Calcium Signaling of Dioleoyl Phosphatidic Acid via Endogenous LPA Receptors: A Study Using HCT116 and HT29 Human Colon Cancer Cell Lines

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Abstract – In the present study, we have tested the effect of dioleoyl phosphatidic acid (PA) on intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in two human colon cancer cell lines (HCT116 and HT29). PA and lysophosphatidic acid (LPA), a bioactive lysolipid, increased $[\text{Ca}^{2+}]_i$ in both HCT116 and HT29 cell lines. Increases of $[\text{Ca}^{2+}]_i$ by PA and LPA were more robust in HCT116 cells than in HT29 cells. A specific inhibitor of phospholipase C (U73122), however, was not inhibitory to the cell responses. Pertussis toxin, a specific inhibitor of $\text{G}_{i/o}$ type G proteins, however, had an inhibitory effect on the responses except for an LPA-induced one in HT29 cells. Ruthenium red, an inhibitor of the ryanodine receptor, was not inhibitory on the responses, however, 2-APB, a specific inhibitor of inositol 1,4,5-trisphosphate receptor, completely inhibited both lipid-induced Ca^{2+} increases in both cell types. Furthermore, by using Ki16425 and VPC32183, two structurally dissimilar specific antagonists for $\text{LPA}_1/\text{LPA}_3$ receptors, an involvement of endogenous LPA receptors in the Ca^{2+} responses was observed. Ki16425 completely inhibited the responses but the susceptibility to VPC32183 was different to PA and LPA in the two cell types. Expression levels of five LPA receptors in the HCT116 and HT29 cells were also assessed. Our data support the notion that PA could increase $[\text{Ca}^{2+}]_i$ in human colon cancer cells, probably via endogenous LPA receptors, G proteins and IP_3 receptors, thereby suggesting a role of PA as an intercellular lipid mediator.

Keywords □ phosphatidic acid; lysophosphatidic acid; G-protein-coupled receptor; calcium; LPA_1 ; LPA_3

INTRODUCTION

Dioleoyl phosphatidic acid (PA) is a minor fraction of the intracellular phospholipid pool. However, PA has been proposed as a second messenger for many stimuli and its action on intracellular signaling molecules such as mTOR has been intensively studied (Fang *et al.*, 2001; Hornberger *et al.*, 2006; Stace and Ktistakis, 2006). On the other hand, exogenous PA has been shown to induce calcium influx in a number of biological tissues including isolated platelets (Gerrard *et al.*, 1978; Ikeda *et al.*, 1979; Imai *et al.*, 1982), the parotid gland (McGhee and Shoback, 1990; Weiss *et al.*, 1982), liver cells (Barritt *et al.*, 1981; Osugi *et al.*, 1984), nerve terminals (Harris *et al.*, 1981), epithelium (Lee *et al.*, 1989), osteoblasts (Kawase and Suzuki, 1988, 1990), and cardiac myocytes (Kurz *et al.*, 1993) and shown to exert potent mitogenic effects (Bashir *et*

al., 1992; Knauss *et al.*, 1990; Krabak and Hui, 1991; Pearce *et al.*, 1994; Siegmann, 1987; Wood *et al.*, 1993). Nevertheless, the findings that lysophosphatidic acid (LPA) is a potent mitogenic lipid (van Corven *et al.*, 1989) and that commercial preparations of PA were contaminated with LPA has shifted importance from PA to LPA (Jalink *et al.*, 1990). Contrary to the above citations, growth factors induce cellular proliferation through PA dependent activation of mitogen-activated protein

Abbreviations used are: PA, dioleoyl phosphatidic acid; LPA, lysophosphatidic acid; VPC32183, (S)-Phosphoric acid mono-{2-octadec-9-enoylamino-3-[4-(pyridine-2-ylmethoxy)-phenyl]-propyl} ester; Ki16425, 3-(4-[4-([1-(2-Chlorophenyl)ethoxy]carbonylamino)-3-methyl-5-isoxazolyl]benzylthio) propanoic acid; PTX pertussis toxin; GPCR, G-protein-coupled receptor; LPA_1 , lysophosphatidic acid receptor type 1 (EDG2); LPA_2 , lysophosphatidic acid receptor type 2 (EDG4); LPA_3 , lysophosphatidic acid receptor 3 (EDG7); LPA_4 , lysophosphatidic acid receptor type 4 (GPR23); LPA_5 , lysophosphatidic acid receptor 5 (GPR92); PLD, phospholipase D

