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Calcium Signaling of Lysophosphatidylethanolamine through LPA_1 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 Ca^{2+} through G-protein-coupled receptor (GPCR) in some cells types. However, GPCRs for lysophosphatidic 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 LPA_1 antagonists showed LPE induced intracellular Ca^{2+} increases in an LPA_1 GPCR-dependent manner. Furthermore, LPE increased intracellular Ca^{2+} through pertussis-sensitive G proteins, edelfosine-sensitive-phospholipase C, 2-APB-sensitive IP_3 receptors, Ca^{2+} release from intracellular Ca^{2+} stores, and subsequent Ca^{2+} influx across plasma membranes, and LPA acted on LPA_1 and LPA_2 receptors to induce Ca^{2+} 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, LPA_1, Lysophosphatidic acid, GPCR, Neuroblastoma, Receptor

INTRODUCTION

Lysophosphatidylethanolamine (LPE) is a metabolic product from phosphatidylethanolamine (a minor constituent of cell membranes) by phospholipase A_2. LPE has an ethanolamine head group linked to a lysophosphatidic acid. LPE is commercially used as a plant bio-regulator to delay leaf and fruit senescence, improve product shelf-life post harvest, and mitigate ethylene-induced process (Cowan, 2009). In addition, LPE appears to have certain roles in organisms other than mammals, for example, in the housefly, LPE has antimicrobial activity (Meylaers *et al*., 2004). Furthermore, LPEs isolated from *Grifola frondosa* were recently reported to exhibit anti-apoptotic activity and to enhance neuronal differentiation via MAPK activation in PC-12 cells (Nishina *et al*., 2006).

LPE has been detected in human serum at concentrations of about several hundreds 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 lysophosphatidic acid (LPA), a serum-derived lipid mediator, have been discovered and named LPA_{1-6} (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 Ca^{2+} concentration ($[Ca^{2+}]_i$) (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 $[Ca^{2+}]_i$ 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 Ca^{2+} signaling has crucial roles in development from fertilization through differentiation to organogenesis (Leclerc *et al*., 2012). In the nervous system, Ca^{2+} signaling plays important roles in the development from neural induction to the proliferation, migration, and differentiation of neural cells (Leclerc *et al*., 2012).

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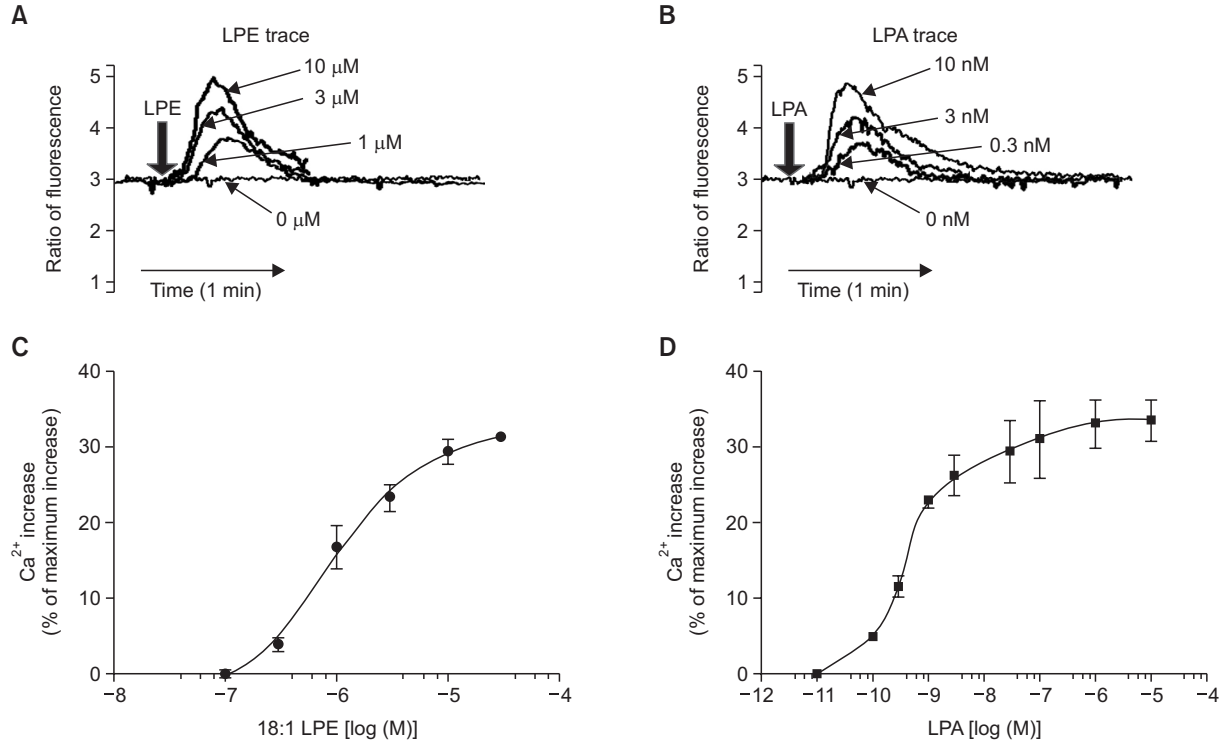

