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Expression of orphan G-protein coupled receptor GPR174 in CHO cells induced morphological changes and proliferation delay via increasing intracellular cAMP

Kazuya Sugita [a], Chiaki Yamamura [a], Ken-ichi Tabata [a], Norihisa Fujita [a,b,*]

[a] Laboratory of Pharmacoinformatics, Graduate School of Ritsumeikan University, Kusatsu, Shiga 525-8577, Japan
[b] School of Pharmacy, Ristumeikan University, Kusatsu, Shiga 525-8577, Japan

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ABSTRACT

We established cell lines that stably express orphan GPCR GPR174 using CHO cells, and studied physiological and pharmacological features of the receptor. GPR174-expressing cells showed cell–cell adhesion with localization of actin filaments to cell membrane, and revealed significant delay of cell proliferation. Since the morphological changes of GPR174-cells were very similar to mock CHO cells treated with cholera toxin, we measured the concentration of intracellular cAMP. The results showed the concentration was significantly elevated in GPR174-cells. By measuring intracellular cAMP concentration in GPR174-cells, we screened lipids and nucleotides to identify ligands for GPR174. We found that lysophosphatidylserine (LysoPS) stimulated increase in intracellular cAMP in a dose-dependent manner. Moreover, phosphorylation of Erk was elevated by LysoPS in GPR174 cells. These LysoPS responses were inhibited by NF449, an inhibitor of $G\alpha_s$ protein. These results suggested that GPR174 was a putative LysoPS receptor conjugating with $G\alpha_s$, and its expression induced morphological changes in CHO cells by constitutively activating adenylyl cycles accompanied with cell conjunctions and delay of proliferation.

1. Introduction

Human and other vertebrate genome sequencing projects revealed that approximately 30% of the total proteins are membrane proteins and more than 800 genes belong to the G-protein coupled receptor (GPCR) superfamily [1,2]. Two thirds of GPCRs are thought to be odorant receptors and, of the remaining third, the endogenous ligand for only half of the GPCRs has been identified [3]. Activation of GPCR induces various physiological and pathological functions depending on the type of G protein. It is well known that one third of marketed medicines exert their pharmacological effects by acting on human GPCRs. However, as mentioned above, nearly 140 GPCRs remain orphan GPCRs of which endogenous ligands are still missing [4]. These GPCRs hold higher potentials for revealing new intercellular interactions that will open new areas of basic research as well as new therapeutic applications. Therefore, it is highly anticipated to identify the endogenous ligands and to elucidate physiological functions of orphan GPCRs.

In our laboratory, we have identified ligands for orphan GPCRs such as leukotriene E4 for $P2Y_{12}$ receptor, LPA for GPR87 and LPA and S1P for $P2Y_{10}$ receptor [5–7]. Concerning $P2Y_{10}$ receptor, recently Inoue et al. reported that using their newly developed assay method, $P2Y_{10}$ receptor was found to be a novel LysoPS receptor [8]. On the other hand, Gyöngyi et al. reported that a new LPA receptor ligand H2L 5547924 inhibited $P2Y_{10}$ receptor response in a high affinity manner [9], indicating that there is still some controversy over whether $P2Y_{10}$ receptor is an LPA receptor or not. Phylogenetic analysis shows that GPR174 belongs to the P2Y family and has 50% sequence homology with $P2Y_{10}$ receptor. In humans, the gpr174 gene is located on chromosome X, region q21, in a cluster consisting of p2y10 and lpa4 genes (Fig. S1A). The gpr174 gene contains an open reading frame of 1258 base pairs encoding 333 amino acids with a calculated molecular mass of ~35 kDa. Recently, its mRNA was reported to be specifically expressed at higher levels in melanoma cells [10]. However, biochemical and pharmacological features are not known. P2Y family consists of 34 receptors that are activated by nucleotides, phospholipids and leukotrienes [1,6]. Therefore, we studied the physiological and pharmacological features of GPR174 with the assumption that it was one of the lipid or nucleotide receptors.

2. Materials and methods

2.1. Preparation of CHO cells expressing GPR174

The cDNA coding human GPR174 was obtained from GFC-Array™ (Origene, Rockville, MD). The gene was cloned into the

Abbreviations: CHO cell, Chinese hamster ovary cell; ERK, extracellular signal-regulated kinase; Epac, exchange proteins activated by cAMP.