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(MAP) kinase, an event resulting from the upstream induction of tyrosine kinase, MAP kinase or MAP kinase kinase (Siddiqui and Yang, 1995) and in such a system, LPA was without effect. Furthermore, several reports have excluded LPA contamination as a cause of the noted PA response (Fernandez *et al.*, 1994; Knauss *et al.*, 1990; Krabak and Hui, 1991; Kurz *et al.*, 1993; Moritz *et al.*, 1992; Ryder *et al.*, 1993; Siddiqui and Yang, 1995). In addition, PA has been shown to behave as a partial agonist of LPA₁, LPA₂ and LPA₃ receptors in recombinant LPA receptor-expressing cells, even though some investigators did not observe an agonistic property in LPA receptor-overexpression systems (An *et al.*, 1998a; An *et al.*, 1998b; Durgam *et al.*, 2006; Fischer *et al.*, 2001; Fitzgerald *et al.*, 2000).

In the previous study, we found PA may act on LPA receptors in rat C6 glioma and mouse L2071 fibroblast cells (Chang *et al.*, 2007). In the present study we used two human colon cancer cell lines (HCT116 and HT29) to test the hypothesis that PA could act as a ligand of G-protein coupled receptor (GPCR) on the plasma membrane and also to determine if such action was mediated through LPA receptors. By employing pharmacological inhibitors of signaling molecules and LPA receptors, we investigated the signaling mechanism of PA-induced increase of [Ca²⁺]_i and the involvement of endogenous LPA receptors in human colon cancer cells.

MATERIALS AND METHODS

Materials

1, 2-Dioleoyl-*sn*-glycero-3-phosphate (PA), 1-oleoyl lyso-phosphatidic acid (LPA) and VPC32183 were purchased from Avanti Polar Lipids (Alsbaster, AL, USA). Fura 2-AM was obtained from Calbiochem (Darmstadt, Germany) while pertussis toxin (PTX), Ki16425, and other materials were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

Human HCT116 and HT29 colon cancer cells were maintained in high glucose DMEM, 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% CO₂ incubator.

Measurement of intracellular Ca²⁺ concentration

Cells were trypsin-digested, allowed to sediment, resus-

pended in Hepes-buffered medium (HBM), consisting of 20 mM Hepes (pH 7.4), 103 mM NaCl, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 0.5 mM CaCl₂, 25 mM NaHCO₃, 15 mM glucose and 0.1% bovine serum albumin (fatty acid free), and then incubated for 40 min with 5 µM fura 2-AM for subsequent Ca²⁺ measurement. The amount of [Ca²⁺]_i was estimated from a change in the fluorescence of the fura 2-loaded cells (Yun *et al.*, 2004). Fluorescence emission at 510 nm wavelength from two excitation wavelengths (340 nm and 380 nm) were measured every 0.1 sec by F4500 fluorescence spectrophotometer (Hitachi, Japan), and the ratio of fluorescence intensities from the two wavelengths was monitored as an estimate of [Ca²⁺]_i (Chang *et al.*, 2006).

Reverse Transcriptase-PCR

To identify expression of LPA receptors in HCT116 and HT29 cells by RT-PCR, first strand cDNA was synthesized with total RNA isolated by using the Promega ImProm-II Reverse Transcription System (Madison, WI, USA). Synthesized cDNA products along with primers for LPA₁₋₅ were used for PCR by using Promega Go-Taq DNA polymerase (Madison, WI, USA). Specific primers for LPA₁ (sense 5'- CAG GAC CCA ATA CTC GGA GA -3', antisense 5'- GTT GAA AAT GGC CCA GAA GA -3'), LPA₂ (sense 5'- TTT CAC TTG AGG GCT GGT TC -3', antisense 5'- CAT GAG CAG GAA GAC AAG CA -3'), LPA₃ (sense 5'- CTC ATG GCC TTC CTC ATC AT-3', antisense 5'- GCC ATA CAT GTC CTC GTC CT -3'), LPA₄ (sense 5'- CTT CGC AAG CCT GCT ACT CT -3', antisense 5'- GGC TTT GTG GTC AAA GGT GT -3') and LPA₅ (sense 5'- TCT CCC GTG TCC TGA CTA CC -3', antisense 5'- TGA GCA TCA GGA AGA TGC AG -3') were used to amplify 317, 317, 321, 341 and 308 bps of fragments of LPA₁₋₅, respectively. The PCR reaction was performed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 2 min in an Eppendorf Mastcycler gradient PCR machine (Hamburg, Germany) (Yun *et al.*, 2004). Ten µl of aliquots were electrophoresed in 1.2% agarose gels and stained with ethidium bromide.

Statistics

The results are expressed as means ± SE for the number of indicated determinations. Statistical significance of the differences was determined by ANOVA with significance accepted when P<0.05.

