Calcium Signaling of Lysophosphatidylethanolamine through LPA₁ in Human SH-SY5Y Neuroblastoma Cells

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Abstract

Lysophosphatidylethanolamine (LPE), a lyso-type metabolite of phosphatidylethanolamine, has been reported to be an intercellular signaling molecule. LPE mobilizes intracellular Ca²⁺ through G-protein-coupled receptor (GPCR) in some cell types. However, GPCRs for lysosphosphatic acid (LPA) were not implicated in the LPE-mediated activities in LPA GPCR overexpression systems or in SK-OV3 ovarian cancer cells. In the present study, in human SH-SY5Y neuroblastoma cells, experiments with LPA₁ antagonists showed LPE induced intracellular Ca²⁺ increases in an LPA₁ GPCR-dependent manner. Furthermore, LPE increased intracellular Ca²⁺ through pertussis-sensitive G proteins, edelfosine-sensitive-phospholipase C, 2-APB-sensitive IP₃ receptors, Ca²⁺ release from intracellular Ca²⁺ stores, and subsequent Ca²⁺ influx across plasma membranes, and LPA acted on LPA₁ and LPA₂ receptors to induce Ca²⁺ response in a 2-APB-sensitive and insensitive manner. These findings suggest novel involvements for LPE and LPA in calcium signaling in human SH-SY5Y neuroblastoma cells.

Key Words: Lysophosphatidylethanolamine, LPA₁, Lysosphosphatic acid, GPCR, Neuroblastoma, Receptor

INTRODUCTION

Lysophosphatidylethanolamine (LPE), a lyso-type metabolite of phosphatidylethanolamine, has been reported to be an intercellular signaling molecule. LPE mobilizes intracellular Ca²⁺ through G-protein-coupled receptor (GPCR) in some cell types. However, GPCRs for lysosphosphatic acid (LPA) were not implicated in the LPE-mediated activities in LPA GPCR overexpression systems or in SK-OV3 ovarian cancer cells. In the present study, in human SH-SY5Y neuroblastoma cells, experiments with LPA₁ antagonists showed LPE induced intracellular Ca²⁺ increases in an LPA₁ GPCR-dependent manner. Furthermore, LPE increased intracellular Ca²⁺ through pertussis-sensitive G proteins, edelfosine-sensitive-phospholipase C, 2-APB-sensitive IP₃ receptors, Ca²⁺ release from intracellular Ca²⁺ stores, and subsequent Ca²⁺ influx across plasma membranes, and LPA acted on LPA₁ and LPA₂ receptors to induce Ca²⁺ response in a 2-APB-sensitive and insensitive manner. These findings suggest novel involvements for LPE and LPA in calcium signaling in human SH-SY5Y neuroblastoma cells.

LPE has been detected in human serum at concentrations of about several hundreds of nanograms per ml (Misra, 1965; Makide et al., 2009), but the physiological significance of plasma LPE remains unknown. LPE has also been shown to play a role in intercellular signaling and in the activation of signaling enzymes (Park et al., 2007b), and has been suggested to act through putative G protein-coupled receptors (GPCRs) (Park et al., 2007b, 2013). Furthermore, GPCRs for lysosphosphatic acid (LPA), a serum-derived lipid mediator, have been discovered and named LPA₁-6 (Choi and Chun, 2013), and these discoveries resulted in intensive knock-out mouse studies and in the developments of selective agonists and antagonists (Im, 2010). However, few studies have been conducted on LPE GPCRs.

In SK-OV3 and OVCAR-3 ovarian cancer cells, LPE induces several responses, which include increasing intracellular Ca²⁺ concentration ([Ca²⁺]i) (Park et al., 2007b), and these responses have been proposed to be mediated through GPCR, but not through GPCRs for LPA (Park et al., 2007b). Actually, LPA GPCRs do not respond to LPE in LPA GPCR overexpression systems (Park et al., 2007b). However, LPE does induce [Ca²⁺] increases through LPA₁ in MDA-MB-231 breast cancer cells and PC-12 pheochromocytoma cells (Park et al., 2013, 2014a; Lee et al., 2015). Intracellular Ca²⁺ signaling has crucial roles in development from fertilization through differentiation to organogenesis (Leclerc et al., 2012). In the nervous system, Ca²⁺ signaling plays important roles in the development from neural induction to the proliferation, migration, and differentiation of neural cells (Leclerc et al., 2012).
In the present study, the relation between LPA-induced Ca\(^{2+}\) response and LPE-induced Ca\(^{2+}\) signaling was studied in human SH-SY5Y neuroblastoma cells.