Fig. 1. Concentration-dependences of LPE- and LPA-induced $[Ca^{2+}]_i$ increases in SH-SY5Y neuroblastoma cells. Representative $[Ca^{2+}]_i$ traces of SH-SY5Y cells treated with various concentrations of 18:1 LPE (A) and 18:1 LPA (B). Arrows indicate when lipids were added. Concentration-response curves for LPE (C) and LPA (D) for $[Ca^{2+}]_i$ increase in cells. Results are presented as the means ± SEs of three independent experiments.

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-oleoyl-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 μg/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_2PO_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 μM 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 $LPA_{1\text{-}6}$ 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 $LPA_{1\text{-}6}$ and GAPDH were as follows: LPA_1 (sense 5'-CAG GAC CCA ATA CTC GGA GA-3', antisense 5'-GTT GAA AAT GGC CCA GAA GA-3'), LPA_2 (sense 5'-TTT CAC TTG AGG GCT GGT TC-3', antisense 5'-CAT GAG CAG GAA GAC AAG CA-3'), LPA_3 (sense 5'-CTC ATG GCC TTC CTC ATC AT-3', antisense 5'-GCC ATA CAT GTC CTC GTC CT-3'), LPA_4 (sense 5'-CTT CGC AAG CCT GCT ACT CT-3', antisense 5'-GGC TTT GTG GTC AAA GGT

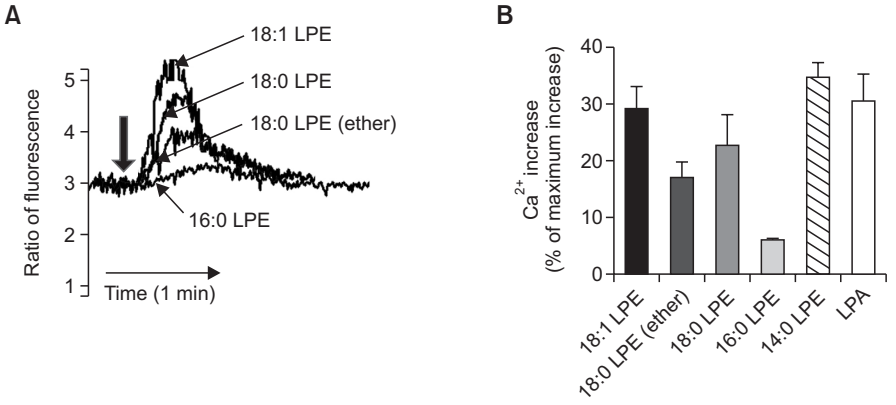

Fig. 2. Effects of synthetic LPEs, that is, 18:1 LPE, 18:0 LPE, 18:0 ether-linked LPE, 14:0 LPE, and 16:0 LPE in SH-SY5Y neuroblastoma cells. Representative $[Ca^{2+}]_i$ traces of SH-SY5Y cells treated with synthetic LPEs (A). Arrows indicate when lipids were added. The results shown are representative of at least three independent experiments. Ca^{2+} responses are presented as the means ± SEs of three independent experiments (B).

