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Effects of Proton on Lysolipid-induced Actions in OGR1-subfamily GPCRs

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Abstract – Lysolipids such as lysophosphatidylcholine (LPC), sphingosylphosphorylcholine (SPC), galactosylsphingosine (psychosine) have been matched as ligands for OGR1-subfamily G-protein-coupled receptors (GPCR), consisted of OGR1, GPR4, G2A, and TDAG8. Recently, those members of GPCRs have been reported as proton-sensing GPCRs. We used Jurkat T cells, which express four members of OGR1 subfamily GPCRs endogenously to investigate effects of proton on lysolipid-induced several cellular events. We found no significant effect of proton on the lysolipid-induced Ca^{2+} increase and ROS production in Jurkat T cells. Further investigation is necessary to clarify the relationship of lysolipid and proton on the OGR1-subfamily GPCRs.

Keywords □ lysolipid; proton, G-protein-coupled receptor, sphingosylphosphorylcholine, lysophosphatidylcholine, psychosine

INTRODUCTION

G-protein-coupled receptor is the largest gene family of human genome. Their importance is glaringly obvious by the fact that more than 50% of drugs on the market are either agonists or antagonists on GPCRs (Im 2002). Many chemical messengers such as hormones and neurotransmitters use their own GPCRs as signal receivers on the target cell membrane. Therefore, positive or negative modulation of GPCRs with drugs has been successful tools to treat many diseases such as allergy, gastric ulcer, and hypertension (Fredriksson et al. 2003; Im 2002, 2004). GPCR activations are evoked by stimuli as diverse as light, Ca^{2+}, odorants, amino acids, nucleotides, proteins, polypeptides, steroids, and fatty acid derivatives.

The completion of the human genome project has identified about 865 GPCR genes (Perez 2005). Except sensory receptors, 367 GPCRs have been considered as receptors for endogenous ligands in the human genome (Vassilatis et al. 2003). OGR1 subfamily is composed of four members (OGR1, GPR4, G2A, and TDAG8) and has previously been identified as receptors for lysolipids; sphingosylphosphorylcholine (SPC), lysophosphatidylcholine (LPC) and galactosylsphingosine (psychosine) (Im *et al.* 2001; Kabarowski *et al.* 2001; Xu *et al.* 2000; Zhu *et al.* 2001). And their importance has been reported as like G2A deficient mouse developed an autoimmune syndrome similar to systemic lupus erythematosus (SLE) (Le et al. 2001), and therapeutic application of LPC for sepsis was proposed in relation with G2A receptor (Yan et al. 2004). Recently, Kim *et al.* reported that GPR4 plays a critial role in SPC-induced angiogenesis and SPC transactivates VEGF receptor 2 in endothelial cells (Kim et al. 2005). Furthermore, Ikeno *et al.* reported that secretary phospholipase A_2 induce neurite outgrowth in PC12 cells through LPC generation and activation of G2A receptor (Ikeno *et al.* 2005).

Nevertheless, confirmation of the ligand matching of OGR1 subfamily with lysolipids has not been fully supported by publications of independent research groups (Im 2004, 2005). A series of publications propose that extracellular proton could be an activator of the OGR1 subfamily GPCRs (Ishii *et al.* 2005; Ludwig *et al.* 2003; Murakami *et al.* 2004; Radu *et al.* 2005; Wang *et al.* 2004). More than two independent groups reported proton-sensing properties of OGR1, GPR4, and TDAG8 (Ishii *et al.* 2005; Ludwig et al. 2003; Mogi *et al.* 2005; Murakami *et al.* 2004; Radu et al. 2005; Wang *et al.* 2004). In the case of G2A, constitutive activation at pH 7.4 has been observed in many transfected cells by many research groups, however, pH-

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dependent activation was supported only by one group and was not fully reproduced by another group (Murakami *et al.* 2004; Radu *et al.* 2005).

Reactive oxygen species (ROS) exert numerous effects on cell functions, including induction of growth and regulation of kinase activity (Finkel 2000; Larsson and Cerutti 1988; Schreck et al. 1991). Oxidative stress plays a prominent role in the functioning of the immune system at both physiological and pathological levels, and also is known to be associated with reduced or abnormal immune function with aging (Lieber 1998), AIDS (Dobmeyer *et al.* 1997), diabetes (Dandona *et al.* 1996), smoking-related pathologies (McAllister-Sistilli *et al.* 1998), and some autoimmune and inflammatory diseases (Frenkel *et al.* 1992).