**MATERIALS AND METHODS**

**Materials**

1-Oleoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine (18:1 LPE), 1-stearoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine (18:0 LPE), 1-octadecyl-2-hydroxy-sn-glycero-3-phosphoethanolamine (ether-linked 18:0 LPE), 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine (16:0 LPE), 1-oleyl-2-hydroxy-sn-glycero-3-phosphate (LPA, sodium salt), and VPC32183 were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Fura 2-AM, EGTA, 2-aminoethoxydiphenylborane (2-APB) and pertussis toxin (PTX) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ki16425 and edelfosine were obtained from Cayman chemical (Ann Arbor, MI, USA). AM-095 was from Chemscene (Monmouth Junction, NJ, USA).

**Cell culture**

Human SH-SY5Y neuroblastoma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured at 37°C in a 5% CO\(_2\) humidified incubator, and maintained in RPMI 1640 medium (GenDEPOT, Barker, TX, USA) supplemented with 10% (v/v) fetal bovine serum, 100 units/mL penicillin, 50 \(\mu\)g/mL streptomycin, 2 mM glutamine, and 1 mM sodium pyruvate.

**Measurement of [Ca\(^{2+}\)]\(_{i}\) concentrations**

Cells were trypsin-digested, allowed to sediment, resuspended in HEPES-buffered medium (HBM), consisting of 20 mM HEPES (pH 7.4), 103 mM NaCl, 4.8 mM KCl, 1.2 mM KH\(_2\)PO\(_4\), 1.2 mM MgSO\(_4\), 0.5 mM CaCl\(_2\), 25 mM NaHCO\(_3\), and 15 mM glucose, and then incubated for 40 min with 5 \(\mu\)M fura 2-AM. [Ca\(^{2+}\)]\(_{i}\) levels were estimated by measuring changes in fura-2 fluorescence at an emission wavelength of 510 nm and excitation wavelengths of 340 nm and 380 nm every 0.1 sec using a F4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) (Park et al., 2013). Ratios of fluorescence intensities (\(\lambda_{340}/\lambda_{380}\)) at these two wavelengths were used as surrogates of [Ca\(^{2+}\)]\(_{i}\), as previously described (Park et al., 2014a).

**Reverse transcriptase-PCR**

To detect the expressions of LPA receptors in SH-SY5Y cells by RT-PCR, first strand cDNA was synthesized using total RNA isolated using Trizol reagent (Invitrogen, Waltham, MA, USA). Synthesized cDNA products and primers for LPA1-6 were subjected to PCR using Promega Go-Taq DNA polymerase (Madison, WI, USA). The primers used to amplify 317, 317, 321, 341, 308, and 247 bps fragments of LPA 1-6 and GAPDH were as follows: 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 AGG CA-3’), LPA3 (sense 5’-TTT CAC TTG AGG GCT GGT TC-3’, antisense 5’-GTT GAA AAT GGC CCA GAA GA-3’), LPA4 (sense 5’-TTT CAC TTG AGG GCT GGT TC-3’, antisense 5’-GTT GAA AAT GGC CCA GAA GA-3’), LPA5 (sense 5’-TTT CAC TTG AGG GCT GGT TC-3’, antisense 5’-GTT GAA AAT GGC CCA GAA GA-3’), LPA6 (sense 5’-TTT CAC TTG AGG GCT GGT TC-3’, antisense 5’-GTT GAA AAT GGC CCA GAA GA-3’).