* Corresponding author at: School of Pharmacy, Ritsumeikan University, 1-1-1 Noji-higashi, Kusatsu, Shiga 525-8577, Japan.
E-mail address: nori@ph.ritsumei.ac.jp (N. Fujita).

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pcDNA5/FRT (Invitrogen, Carlsbad), and transfected into Flp-In CHO cells (Invitrogen). CHO cells stably expressing GPR174 were selected in Ham's F-12 medium (Wako Pure Chemical Inc., Osaka) supplemented with 10% fetal bovine serum (Gibco-BRL, Gaithersburg, MD), 100 U/ml penicillin (Sigma–Aldrich, St. Louis, MO), 100 μg/ml streptomycin (Sigma–Aldrich) and 0.4 mg/ml hygromycin B (Invitrogen). The expression of the GPR174 was confirmed by RT-PCR method and Western Blot analysis with anti-GPR174 antibody.

2.2. RT-PCR analysis

Total RNA was isolated from GPR174-cells, mock CHO cells, and tissues from BALB/c mice of 8 week-old (Shimizu Experimental Materials, Kyoto) using TRIzol@ reagent (Invitrogen). Each RNA preparation was used to perform reverse transcription by the reverse transcriptase, ReverTra Ace (TOYOBO, Osaka). PCR amplification was done with a forward primer (5′-TTGGTTTCTCA TGTA CCCCTTTCGC-3′) and a reverse primer (5′-AAAACCGTCTTCCAGG TACAATAT AGGACA-3′) suitable to amplify 279 bp of hGPR174, or a forward primer (5′-ATGATTCTGA CCTGTGCAGGGT-3′) and a reverse primer (5′-TTAACATAGTTCAGCTGCCACGGTA GAAGT-3′) suitable to amplify 318 bp region of mouse GPR174 using Taq DNA polymerase (New England BioLabs, Ipswich MA).

2.3. Staining of actin filaments of cells

GPR174 expressing cells and mock cells were immobilized by 4% paraformaldehyde solution (WAKO Bioproducts, Tokyo) and membrane-permeabilized by 0.2% TritonX-100 containing PBS buffer. Thereafter, they were stained with 100 nM rhodamine–phalloidin in PBS solution. The stained cells were observed by a confocal laser scanning microscope (Olympus FV-1000, Tokyo). To examine the effects of $G\alpha_s$ protein on the mock CHO cells, we added cholera toxin (100 ng/ml) to the medium and incubated cells for 48 h. The morphological change and actin distribution were observed with a microscope (Olympus IX81, Tokyo). Furthermore, we used N_6-benzoyladenosine-3′,5′-cyclic mono-phosphate (Life Science Institute, Bremen Germany) and 8-chlorophenylthio-2′-*O*-methyladenosine-3′,5′-cyclic mono-phosphate (Life Science Institute) to investigate the effects of membrane permeable cAMP analogs on the mock cells.

2.4. Measurements of MAPKs and Akt activities

Cells were washed twice with ice cold PBS buffer, and disrupted by lysis buffer (62 mM Tris–HCl pH 6.8, 1.5% SDS, 75 mM DTT, 7.5% glycerol). Proteins were separated on 10% SDS polyacrylamide gels and transferred to Immobilon-P@ (Millipore). Phosphorylated- and nonphosphorylated-MAPKs or Akt were detected by Western Blot with antibodies that recognize target proteins or their phosphorylated sites. After treatment with the first antibodies, filters were washed with TBS buffer containing 0.1% TritonX-100, then soaked in the secondary antibody solution containing 5% skim milk and HRP-linked anti-rabbit IgG. Subsequently, filters were incubated in ImmunoStar LD@ (Wako Pure Chemical Inc.) for 5–10 min and each protein band was detected by LAS3000 (Fujifilm, Tokyo). All antibodies except anti-GPR174 (Abchem, Cambridge MA) were purchased from CalbioChem.