Calcium is a ubiquitous second messenger controlling a broad range of cellular functions, and an increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) plays an important role in cellular functions such as cell proliferation and insulin secretion (Himmel *et al.* 1998; Lipskaia and Lompre 2004).

Lysophospholipids regulate a variety of biological processes including cell proliferation, tumor cell invasiveness, and inflammation (Moolenaar 1999; Spiegel and Milstien 1995). Jurkat T cell line is homogeneous T lymphocytes and possess membrane markers that characterize normal T lymphocytes (Konikova et al. 1992). In the previous study, LPC was found to induce ROS generation and $[Ca^{2+}]_i$ increase in human lymphocytic Jurkat T cells (Im *et al.* 2006). Furthermore, four members of OGR1 subfamily GPCRs are expressed endogenously (Im *et al.* 2006). Because all four members of OGR1 subfamily GPCR were detected at mRNA level in Jurkat T cells, we applied this cell line as a model.

MATERIALS AND METHODS

Materials

1-palmitoyl (C16:0) LPC, 1-oleoyl lysophosphatidic acid (LPA), sphingosyl phosphorylcholine (SPC), sphingosine 1-phosphate (S1P), and galactosyl sphingosine (psychosine, Psy) were purchased from Avanti Polar Lipids (Alabaster, AL, USA); fura 2/acetoxymethyl ester was from Calbiochem (Darmstadt, Germany). 2',7' dichlorofluorescin diacetate (DCFDA) were from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

Jurkat T cells were maintained in RPMI 1640 containing 10 % (v/v) fetal bovine serum, 100 units/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine, and 1 mM sodium pyruvate at 37°C in a humidified 5% CO incubator.

Measurement of ROS

Jurkat T cells were sedimented, resuspended in Hepes-buffered media (HBM) consisting of 20 mM Hepes (pH 7.4), 103 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 0.5 mM $CaCl_2$, 25 mM $NaHCO_3$ and 15 mM glucose, and then incubated for 20 min with 10 μM DCFDA at pH 7.4. Then, the cells were used for the measurements. Fluorescence was measured in DCFDA-loaded cells. Fluorescence emission at 520 nm wavelength from 488 nm excitation wavelength was measured every 0.1 sec by an F4500 fluorescent spectrophotometer (Hitachi, Japan). The extracellular pH was adjusted by addition of pre-calculated amounts of 1 N HCl for each pH like 7.1, 6.8, and 6.5. After 15 sec, fluorescent intensity was recorded at the first 60 sec, 60 sec after lipid addition and 10 min later. Individual histogram was made by the percent of control level, and ROS was measured more than twice (Im *et al.* 2006).

Measurement of intracellular Ca^{2+} concentration

Different pHs of HBM were adjusted just before the experiments by addition of 1 N HCl. The Jurkat T cells were sedimented, resuspended in the HBM (pH 7.4), and then incubated for 40 min with 5 μM fura 2/acetoxymethyl ester at pH 7.4. Fura 2-loaded cells were washed twice with each HBM with different pH and resuspended in the same media. Fluorescence emission at 510 nm wavelength from two excitation wavelengths (340 nm and 380 nm) were measured every 0.1 sec (F4500, Hitachi, Japan). The ratio of fluorescence intensities from the two wavelengths was monitored as an estimate of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Lee *et al.* 2006).

Data presentation and statistical analysis

Representative traces for intracellular Ca^{2+} concentration were chosen from 3-5 separate experiments and are shown in Figs 1 and 2. In Fig 3, results from two separate experiments were shown by the percent of control level.

RESULTS

Effect of extracellular pH on LPC-induced $[Ca^{2+}]_i$ increase

Because all four members of OGR1 subfamily GPCR were detected at mRNA level in Jurkat T cells, we applied this cell line as a model to study functions of endogenous OGR1 GPCRs. Recent data show that LPC elevates the intracellular