**Fig. 1.** Concentration-dependences of LPE- and LPA-induced [Ca\(^{2+}\)]\(_{i}\) increases in SH-SY5Y neuroblastoma cells. Representative [Ca\(^{2+}\)]\(_{i}\) traces of SH-SY5Y cells treated with various concentrations of 18:1 LPE (A) and 18:1 LPA (B). Arrows indicate when lipids were added. Concentration-response curves for LPE (C) and LPA (D) for [Ca\(^{2+}\)]\(_{i}\) increases in cells. Results are presented as the means ± SEs of three independent experiments.
GT-3’), LPA5 (sense 5’-TCT CCC GTG TCC TGA CTA CC-3’, antisense 5’-TGA GCA TCA GGA AGA TGC AG-3’), and LPA6 (sense 5’-TGC TCA GTA GTG GCA GCA GT-3’, antisense 5’-CAG GCA GCA GAT TTG TC-3’), and GAPDH (sense 5’-GAG TCA ACG GAT TTG GTC GT-3’, antisense 5’TGG ATT TTG GAG GGA TCT CG-3’). PCR reactions were performed over 30 cycles of 95°C for 30 s (denaturation), 57°C for 30 s (annealing) for LPA1-6, and 72°C for 30 s (elongation) for GAPDH in an Eppendorf Mastercycler gradient unit (Hamburg, Germany) (Park et al., 2014b). Aliquots of the PCR products (7 μl) obtained were electrophoresed in 1.2% agarose gels and stained with ethidium bromide.

**Statistics**

The results are expressed as means ± SEs for the indicated numbers of determinations. Significances of differences were determined using the student t test, and significance was accepted for *p*-values < 0.05.

**RESULTS**

LPE increased [Ca²⁺] in SH-SY5Y neuroblastoma cells

Synthetic oleoyl LPE (18:1 LPE) increased [Ca²⁺] levels in SH-SY5Y neuroblastoma cells (Fig. 1A) in a concentration-dependent manner (Fig. 1C), and response to LPA was greater than to LPE, but in SH-SY5Y cells LPA and LPE had similar
investigated homologous and heterologous desensitizations of LPE action (Fig. 3C). These results suggest that LPE acts on LPA1 expressed, whereas the other four LPA receptors were not desensitized in desensitization experiments. LPE or LPA were pre-treated for various times before addition of the agonist in MB-231 cells, in which oleoyl LPE (18:1 LPE) was the only active LPE. Structure-activity relationships in LPE-responsive MB-231 cells, in which oleoyl LPE (18:1 LPE) was the only active LPE, were also studied using structurally different LPEs, that is, stearoyl LPE (18:0 LPE), octadecanoyl LPE (ether-linked 18:0 LPE), and palmitoyl LPE (16:0 LPE). As shown in Fig. 2, 18:1 LPE, 18:0 LPE, ether-linked 18:0 LPE, and 16:0 LPE induced a [Ca2+]i increase in SH-SY5Y cells, which contrasted to that observed in MDA-MB-231 cells, in which oleoyl LPE (18:1 LPE) was the only active LPE. Structure-activity relationships in LPE-responsive cells are addressed in the Discussion.

**Heterologous desensitization between LPE- and LPA-induced [Ca2+]i responses**

Because previous studies have implicated LPA receptor in LPE-induced Ca2+ signaling in certain cell types, we investigated homologous and heterologous desensitizations of LPE- and LPA-induced [Ca2+]i increases in SH-SY5Y cells. In desensitization experiments, LPE or LPA were pre-treated for 1 min before adding LPE (10 μM) or LPA (10 μM). As shown in Fig. 3A, 3B, LPE pre-treatment blocked LPE-induced [Ca2+]i response by 100%, and LPA pre-treatment attenuated LPA-induced [Ca2+]i response by 90%, implying homologous desensitization. In addition, LPA pre-treatment attenuated LPE-induced [Ca2+]i response by 90%, and LPE pre-treatment attenuated LPA-induced [Ca2+]i response by 63%, implying heterologous desensitization (Fig. 3). In addition, we examined the expression levels of the six known LPA receptors by RT-PCR in human SH-SY5Y cells. LPA1 and LPA2 were found to be strongly expressed, whereas the other four LPA receptors were not detected (Fig. 3C). These results suggest that LPE acts on LPA1 and/or LPA2 receptors in SH-SY5Y cells.