2.5. Measurements of cAMP concentration

GPR174- or mock cells were plated on 96-well plates at 5×10^4 cells/well and cultured overnight in serum-free medium. The cells were washed three times with 0.5 ml of assay buffer (Hanks' buffered salt solution (pH 7.4), 0.2 mM 3-isobutyl-1-methylxanthine (Wako Bioproducts), 0.05% bovine serum albumin, and 20 mM HEPES). After washing, a test compound in 0.5 ml of assay buffer was added to the cells, and the cells were incubated at 37 °C for 30 min. cAMP synthesis in the cells was stopped by addition of 0.1 ml of 0.1 M HCl, and intracellular cAMP was extracted at room temperature for 10 min. The amount of extracted cAMP was measured using an enzyme-linked immunoassay kit (Cell Signaling Technology, Danvers, MA). 1-oleoyl-2-hydroxy-sn-glycero-3-phospho-L-serine, 1-stearoyl-2-hydroxy-sn-glycero-3-phospho-L-serine, and 1-palmitoyl-2-hydroxy-sn-glycero-3-phospho-L-serine, sphingosine-1-phosphate (d18:1) and 1-oleoyl-2-hydroxy-sn-glycero-3-phospate were from Avanti Polar Lipids, Inc. (Alabaster, Alabama). Unless otherwise noted, we used 1-stearoyl-2-hydroxy-sn-glycero-3-phospho-L-serine as LysoPS and 1-oleoyl-2-hydroxy-sn-glycero-3-phospate as LPA.

3. Results

First we examined expression levels of GPR174 in mouse tissues, and found that GPR174 was expressed abundantly in spleen, faintly in brain, but not in any other tissues (Fig. S1B). This expression pattern was very different from that of $P2Y_{10}$ receptor [5], which consist a gene cluster with GPR174 (Fig. S1A). In order to establish the cell lines that stably express GPR174, we used Flip-in system with CHO cells. We could detect mRNA coding human GPR174 by the RT-PCR analysis in the transformed cells, but not in mock cells (Fig. S1C). Furthermore, Western Blot analysis also showed that the transformed cells expressed GPR174, but mock cells did not (Fig. S1D).

As shown in Fig. 1, the GPR174-expressing cells presented morphological changes and showed delay of proliferation rate. It was clear that the GPR174-cells revealed spindle-shaped form and tended to form cell–cell adhesions. When mock cells were treated with cholera toxin that constitutively activates $G\alpha_s$ protein by ADP-ribosylation, the cells showed similar morphological changes to GPR174-cells (Fig. 1A).

To measure the cell proliferation rate, we seeded 1×10^5 GPR174-expressing cells or mock cells in each well, and counted the number of growing cells every 24 h. As a result, significant differences appeared in the number of cells from the third day. The proliferation rate of cells expressing GPR174 was clearly slow (Fig. 1B). In addition, when actin filaments were stained with rhodamine–phalloidin reagents, stress fibers were observed in entire regions of mock cells, while actin filaments were localized to the cell membrane in GPR174 expressing cells as well as cholera toxin-treated CHO cells (Fig. 1A).

Since the morphological changes of GPR174 cells were similar to cholera toxin-treated cells, it was predicted that the concentration of intracellular cAMP was increased in GPR174-cells. Therefore, we measured intracellular cAMP concentration in the GPR174 cells incubated in normal medium, using ELISA kit. While the concentration of cAMP in 2×10^4 mock cells was about 20 pg, it was about 60 pg in the same number of GPR174-cells. These results suggested that expression of GPR174 constitutively increased in intracellular cAMP concentration (Fig. 2A).

Subsequently, we screened nucleotides and phospholipids to identify a ligand to GPR174 by examining whether a compound stimulated increase in intracellular cAMP in GPR174-cells pre-incubated in serum-free medium. Then we found that the amount of cAMP in GPR174-cells incubated with more than 0.1 μM LysoPS for 30 min was significantly higher than that incubated with vehicle, while no significant difference was observed in mock cells. The LysoPS-stimulated increase in cAMP was in a dose-dependent manner and the increase was inhibited in the presence of 20 μM NF449, an inhibitor of $G\alpha_s$ protein (Fig. 2B). We examined the ef-