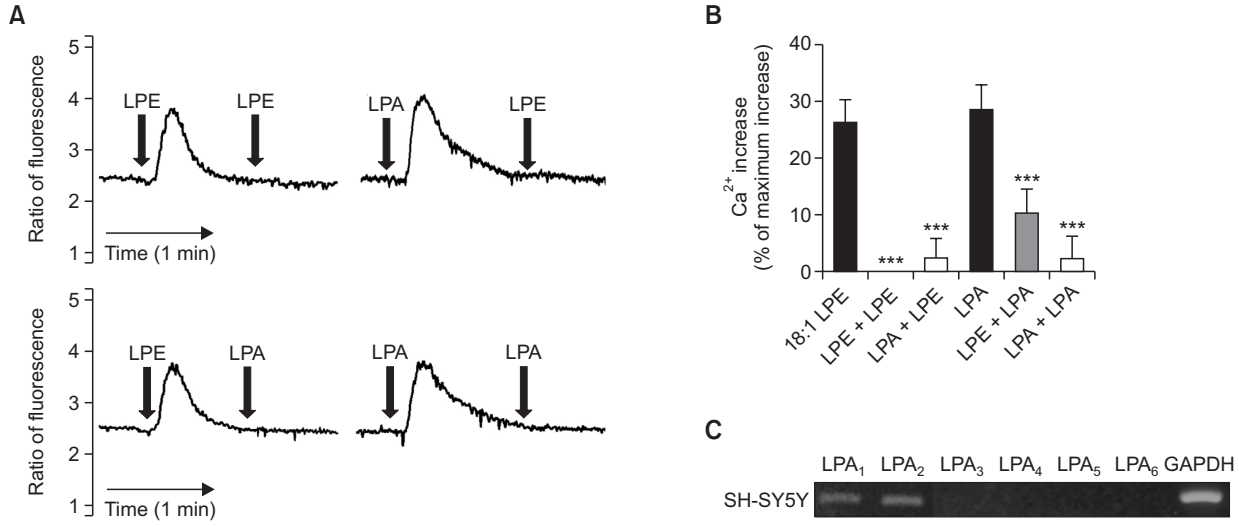

Fig. 3. Desensitization of LPE- or LPA-induced $[Ca^{2+}]_i$ increase by LPE or LPA and expression analysis of six LPA receptors in SH-SY5Y neuroblastoma cells. $[Ca^{2+}]_i$ levels in SH-SY5Y cells pre-treated with 10 μM of LPE or 10 μM LPA were monitored after treating them with 10 μM LPE or 10 μM LPA (A). Arrows indicate when lipids were added. Results are representative of at least three independent experiments. $[Ca^{2+}]_i$ increases induced by 10 μM of LPE or 10 μM LPA alone and $[Ca^{2+}]_i$ increases induced by lipids after pretreating SH-SY5Y cells with LPE or LPA. Results are the means ± SEs of three independent experiments (B). Statistical significance: ***p<0.001 vs. non-pretreated cells. (C) RT-PCR was performed using total mRNA from SH-SY5Y cells. Results are representative of three independent experiments that yielded similar results.