Fig. 1. Concentration-dependence of LPA- and PA-induced Ca^{2+} increases in HCT116 and HT29 human colon cancer cells. Representative Ca^{2+} traces with different concentrations of PA (upper panel) and LPA (lower panel) in HCT116 cells (A) or in HT29 human colon cancer cells (B). Each arrow indicates when the lipid was added. The data shown are representative of three independent experiments.

RESULTS

PA and LPA increase intracellular Ca^{2+} concentration in HCT116 and HT29 human colon cancer cells.

PA and LPA increased intracellular Ca^{2+} concentrations in both cell lines and in a concentration-dependent manner (Fig.

1). HCT116 cells showed a greater response to PA and LPA than did HT29 cells (Fig. 1). As shown Fig. 1, LPA was more potent than PA by increasing intracellular Ca^{2+} concentrations in both cell types.

Effects of U73122, PTX, 2-APB, and ruthenium red on PA- and LPA-induced Ca^{2+} responses.

To characterize the intracellular Ca^{2+} responses to both lipids, we treated cells with specific inhibitors (U73122, PTX, 2-APB, ruthenium red) of phospholipase C, $\text{G}_{i/o}$ -type G proteins, inositol 1,4,5-trisphosphate (IP_3) receptor, and ryanodine receptor, respectively. As shown in Fig. 2, U73122 and ruthenium red were not inhibitory on the responses of PA and LPA in the two cell types. However, PTX, a specific inhibitor of $\text{G}_{i/o}$ type G proteins was inhibitory on the responses except for an LPA-induced effect in the HT29 cells. In addition, 2-APB, a specific inhibitor of the IP_3 receptor, was seen to completely inhibit both lipid-induced Ca^{2+} increases in the two cell types. These results suggest that both LPA and PA mobilize Ca^{2+} through PTX-sensitive G proteins and IP_3 receptors in the HCT116 cells. On the other hand, PA mobilizes Ca^{2+} through moderately PTX-sensitive G proteins (about 60%) and IP_3 receptors in HT29 cells. However, LPA mobilizes Ca^{2+} through PTX-insensitive G proteins and IP_3 receptors in the HT29 cells.

Fig. 2. Effects of U73122, PTX, 2-APB, ruthenium red on PA- and LPA-induced Ca^{2+} increases. Cells treated with U73122 (5 μM , 10 min), PTX (100 ng/mL, 24 hr), 2-APB (100 μM , 10 min), ruthenium red (20 μM , 10 min) or with nothing were used for Ca^{2+} measurements with 100 nM of LPA or 5 μM of PA in HCT116 (A) or 800 nM of LPA or 30 μM of PA in HT29 cells (B). Data are presented as means $\pm$ SE of three independent experiments (A and B). Statistical significance: ** $P < 0.01$ vs. non-treated cells.

Fig. 3. Effects of Ki16425 and VPC32183 on PA- and LPA-induced Ca²⁺ increases. Cells treated with VPC32183 (20 μ M), Ki16425 (100 μ M) or without were used for Ca²⁺ measurements with 100 nM of LPA or 5 μ M of PA in HCT116 (A) or 800 nM of LPA or 30 μ M of PA in HT29 (B). Data are presented as means $\pm$ SE of three independent experiments (A and B). Statistical significance: ** P <0.01 vs. non-treated cells.

Effects of Ki16425 and VPC32183 on PA- and LPA-induced Ca²⁺ responses

We tested the effects of Ki16425 and VPC32183, two structurally-different antagonists of LPA₁/LPA₃ receptors to determine a possible involvement of endogenously expressed LPA receptors in the Ca²⁺ responses. As shown in Fig. 3, Ki16425 completely inhibited both LPA- and PA-induced Ca²⁺ responses in both HCT116 and HT29 cells. On the other hand, VPC32183, a structurally-different specific inhibitor of LPA₁/LPA₃ receptors, inhibited LPA- and PA-induced Ca²⁺ responses by nearly 70% in HCT116 cells (Fig. 3) and for PA-induced Ca²⁺ increase of 40% but not at all for an LPA-induced one in HT29 cells (Fig. 3). Therefore, PA and LPA appear to increase [Ca²⁺]_i through Ki16425/VPC32183-sensitive LPA receptors coupled to PTX-sensitive G proteins mainly in HCT116 cells. In the HT29 cells, however, LPA increases [Ca²⁺]_i through a Ki16425-sensitive and VPC32183-insensitive LPA receptor population coupled to PTX-insensitive G proteins, whereas PA utilized Ki16425-sensitive and partially VPC32183-sensitive LPA receptors where those LPA receptors are coupled to PTX-sensitive and insensitive G proteins.