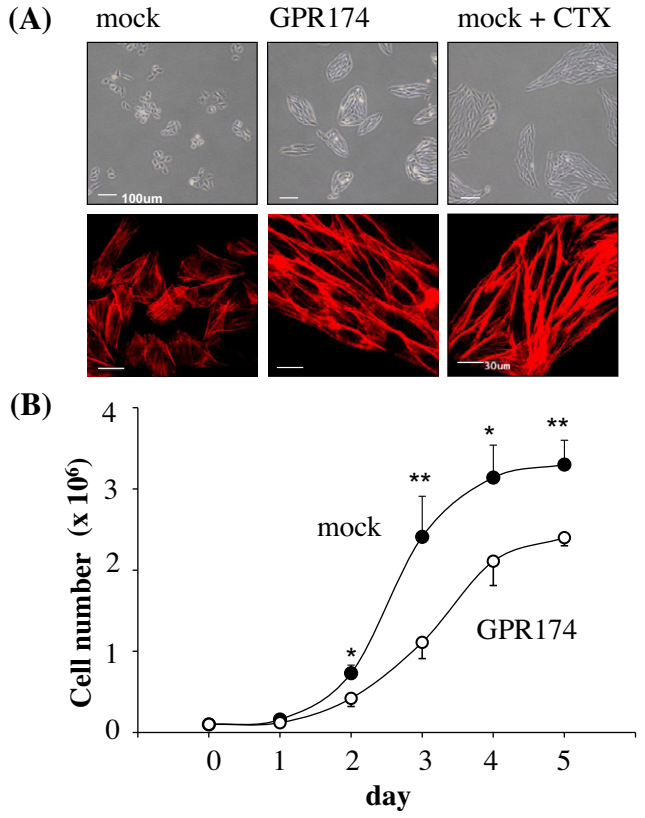

Fig. 1. Morphological changes and growth rate of GPR174 expressing CHO cells. (A) Morphological changes and reorganization of actin filaments. Left, mock CHO cells; Middle, GPR174 expressing cells; Right, mock cells treated with cholera toxin. Upper; phase contrast, Bottom; rhodamine–phalloidin staining. (B) Time courses of cell growth. The growth rate of each cell line was calculated by counting total number of cells in triplicated wells every 24 h for 5 days. Statistical comparison between two groups has been performed with Bonferroni-test. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to mock cells.

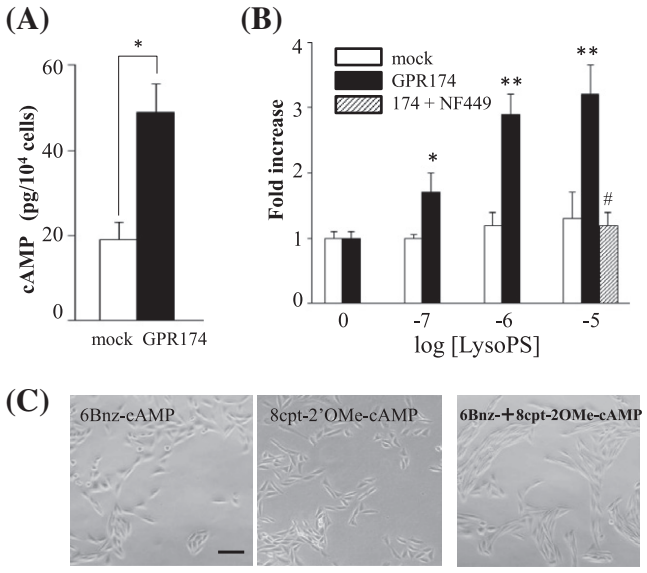

Fig. 2. LysoPS-evoked increase in intracellular cAMP via GPR174. (A) The levels of intracellular cAMP were measured by ELISA as described in the section of methods. $^{*}P < 0.01$. (B) Dose dependency for LysoPS-stimulated cAMP increases. The results from the cells expressing GPR174 (closed symbols) and mock CHO cells (open symbols). Individual bars represent the means ± SD of 4–6 experiments each measuring at least three times. Hatched bar presents the result in the presence of 20 μM NF449. Phosphorylation levels obtained with vehicle (PBS) were regarded as one. $^{*}P < 0.01$, $^{**}P < 0.01$ compared to vehicle (PBS). $^{\#}P < 0.01$ compared to 10 μM LPA. (C) The effect of cAMP analogs on mock CHO cells. 200 μM 6Bnz-cAMP (left), 200 μM 8-pCPT-2′OMe-cAMP (center) and 100 μM 6Bnz-cAMP + 100 μM 8-pCPT-2′OMe-cAMP (right) were added to the medium and incubated cells for 48 h.

fects of fatty acid moiety of LysoPS on GPR174 activation using cAMP assay with GPR174 cells, and found that GPR174 was fully activated by oleoyl or stearoyl acyl group on LysoPS, but inefficiently by palmitoyl LysoPS. On the other hand, other phospholipids, including sphingosine-1-phosphate and LPA as well as nucleotides (at 10 μM), did not show significant increase in intracellular cAMP (data not shown).