**Effects of LPA antagonists on LPE- or LPA-induced [Ca2+]i responses**

Three pharmacological tools were applied to investigate the involvements of LPA receptors in SH-SY5Y cells, that is, structurally different antagonists of LPA1 and LPA3 (Ki16425 and VPC32183) (Heise et al., 2001; Ohta et al., 2003) and a selective LPA1 antagonist, AM-095 (Castelino et al., 2011; Swaney et al., 2011). As shown in Fig. 4, Ki16425 (10 μM), VPC32183 (1 μM), and AM-095 (500 nM) completely inhibited LPE-induced [Ca2+]i response, whereas Ki16425 and AM-095 inhibited it by more than 50%, and VPC32183 had no effect (Fig. 4). Thus, it appeared LPE induced [Ca2+]i through LPA1 receptors in SH-SY5Y cells, whereas LPA increased [Ca2+]i through AM-095/Ki16425-sensitive LPA1, and AM-095/Ki16425-insensitive LPA2 receptors.

**Effects of PTX, edelfosine, 2-APB, EGTA or HA130 on LPE- or LPA-induced [Ca2+]i responses**

To investigate cascades signaling LPE and LPA [Ca2+]i responses, SH-SY5Y cells were treated with specific inhibitors or blockers of Gαi/o-type G proteins, phospholipase C, inositol 1,4,5-trisphosphate receptor (IP3R), extracellular Ca2+, or autotaxin, that is, pertussis toxin (PTX), edelfosine, 2-APB, EGTA, and HA130, respectively (Park et al., 2007b; Melchior and Frangos, 2012; Zhang et al., 2012). As shown in Fig. 5, PTX, a specific inhibitor of Giα type G proteins, inhibited [Ca2+]i responses to LPE by 84% and to LPA by 67%, suggesting the involvements of Giα proteins in [Ca2+]i responses to LPE and LPA (Fig. 5). In addition, edelfosine (a specific inhibitor of phospholipase C) also partially inhibited responses to LPE and LPA, suggesting the involvement of phospholipase C in these responses (Fig. 5). Next, the involvement of IP3 receptor on Ca2+ release from endoplasmic reticulum (ER) was tested using 2-APB, a specific inhibitor of IP3R. Pretreatment with 2-APB inhibited completely LPE-induced [Ca2+]i increase, but only partly inhibited LPA-induced [Ca2+]i increase (Fig. 5). To investigate the possibility that Ca2+ influx across the plasma membrane contributed to Ca2+ response, we pretreated SH-SY5Y cells with EGTA (an extracellular Ca2+ chelator). EGTA
partially inhibited LPE- and LPA-induced \([\text{Ca}^{2+}]\) increases, suggesting that \(\text{Ca}^{2+}\) influx across the plasma membrane contributed to observed \([\text{Ca}^{2+}]\) increases. Because LPE could not induce \(\text{Ca}^{2+}\) increase even when \(\text{Ca}^{2+}\) ions were present in extracellular media in the presence of 2-APB, we supposed LPE-induced \(\text{Ca}^{2+}\) influx was mediated solely by IP₃ receptor-mediated \(\text{Ca}^{2+}\) release. However, the partial inhibition of LPA-induced \(\text{Ca}^{2+}\) increase by 2-APB suggested another component, insensitive to 2-APB, signaled \(\text{Ca}^{2+}\) influx for LPA. These results suggest involvements of \(G_{\text{i/o}}\)-type proteins, phospholipase C, IP₃ receptors, \(\text{Ca}^{2+}\) release from intracellular \(\text{Ca}^{2+}\) stores, and \(\text{Ca}^{2+}\) influx across the plasma membrane in LPE- and LPA-induced \([\text{Ca}^{2+}]\) increases in SH-SY5Y cells.

To determine whether LPE is converted to LPA by autotaxin (also known as lysophospholipase D), and this LPA mediates the action of LPE, we pretreated SH-SY5Y cells with HA130 (a specific inhibitor of autotaxin). However, HA130 did not inhibit LPE-induced \(\text{Ca}^{2+}\) increase, indicating that autotaxin was not responsible for the observed effects of LPE (Fig. 6).