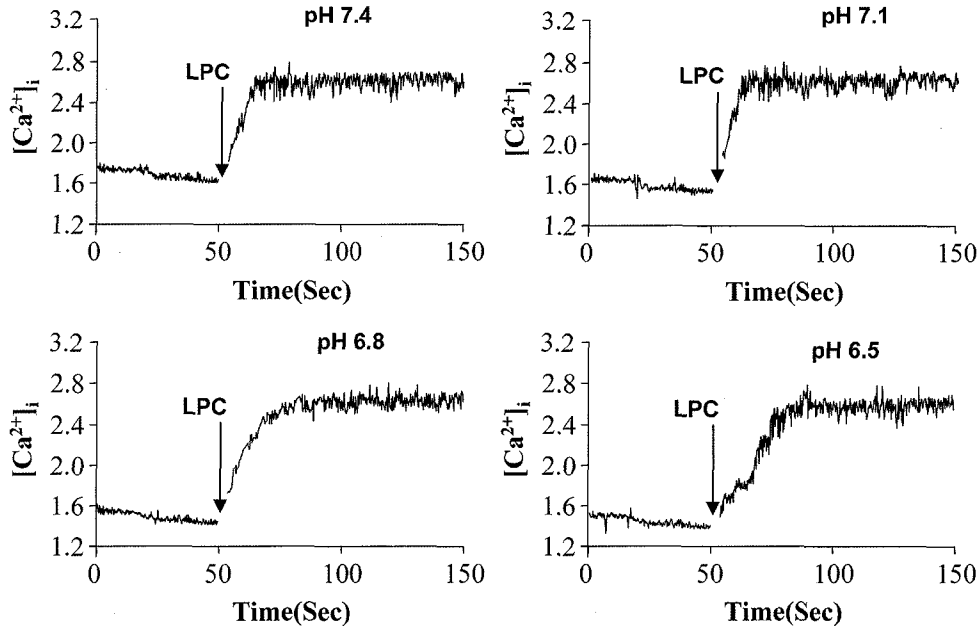

Fig. 1. Effect of proton on LPC-induced increase of intracellular Ca^{2+} concentration. After loading Fura-2/AM into Jurkat T cells (1.5 $\times 10^6$/cells/ml) described in Method section, cells suspended in HBM with different pHs without BSA were divided into 2 ml cuvette. Each trace was monitored and saved for F4500 fluorescent spectrophotometer. Representative Ca^{2+} traces with 20 μM of LPC in Jurkat T cells at different extracellular pH. LPC was added at the arrow indicated. The data shown are representative of three independent experiments.

Ca^{2+} concentration in Jurkat T cells (Im *et al.* 2006; Legradi *et al.* 2004). As shown in Fig. 1, 20 μM concentration of LPC sharply increased $[Ca^{2+}]_i$ in Jurkat T cells. The effect of extracellular pH on LPC-induced increase of $[Ca^{2+}]_i$ was studied. As shown in Fig 1, the increase was not influenced by lowering extracellular pH upto 6.5. Further, we tested effects of other lysolipids including SPC, Psy, LPA, and S1P. Those lipids did not increase the intracellular Ca^{2+} concentration in different extracellular pHs (Fig. 2).

Effect of extracellular pH on LPC stimulated ROS generation in Jurkat T cells

Previously, we reported that LPC rapidly and significantly increased reactive oxygen species (ROS) level in Jurkat T cells (Im *et al.* 2006). We measured changes of ROS levels by lysolipids and influences of extracellular pH on the effect. As

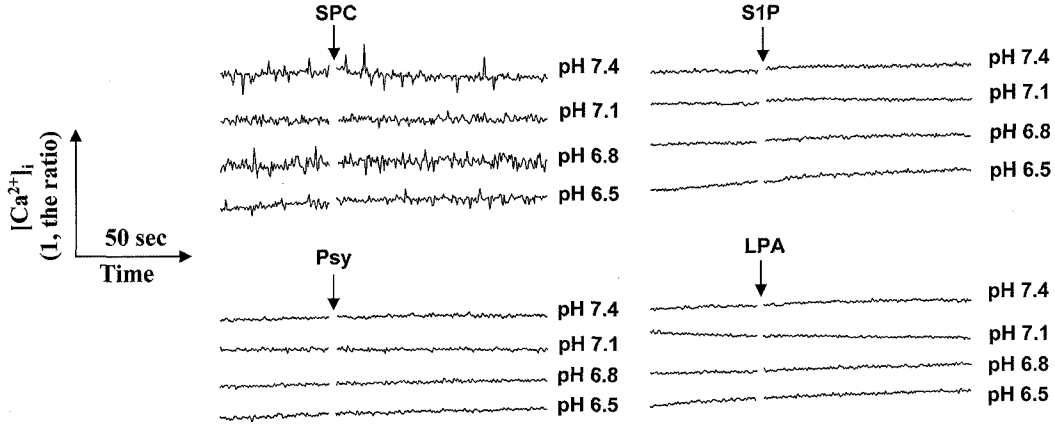

Fig. 2. Effect of lysolipid on intracellular Ca^{2+} concentration at different extracellular pH. Representative Ca^{2+} traces with 20 μM of SPC, S1P, Psy, or LPA in Jurkat T cells at different extracellular pH. Each lipid was added at the arrow indicated. The data shown are representative of three independent experiments.