GT-3'), LPA_5 (sense 5'-TCT CCC GTG TCC TGA CTA CC-3', antisense 5'-TGA GCA TCA GGA AGA TGC AG-3'), and LPA_6 (sense 5'-TGC TCA GTA GTG GCA GCA GT-3', antisense 5'-CAG GCA GCA GAT TCA TTG TC-3'), and GAPDH (sense 5'-GAG TCA ACG GAT TTG GTC GT-3', antisense 5'-TTG 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 LPA_{1-6}, and 72°C for 30 s (elongation) for GAPDH in an Eppendorf Mastcycler 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 $[Ca^{2+}]_i$ in SH-SY5Y neuroblastoma cells

Synthetic oleoyl LPE (18:1 LPE) increased $[Ca^{2+}]_i$ 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

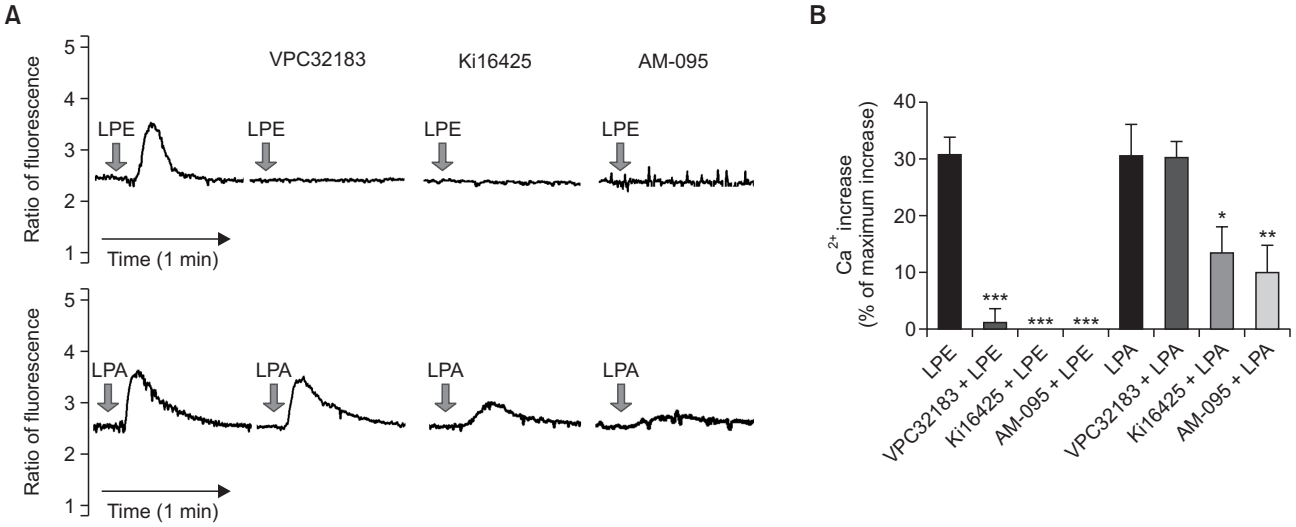

Fig. 4. Effects of Ki16425, VPC32183, and AM-095 on LPE- or LPA-induced $[Ca^{2+}]_i$ increase in SH-SY5Y neuroblastoma cells. Representative $[Ca^{2+}]_i$ traces of SH-SY5Y cells treated with 10 μM of LPE or 10 μM LPA in the presence of Ki16425, VPC32183, AM-095, or vehicle (A). Arrows indicate when lipids were added. The results shown are representative of more than three independent experiments. Increases in $[Ca^{2+}]_i$ by LPE (10 μM) or LPA (10 μM) were observed in cells pre-treated with or without Ki16425 (10 μM), VPC32183 (1 μM), or AM-095 (500 nM). 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.

efficacies (Fig. 1B, 1D). Responses were also studied using structurally different LPEs, that is, stearoyl LPE (18:0 LPE), octadecanyl 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 $[Ca^{2+}]_i$ increase in SY-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 $[Ca^{2+}]_i$ responses

Because previous studies have implicated LPA receptor in LPE-induced Ca^{2+} signaling in certain cell types, we investigated homologous and heterologous desensitizations of LPE- and LPA-induced $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ response by 100%, and LPA pre-treatment attenuated LPA-induced response by 90%, implying homologous desensitization. In addition, LPA pre-treatment attenuated LPE-induced $[Ca^{2+}]_i$ response by 90%, and LPE pre-treatment attenuated LPA-induced $[Ca^{2+}]_i$ 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. LPA_1 and LPA_2 were found to be strongly expressed, whereas the other four LPA receptors were not detected (Fig. 3C). These results suggest that LPE acts on LPA_1 and/or LPA_2 receptors in SH-SY5Y cells.