Elevation of intracellular cAMP concentration regulates many biological processes such as cell morphology, proliferation, and differentiation. Recently, it has been well established that cAMP mediates its effects through not only protein kinase A (PKA) but also Epac [11,12]. To confirm the involvement of PKA and Epac in cAMP-induced morphological changes in CHO cells, we examined the effects of membrane-permeable cAMP analogs, N_6-benzoyladenosine-3′,5′-cyclic mono-phosphate (6Bnz-cAMP) and 8-chlorophenylthio-2′-*O*-methyladenosine-3′,5′-cyclic mono-phosphate (8-pCPT-2′OMe-cAMP) on mock cells. 200 μM 6Bnz-cAMP, which specifically activates PKA, moderately changed cell shapes (Fig. 2C). Epac-specific cAMP analog 8-pCPT-2′OMe-cAMP slightly induced morphological changes in CHO cells at a concentration of 200 μM. However, as illustrated in Fig. 2C, combined stimulation elicited considerable morphological changes and cell–cell adhesions in the cells.

As GPR174-cells showed proliferation delay, we investigated activities of MAPKs and Akt by Western Blot analyses. Each amount of phosphorylation of MAPKs was found to be unchanged in the absence of serum (Fig. 3A). On the other hand, while no significant change was observed on phosphorylation of p38, JNK or Akt in the presence of serum (Fig. 3A), Erk was highly phosphorylated by a factor of four (Fig. 3B).

Subsequently, we examined whether LysoPS stimulated the phosphorylation of Erk in GPR174-cells, and we confirmed that LysoPS promoted the phosphorylation of ERK at the concentration of more than 0.1 μM in GPR174-expressing cells, but not in mock cells (Fig. 4A). This effect of LysoPS was abolished in the presence of NF449 (Fig. 4B).

4. Discussion

This study was conducted to elucidate physiological function of orphan GPCR GPR174 and identify a ligand for the receptor. By over-expressing GPR174 in CHO cells, cell–cell adhesion with reorganization of actin filaments to the vicinity of the cell membrane was observed (Fig. 1A). As the similar morphological changes were observed in CHO cells treated with cholera toxin, it was suggested that these changes were the result of increased concentration of intracellular cAMP. In fact, we confirmed the levels of cAMP significantly elevated in GPR174-cells.

Based on these results, we searched endogenous ligands for GPR174. Since GPR174 is phylogenetically a member of P2Y family, we examined whether some of phospholipids or nucleotides stimulated increase in cAMP in GPR174-cells starved prior to assay. Consequently, we found that LysoPS increased in cAMP in a dose dependent manner at a range of 0.1–10 μM. Mammalian serum contains several lysophospholipids [13], and the concentration of LPA in 10%(v/v) fetal calf serum has been estimated to be a few μM [14]. Since the concentration of LysoPS in serum is considered to be at a similar level to LPA [15], the dose dependency of LysoPS in the GPR174-cells was in a proper range and the expressed GPR174 was constitutively activated in the serum-containing medium.