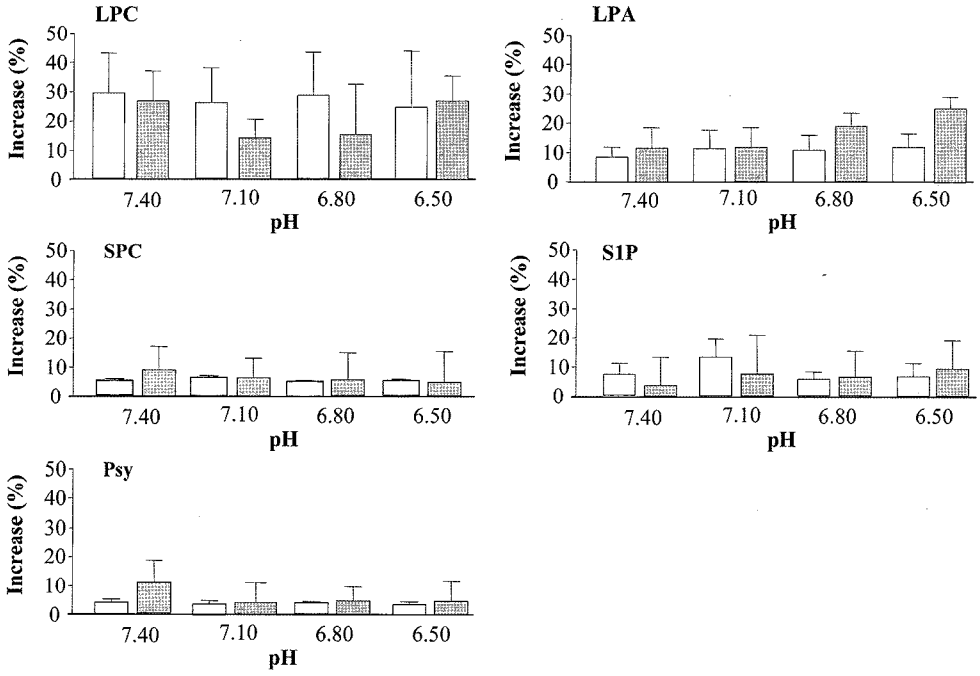

Fig. 3. Effect of proton on lysolipid-induced increase of ROS generation. After loading DCFDA into Jurkat T cells (1.5×10^6 cells/ml) as described in Method section, cells suspended in HBM without BSA were divided into 2 ml cuvette. Each trace was monitored and saved for F4500 fluorescent spectrophotometer. After adjusting the pH by HCl, fluorescent intensity was monitored at the first 60 sec, 60 sec after lipid addition and 10 min later. The first ten points of each step of times were averaged, and individual histogram was made by the percent of control level. Open column means ROS level after lysolipid addition and filled column means ROS level 10 min after lysolipid addition.

shown in Fig. 3, LPC increased ROS most prominently and the increase was followed by LPA and S1P. Effect of proton on lysolipid-induced ROS generation was tested by changing extracellular pH (Fig. 3). However, decrease of extracellular pH, in other word increase of proton in the media, did not affect on the increase of ROS by each lipid (Fig. 3).

DISCUSSION

In the present study, we were not able to find any effect of proton on lysolipid-mediated responses such as Ca^{2+} increase and ROS generation. Proton itself also did not induce any change in Ca^{2+} increase in Jurkat T cells (data not shown). In the previous study, we concluded that LPC-induced Ca^{2+} increase and ROS generation may not be mediated through GPCRs in Jurkat T cells. In this study, we found no effect of proton on lysolipid-induced responses. Even though four members of OGR1 subfamily GPCRs are expressed in Jurkat T cells, no significant change was observed by lysolipid and proton. Three possibilities could be considered; 1) different signalings of OGR1 subfamily GPCRs triggered by proton in the Jurkat T cells to signalings of proton reported in overexpression system of the GPCRs, 2) dysfunction of four OGR1 subfamily GPCRs in the Jurkat T cells, and 3) the idea of proton-sensing GPCR of OGR1 subfamily GPCRs may not be correct as like lysolipid.

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