Effects of LPA antagonists on LPE- or LPA-induced $[Ca^{2+}]_i$ responses

Three pharmacological tools were applied to investigate the involvements of LPA receptors in SH-SY5Y cells, that is, structurally different antagonists of LPA_1 and LPA_3 (Ki16425 and VPC32183) (Heise *et al*., 2001; Ohta *et al*., 2003) and a selective LPA_1 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 $[Ca^{2+}]_i$ response, whereas Ki16425 and AM-095 inhibited it by more than 50%, and VPC32183 had no effect (Fig. 4). Thus, it appeared LPE increased $[Ca^{2+}]_i$ through LPA_1 receptors in SH-SY5Y cells, whereas LPA increased $[Ca^{2+}]_i$ through AM-095/Ki16425-sensitive LPA_1 and AM-095/Ki16425-insensitive LPA_2 receptors.

Effects of PTX, edelfosine, 2-APB, EGTA or HA130 on LPE- or LPA-induced $[Ca^{2+}]_i$ responses

To investigate cascades signaling LPE and LPA $[Ca^{2+}]_i$ responses, SH-SY5Y cells were treated with specific inhibitors or blockers of $G_{i/o}$-type G proteins, phospholipase C, inositol 1,4,5-trisphosphate receptor (IP_3R), extracellular Ca^{2+}, 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 $G_{i/o}$ type G proteins, inhibited $[Ca^{2+}]_i$ responses to LPE by 84% and to LPA by 67%, suggesting the involvements of $G_{i/o}$ proteins in $[Ca^{2+}]_i$ 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 IP_3 receptor on Ca^{2+} release from endoplasmic reticulum (ER) was tested using 2-APB, a specific inhibitor of IP_3R. Pretreatment with 2-APB inhibited completely LPE-induced $[Ca^{2+}]_i$ increase, but only partly inhibited LPA-induced $[Ca^{2+}]_i$ increase (Fig. 5). To investigate the possibility that Ca^{2+} influx across the plasma membrane contributed to Ca^{2+} response, we pretreated SH-SY5Y cells with EGTA (an extracellular Ca^{2+} chelator). EGTA