**DISCUSSION**

In the present study, LPE-induced \([\text{Ca}^{2+}]\) increase was found to be mediated through LPA₁ in SH-SY5Y cells. Five results sustain this finding: 1) the observed heterologous desensitization found for LPE- and LPA-induced \([\text{Ca}^{2+}]\) increases, 2) the abrogation of LPE-induced response by the LPA₁ antagonist Ki16425 supported the involvements of LPA₁ and/or LPA₁; 3) the complete inhibition of LPE-induced

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**Fig. 5.** Effects of EGTA, PTX, 2-APB, and edelfosine on LPE- and LPA-induced \([\text{Ca}^{2+}]\) increases in SH-SY5Y neuroblastoma cells. \([\text{Ca}^{2+}]\) levels in cells pre-treated with or without EGTA (5 mM, 1 min), PTX (100 ng/mL, 24 h), 2-APB (100 μM, 15 min), or edelfosine (10 μM, 6 h) were monitored after being treated with LPE (10 μM) or LPA (10 μM). (A, C) Representative \([\text{Ca}^{2+}]\) traces of SH-SY5Y cells treated with 10 μM of LPE or 10 μM LPA in the presence of PTX, edelfosine, 2-APB, EGTA, or vehicle. Arrows indicate when lipids were added. The results shown are representative of more than three independent experiments. (B, D) Increases in \([\text{Ca}^{2+}]\) by LPE (10 μM) or LPA (10 μM) were observed in cells pre-treated with or without PTX, edelfosine, 2-APB, or EGTA. Results are presented as the means ± SEs of three independent experiments (B). Statistical significance: *\(p<0.05\), **\(p<0.01\), ***\(p<0.001\) vs. non-treated cells.
response by the LPA1 antagonist, AM-095, 4) the observation that LPA1 was expressed in SH-SY5Y cells, and 5) the G\textsubscript{i/o}-coupling character of LPA1 and the PTX-sensitivity of LPE-induced [Ca\textsuperscript{2+}]\textsubscript{i} increase. LPE-induced [Ca\textsuperscript{2+}]\textsubscript{i} increases have been previously observed in ovarian and breast cancer cells and in pheochromocytoma cells (Park \textit{et al.}, 2007b, 2013, 2014a; Lee \textit{et al.}, 2015). Table 1 summarizes the responses observed in SH-SY5Y cells, PC-12 cells, and ovarian (SKOV3) and breast cancer (MDA-MB-231) cells.

In ovarian cancer cells, LPE-induced [Ca\textsuperscript{2+}]\textsubscript{i} increase was not found to be mediated through Ki16425, VPC32183, or AM-095-sensitive receptors (Park \textit{et al.}, 2007a, 2013), and heterologous desensitization was not observed, although homologous desensitization was observed for LPE- and LPA-induced [Ca\textsuperscript{2+}]\textsubscript{i} increases (Park \textit{et al.}, 2013). Therefore, it appears LPE-induced response in SK-OV3 ovarian cancer cells differs from that in SH-SY5Y, MDA-MB-231, and PC-12 cells. On the other hand, in MDA-MB-231 breast cancer cells and PC-12 cells, LPE-induced [Ca\textsuperscript{2+}]\textsubscript{i} response was inhibited by Ki16425, VPC32183, or AM-095, and heterologous desensitization was observed, indicating intermediation of LPE-induced response in MDA-MB-231 and PC-12 cells by LPA1 (Park \textit{et al.}, 2013). Consequently, LPE-induced [Ca\textsuperscript{2+}]\textsubscript{i} response in SH-SY5Y cells is similar to that in MDA-MB-231 cells and PC-12 cells in terms of LPA1 involvement.