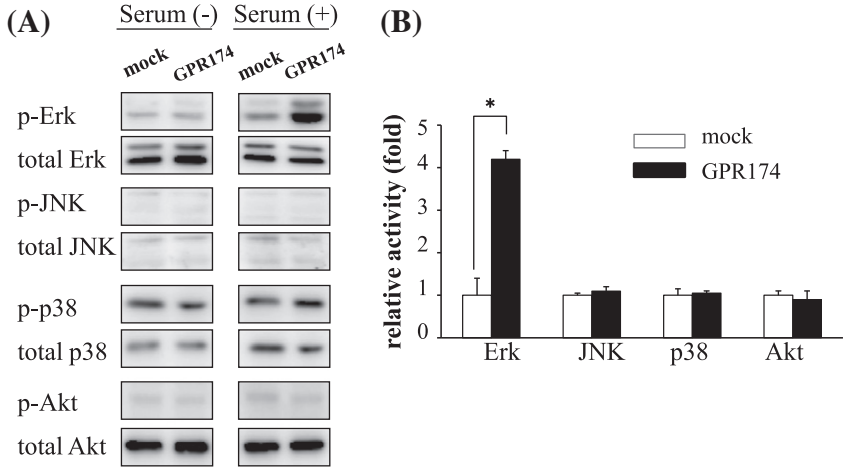

Fig. 3. The MAPKs and Akt activities in GPR174 expressing and mock CHO cells. (A) Phosphorylation levels of MAPKs and Akt were examined as described in the section of methods. $^{*}P < 0.01$, compared to each control. (B) Relative activities of MAPKs and Akt in the presence of serum. Data shown in (A) were used. Each band density was calculated with LAS3000 and the values in mock cells were regarded as one.

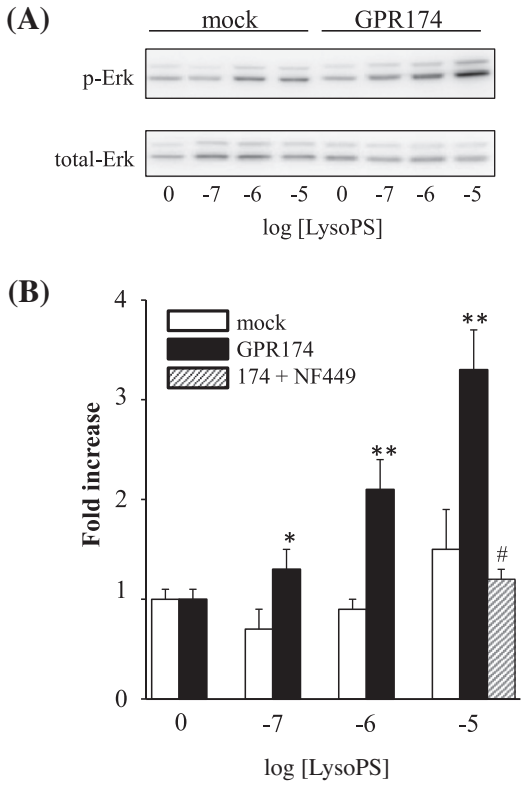

Fig. 4. The effects of LysoPS and NF449 on Erk activity in GPR174-cells preincubated without serum for 24 h. Cells were treated with different concentrations of LysoPS for 10 min and each Erk activity was determined by Western Blotting assay. Hatched bar represents the result of 10 μM LPA in the presence of 20 μM NF449. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to vehicle (PBS). $^{\#}P < 0.01$ compared to 10 μM LPA.

The LysoPS-induced activation of adenylyl cyclase in GPR174 cells was abolished in the presence of $G\alpha_s$ inhibitor, NF449 (Fig. 2B). Similarly, LysoPS dose-dependently increased Erk phosphorylation which was inhibited by NF449 in the cells (Fig. 4A and B). These results indicated that GPR174 was a LysoPS receptor that conjugated with $G\alpha_s$ protein. However, as no LysoPS receptor antagonist is available at present, we need further investigation to confirm these results. To date, GPR34 has been reported to be a LysoPS receptor [16,17]. In contrast to GPR174, GPR34 conjugates with $G\alpha_i$ protein in CHO cells genetically engineered to express GPR34, and its activation suppresses an increase in intracellular cAMP.

Initially, all effects of cAMP were attributed to the activation of PKA, but Epac, the other target, has been shown to be directly regulated by cAMP and exhibits guanine nucleotide exchange factor for Rap1/2, a cAMP-regulated GTPase [11,12,18]. Epac exerts diverse effects on cellular functions including cell junction, hormone/transmitter secretion, intracellular Ca^{2+} mobilization and so on [19–21]. The morphological changes, which were observed in GPR174 cells, appeared in mock CHO cells that were treated with cholera toxin (Fig. 1A) or a mixture of cAMP analogs, 100 μM 6Bnz-cAMP and 100 μM 8-pCPT-2′OMe-cAMP (Fig. 2C).