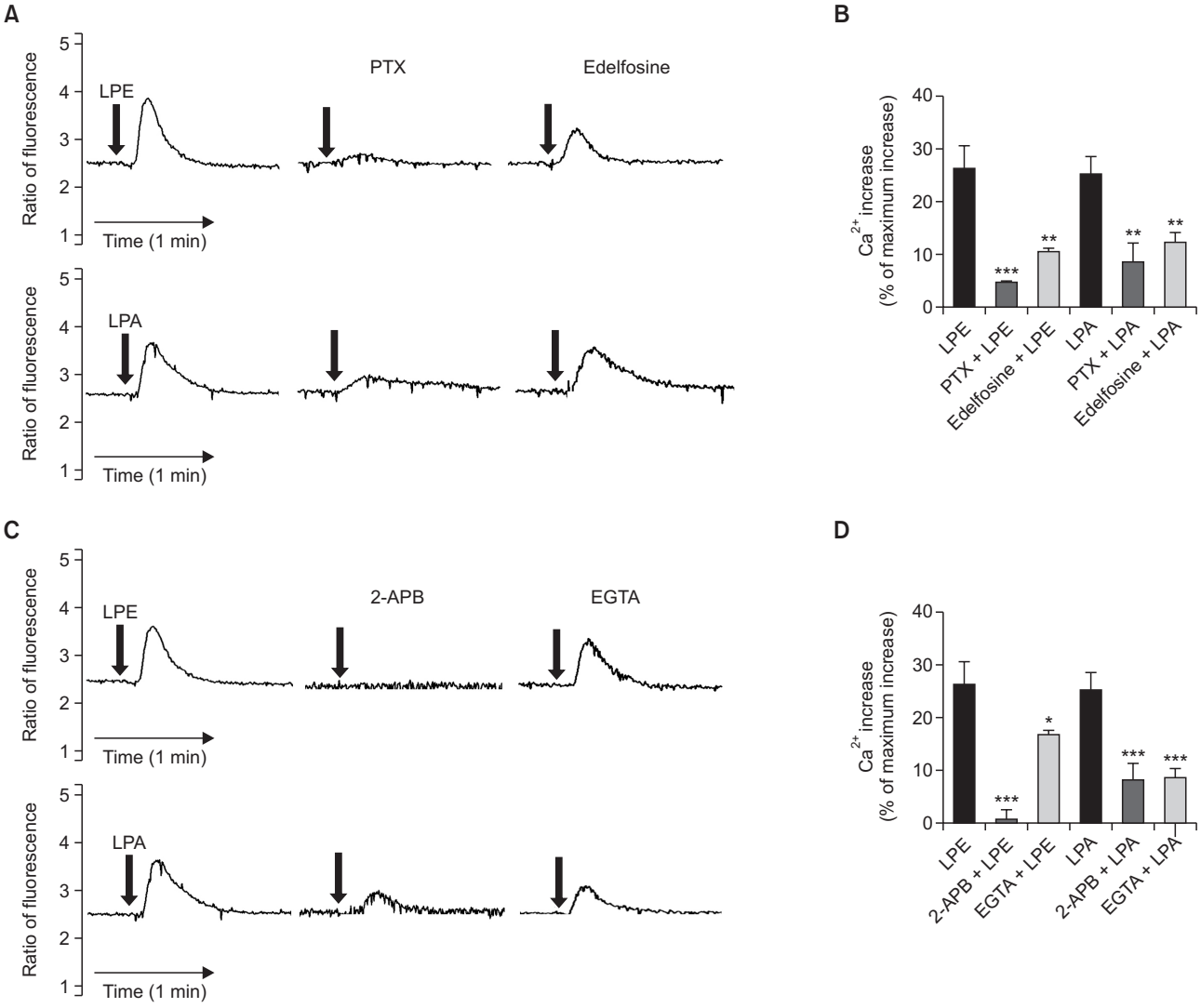

Fig. 5. Effects of EGTA, PTX, 2-APB, and edelfosine on LPE- and LPA-induced $[Ca^{2+}]_i$ increases in SH-SY5Y neuroblastoma cells. $[Ca^{2+}]_i$ levels in cells pre-treated with or without EGTA (5 mM, 1 min), PTX (100 ng/mL, 24 h), 2-APB (100 μM, 15 min), or edelfosine (10 μM, 6 h) were monitored after being treated with LPE (10 μM) or LPA (10 μM). (A, C) Representative $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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.

partially inhibited LPE- and LPA-induced $[Ca^{2+}]_i$ increases, suggesting that Ca^{2+} influx across the plasma membrane contributed to observed $[Ca^{2+}]_i$ increases. Because LPE could not induce Ca^{2+} increase even when Ca^{2+} ions were present in extracellular media in the presence of 2-APB, we supposed LPE-induced Ca^{2+} influx was mediated solely by IP_3 receptor-mediated Ca^{2+} release. However, the partial inhibition of LPA-induced Ca^{2+} increase by 2-APB suggested another component, insensitive to 2-APB, signaled Ca^{2+} influx for LPA. These results suggest involvements of $G_{i/o}$-type proteins, phospholipase C, IP_3 receptors, Ca^{2+} release from intracellular Ca^{2+} stores, and Ca^{2+} influx across the plasma membrane in LPE- and LPA-induced $[Ca^{2+}]_i$ 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 Ca^{2+} increase, indicating that autotaxin was not responsible for the observed effects of LPE (Fig. 6).