Expression of LPA receptors in HCT116 and HT29 cells

RT-PCR was conducted to analyze expression levels of five LPA receptors, including the recently-identified LPA receptors, LPA₄ (GPR23) and LPA₅ (GPR92) (Kotarsky *et al.*, 2006; Lee *et al.*, 2006; Lee *et al.*, 2007; Noguchi *et al.*, 2003; Yanagida *et al.*, 2007). LPA₁ and LPA₄ were strongly expressed in human

Fig. 4. Expression analysis of five LPA receptors in the HCT116 and HT29 human colon cancer cells. RT-PCR was performed using mRNAs extracted from HCT116 and HT29 cells. The data is representative of three independent experiments which have yielded similar results.

colon cancer cells and LPA₅ was moderately detected; LPA₂ and LPA₃ were barely detected in either cell line (Fig. 4).

DISCUSSION

Although much research has focused on the second messenger roles of PA as a signaling product of phospholipase D (PLD) (Fang *et al.*, 2001; Hornberger *et al.*, 2006; Stace and Ktistakis, 2006), a first messenger role of PA on GPCR needs to be considered as well (English *et al.*, 1996), since there are several metabolic enzymes for PA (e.g. glycosylphosphatidylinositol-specific PLD in serum (Huang *et al.*, 1991) and ecto-phosphatidic acid phosphohydrolase (English *et al.*, 1997) and PA-specific phospholipase A₂ (Hiramatsu *et al.*, 2003; Sonoda *et al.*, 2002) in the plasma membrane which suggests production and degradation of PA in the extracellular milieu.

In recombinant LPA receptor-expressing cells, PA has been shown to be a partial agonist for LPA₁, LPA₂ and LPA₃ receptors (An *et al.*, 1998a; An *et al.*, 1998b; Fitzgerald *et al.*, 2000). However, partial agonism of PA in LPA receptor-overexpression systems remains largely controversial (Durgam *et al.*, 2006; Fischer *et al.*, 2001). Previously, we found an action of PA on endogenous LPA receptors in rat C6 glioma and mouse L2071 fibroblasts (Chang *et al.*, 2007). Thus, we have questioned whether PA could act as a ligand of GPCR on the plasma membrane and also whether the action is mediated through endogenous LPA receptors in human colon cancer cell lines. By employing pharmacological inhibitors of signaling molecules and LPA receptors, we have determined that PA increased [Ca²⁺]_i through endogenously expressed LPA receptors in the

cancer cell lines. We also observed differential susceptibility of PA- and LPA-induced responses in the HCT116 and HT29 cells to treatment with PTX, 2-APB, Ki16425, and VPC32183. PTX-sensitivity, VPC32183-sensitivity, and complete inhibitory effects by 2-APB and Ki16425 were observed in HCT116 cells, however, differential sensitivity to PTX and VPC32183 and complete inhibition by 2-APB and Ki16425 were observed in HT29 cells. It seems likely that mainly PTX- and VPC32183-insensitive LPA receptor type mediates Ca^{2+} increase by LPA in HT29 cells. However, PTX- and VPC32183-sensitive LPA receptor type is somewhat involved in the PA-induced Ca^{2+} increase. On the other hand, in HCT116 cells PTX-, Ki16425-, and the VPC32183-sensitive LPA receptor type primarily mediates the LPA- and PA-induced Ca^{2+} responses with but a small portion of the response mediated via PTX- and VPC32183-insensitive LPA receptors. The expression levels of LPA receptors in the two cancer cell lines show no significant differences. Thus, the disparity of antagonist susceptibilities of PA- and LPA-induced Ca^{2+} responses from the two cell lines can not be explained by expression of subtype LPA receptors. Differential coupling of LPA receptors and G proteins, however, could be the explanation (Im *et al.*, 2005; Kajiyama and Ui, 1994). Unfortunately, existing information on the susceptibility of the recently-identified LPA receptors (LPA_4 and LPA_5) to both antagonists has not been made available, thereby limiting a more complete assessment of our results (Kotarsky *et al.*, 2006; Lee *et al.*, 2006; Lee *et al.*, 2007; Noguchi *et al.*, 2003; Yanagida *et al.*, 2007).

Although further investigation is necessary to confirm production of PA in the extracellular milieu and antagonist susceptibilities of the LPA_4 and LPA_5 , our present results regarding a first messenger role of PA and its agonistic effects on endogenous LPA receptors in human colon cancer cells would strongly suggest it to be an intercellular bioactive lipid mediator in human being.

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