions (Fig. 4A). Thus, the mutant receptors seem to be expressed in plasma membranes; however, they are somehow damaged in their ability to couple to G proteins in response to extracellular ligands. The postulated Ca^{2+} signaling pathways by OGR1 and different modes of WT-OGR1 and L74P-OGR1 receptors are shown in Supplementary Fig. 3.

Based on mutagenesis experiments on certain histidine residues to phenylalanine, Ludwig et al. first proposed that hydrogen bonding between histidine residues stabilizes the inactive state of the receptors at a slightly alkaline pH and that the destruction of their bonding by protonation at a slightly acidic pH induces active conformational change in the molecular structure of the receptor in favor of coupling to G_q proteins [2]. Similarly to protons, the response to metal ions has also been reduced by the mutagenesis of the same histidine residues as those sensitive to protons [8, 9]. Thus, nickel ions as well as protons have been speculated to interact with certain histidine residues critical for the

conformational change of the receptors and the subsequent stimulation of the signaling machinery. How does the mutation of L74P damage protons and metal ions-induced receptor activation through histidine residues? We have provisionally speculated that the L74P mutation immediately adjacent to another proline residue was considered likely to destabilize the secondary structure of the second transmembrane helix of OGR1 and severely alter the functioning of the protein [10]. In the present study, we employed a secondary structure prediction for human OGR1 by PSIPRED V4.0 (Supplementary Fig. 1), which again showed that the replacement of L74P in the OGR1 receptors seems to cause an alteration of the helix structure in the second transmembrane domain (Supplementary Fig. 2). Thus, we speculate that the secondary structure of the second transmembrane domain of the variant OGR1 is disintegrated to keep the normal receptor conformation, thereby disturbing the proton or metal ion interaction to the histidine residues critical for proton or metal ion sensing and/or the subsequent receptor activation processes after the ligand interaction.

In conclusion, we characterized the L74P mutant of the OGR receptor structure and function coupling to the Ca^{2+} signaling in response to extracellular protons and NiCl_2 .

Our results suggest that the L74P in the second transmembrane domain in OGR1 may cause abnormal receptor conformational changes, and thereby the uncoupling of extracellular signals to intracellular signaling pathways through G proteins.

Conflict of Interest

The authors have no financial conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at .

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(Figure Legends)

Fig. 1. OGR1 mRNA expression in HEK 293 cells by RT-PCR. OGR1 mRNA products for WT-OGR1-HA or L74P -OGR1-HA were evaluated by RT-PCR. The cDNAs were synthesized from DNase I-treated RNA samples with or without reverse transcriptase (RT). RT-PCR was performed with specific primers as shown in Supplementary Table 1. The results are the representative picture of two independent experiments.

Fig. 2. $[Ca^{2+}]_i$ responses to LPA, acidic pH, and $NiCl_2$ in receptor-transfected HEK293 cells. (A-C) Representative traces of $[Ca^{2+}]_i$ change by 1 μM LPA, acidic pH of 6.7, or 100 μM $NiCl_2$ in control vector cells (Vector), WT-OGR1-HA cells, and L74P OGR1-HA cells are shown. The cells were pre-incubated in HEPES-buffered medium (pH 7.8), and at the arrow, the indicated ligand was added. (D) The results of net $[Ca^{2+}]_i$ changes induced by the indicated ligands are summarized. Differences between peak and basal values are shown as the means \pm SEM of three separate experiments. Ca^{2+} responses were

apparently induced by either pH 6.7, NiCl₂, or LPA in WT-OGR1-HA cells but induced only by LPA in vector and L74P OGR1-HA cells (***p*<0.01 from vector cells).

Fig. 3. Dose dependency of acidic pH and NiCl₂ on [Ca²⁺]_i changes and the involvement of G_{q/11}-proteins in their actions. (A, B) Dose-dependent effect of acidic pH and NiCl₂ on [Ca²⁺]_i changes, respectively. After the cells were pre-incubated in HEPES-buffered medium (pH 7.8), the cells were stimulated for 2 min by the indicated pH or concentrations of NiCl₂. Differences between peak and basal values were shown as the means ± SEM of three separate experiments. Ca²⁺ responses were apparently induced by acidic pH and NiCl₂ in WT-OGR1-HA cells (**p*<0.05 or ***p*<0.01), but those were not significant in L74P-OGR1-HA cells or vector cells. (C, D) The effect of Ca²⁺ chelation with EGTA (C) or YM-2514890 (D) in [Ca²⁺]_i in WT-OGR1-HA cells. After cells were pre-incubated in HEPES-buffered medium (pH 7.8), the cells were treated for 1 min with 2.5 mM EGTA or water as a vehicle for (C) and for 2 min with 100 nM YM-2514890 or DMSO as a vehicle for (D), and stimulated for 2 min by 1N HCl (final pH 6.8) or 100

$\mu\text{M NiCl}_2$. The effect by EGTA was not significant in C, but that of YM-2514890 is significant (** $p < 0.01$) in D.

Fig. 4. Internalization of OGR1 and morphological change of the cells in response to extracellular acidification and NiCl_2 . (A) Localization of OGR1 receptors. HEK293 cells transfected with WT-OGR1-HA or with L74P-OGR1-HA were incubated for 0.5 h with or without 1N HCl (final pH 6.8) or 100 $\mu\text{M NiCl}_2$ in HEPES-buffered RPMI-1640 containing 0.1% BSA to monitor receptor internalization. After fixation, immunostaining and the localization of receptor-HA (green) and nucleus (red) were monitored. The results are representative of two separate experiments. The large square for WT-OGR1-HA (i-iii) shows the higher magnification of the representative cells. (B) The morphological change in cells. The photographed cells as shown in A were analyzed by Fit Ellipse using ImageJ software. The cell morphological change was evaluated as the difference in the ratio of major and minor axes of the cells and the results were expressed as a distribution of observed frequencies of the ratio (fold), $n=40$ for each group.