In the present study, LPE-induced Ca\textsuperscript{2+} responses of synthetic LPE analogues were cell type dependent. In particular, ether-linked 18:0 LPE and ester-linked 18:0 LPE produced more than 50% of the response elicited by ester-linked 18:1 LPE in SK-OV3, SH-SY5Y, and PC-12 cells, but did not produce any response in MDA-MB-231 cells (Park \textit{et al.}, 2007b, 2013, 2014a; Lee \textit{et al.}, 2015) (Table 1). It has been previously reported that 16:0 LPE does not induce [Ca\textsuperscript{2+}]\textsubscript{i} response in SK-OV3 cells, MDA-MB-231 cells, or PC-12 cells (Park \textit{et al.}, 2013, 2014a; Lee \textit{et al.}, 2015). However, in the present study, 16:0 LPE induced [Ca\textsuperscript{2+}]\textsubscript{i} response in SH-SY5Y cells (Table 1). In addition, in previous studies, 14:0 LPE induced similar [Ca\textsuperscript{2+}]\textsubscript{i} responses to LPA in all four cell types (Park \textit{et al.}, 2014a; Lee \textit{et al.}, 2015). However, 14:0 LPE-induced [Ca\textsuperscript{2+}]\textsubscript{i} response may be driven by a different mechanism to 18:1 LPE-induced [Ca\textsuperscript{2+}]\textsubscript{i} response, because 14:0 LPE induced Ca\textsuperscript{2+} response in cells non-responsive to 18:1 LPE. These findings show that LPE-induced [Ca\textsuperscript{2+}]\textsubscript{i} responses have similar and dissimilar features in these four cell types; that is, MDA-MB-231, PC-12 and SH-SY5Y cells all exhibit LPA1 involvement in responses, whereas their responses to different LPE structural types differ (Park \textit{et al.}, 2007a, 2013, 2014a; Lee \textit{et al.}, 2015) (Table 1).

**Table 1. LPE-induced responses in SH-SY5Y, PC-12, MDA-MB-231, and SK-OV3 cells**

<table>
<thead>
<tr>
<th>Inhibition by PTX</th>
<th>Responses to different LPEs</th>
<th>Inhibition by LPA1 antagonists</th>
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<tr>
<td></td>
<td>18:1</td>
<td>18:0</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>PC-12</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td>MDA-MB-231</td>
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<td>Yes</td>
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<tr>
<td>SK-OV3</td>
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**Fig. 6.** Effects of HA130 on LPE- and LPA-induced Ca\textsuperscript{2+} responses. [Ca\textsuperscript{2+}]\textsubscript{i} levels in SH-SY5Y cells pre-treated with or without HA130 (5 μM, 5 min; an autotaxin inhibitor) were monitored after being treated with LPE (10 μM) or LPA (10 μM) (A). Arrows indicate when lipids were added. The results shown are representative of more than three independent experiments. (B) Increases in [Ca\textsuperscript{2+}]\textsubscript{i} by LPE (10 μM) or LPA (10 μM) were observed in cells pre-treated with or without HA130 (5 μM, 5 min). Results are presented as the means ± SEs of three independent experiments. NS, statistical non-significant.
Therefore, in SH-SY5Y cells, LPE was found to act on LPA1 to induce \( [\text{Ca}^{2+}]_i \) increase via G\(_i/o\) proteins, phospholipase C, and IP\(_3\), and LPA was found to use LPA1 and LPA2 to mobilize Ca\(^{2+}\) (Fig. 7). Significance of this study is not only LPE action on LPA1 in SH-SY5Y cells but also involvement of G\(_i/o\) proteins and phospholipase C in LPA2\(^{Ca^{2+}}\) signaling. In previous studies using SH-SY5Y cells, LPA-induced Ca\(^{2+}\) mobilization was shown to be independent on phosphoinositide signaling and not mediated through pertussis toxin-sensitive G\(_i/o\) proteins (Young et al., 1999, 2000). Activation of sphingosine kinase and its product sphingosine 1-phosphate was proposed as a second messenger for LPA-induced Ca\(^{2+}\) signaling (Young et al., 1999, 2000). However, in the present study, involvement of pertussis toxin-sensitive G\(_i/o\) proteins and edelfosine-sensitive phospholipase C were shown in the LPA-induced Ca\(^{2+}\) signaling. Further investigation of the physiological roles of LPE in neuronal cells is required.

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