Spindler et al. reported that activated Epac caused cell–cell adhesion by accumulating the adhesion molecules actin and cadherin in the periphery of cell membrane, through the activation of Rap1, in vascular endothelial cells [22]. When f-actin acts a scaffolding protein to conjugate catenin with cadherin, cell–cell adhesions were enhanced [23–25]. However, in our present study, 200 μM 8-pCPT-2′OMe-cAMP alone induced only low-degree morphological changes in CHO cells. Similarly, 6Bnz-cAMP, which can activate PKA but not Epac slightly induced morphological changes and cell conjunction at a concentration of 200 μM. On the other hand, in the presence of 100 μM 8-pCPT-2′OMe-cAMP, 100 μM 6Bnz-cAMP did induce morphological changes and cell conjunctions of CHO cells in a large quantity (Fig. 2C). These results indicated that the cAMP-induced morphological changes required activations of both PKA and Epac in CHO cells, although their cross-talk cascades are not clear.

As the proliferation rate was decreased in GPR174-cells (Fig. 1B), we had predicted that p38 and/or JNK were activated, or Erk was inactivated in the cells. However, as shown in Fig. 3, phosphorylation levels of JNK, p38 and Akt were not changed but Erk was spontaneously phosphorylated in GPR174-cells being cultured in serum-containing medium. Moreover, it was activated by LysoPS at physiological concentrations (Fig. 4A and B). These results demonstrated that the proliferation delay was not due to the loss of Erk activity. Previously, Sugo et al. reported that LysoPS induced concentration-dependent increase in Erk activity through activation of GPR34, which conjugates with $G\alpha_i$ protein in CHO cells [16]. The negative control of Erk activity by cAMP was also reported with pigment epithelial cells [26]. On the other hand, cAMP is well known to induce activation of Ras family, which induces

phosphorylation of Erk via activating PKA [27]. We confirmed that PKA inhibitor H89 prevented the spontaneous and LysoPS-induced activation of Erk in GPR174 cells (data not shown). Therefore, it is possible that activation of GPR174 resulted in increase in Erk activity via activating PKA.

Concerning the effects of cAMP on cell proliferation, cAMP suppressed it in vascular smooth muscle cells and human lung fibroblasts, but increased in ovarian cancer cells and NIH3T3 cells [28–30]. Therefore, it is currently accepted that cAMP possesses ability to inhibit proliferation in some cell types, and stimulate proliferation in others. In PC12 cells, PKA-dependent activation of ERK1/2 stimulated cell proliferation, and cAMP-activated Epac extended the duration of activation of ERK1/2 that converted cAMP from a proliferative to an anti-proliferative signal [31–33]. Therefore, our present results in lower proliferation rate of GPR174 cells may be caused by the similar cAMP-mediated signal pathway in PC12 cells. As the characterization of cAMP-mediated signaling in many cell systems has revealed complex spatiotemporal regulation [34], the molecular mechanism and its functional roles of cAMP signal should be explored more precisely and spatiotemporally in GPR174 cells and mock cells.

LysoPS is secreted by the immune system in vivo, and acts a lipid mediator that regulates immune system processes, such as degranulation of mast cells [35,36] and inhibition of T-cell proliferation [37,38]. As mentioned above, proliferation of smooth muscle cells and fibroblast cells is inhibited by cAMP [28,29] so we can infer that GPR174 negatively regulates immune-cell proliferation by increasing intracellular cAMP. Moreover, it has been postulated that migration was induced by LysoPS in glioma cells [39] and NGF-induced neurite extension was potentiated by LysoPS in PC12 cells [40]. Since GPR174 was found to be expressed in mouse brain, GPR174 may play important roles in the central nervous system as a LysoPS receptor.

Very recently, Inoue et al. showed that GPR174 was activated by LysoPS and stimulated transforming growth factor-α ectodomain shedding, through activating $G\alpha_{12/13}$ protein [8]. Our present results corresponded with their results except conjugated G_α proteins. Perhaps this discrepancy is due to the difference in receptor assay system. We need further investigation to examine if GPR174 couples with multiple G proteins that can be commonly observed in lysophospholipid receptors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.11.046.

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