DISCUSSION

In the present study, LPE-induced $[Ca^{2+}]_i$ increase was found to be mediated through LPA_1 in SH-SY5Y cells. Five results sustain this finding: 1) the observed heterologous desensitization found for LPE- and LPA-induced $[Ca^{2+}]_i$ increases, 2) the abrogation of LPE-induced response by the LPA_1 and LPA_3 antagonist Ki16425 supported the involvements of LPA_1 and/or LPA_3, 3) the complete inhibition of LPE-induced

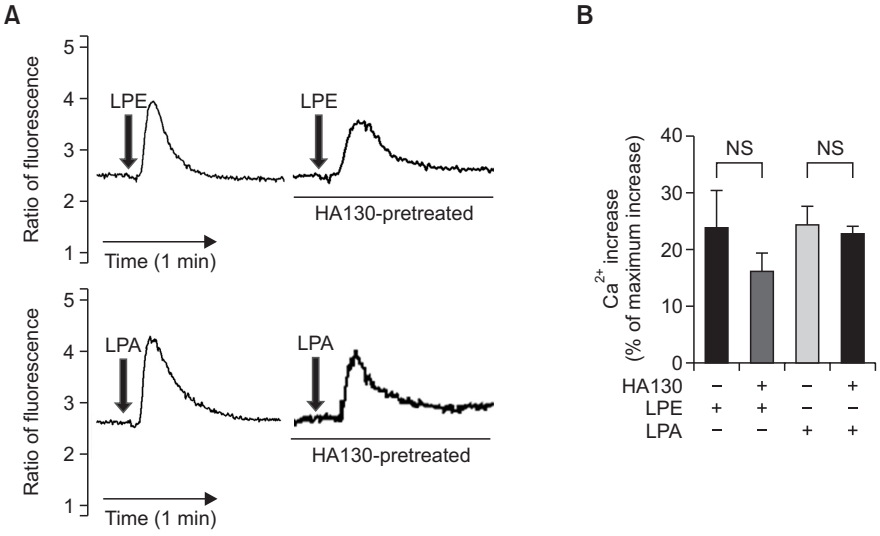

Fig. 6. Effects of HA130 on LPE- and LPA-induced Ca^{2+} responses. $[Ca^{2+}]_i$ levels in SH-SY5Y cells pre-treated with or without HA130 (5 μM, 5 min; an autotaxin inhibitor) were monitored after being treated with LPE (10 μM) or LPA (10 μM) (A). Arrows indicate when lipids were added. The results shown are representative of more than three independent experiments. (B) Increases in $[Ca^{2+}]_i$ 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.

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

	Inhibition by PTX	Responses to different LPEs				Inhibition by LPA_1 antagonists
		18:1	18:0	16:0	14:0	
SH-SY5Y	Yes	Yes	Yes	Yes	Yes	Yes
PC-12	Yes	Yes	Yes	No	Yes	Yes
MDA-MB-231	Yes	Yes	No	No	Yes	Yes
SK-OV3	Yes	Yes	Yes	No	Yes	No

response by the LPA_1 antagonist, AM-095, 4) the observation that LPA_1 was expressed in SH-SY5Y cells, and 5) the $G_{i/o}$-coupling character of LPA_1 and the PTX-sensitivity of LPE-induced $[Ca^{2+}]_i$ increase. LPE-induced $[Ca^{2+}]_i$ increases have been previously observed in ovarian and breast cancer cells and in pheochromocytoma cells (Park *et al*., 2007b, 2013, 2014a; Lee *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 $[Ca^{2+}]_i$ increase was not found to be mediated through Ki16425, VPC32183, or AM-095-sensitive receptors (Park *et al*., 2007a, 2013), and heterologous desensitization was not observed, although homologous desensitization was observed for LPE- and LPA-induced $[Ca^{2+}]_i$ increases (Park *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 $[Ca^{2+}]_i$ 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 LPA_1 (Park *et al*., 2013). Consequently, LPE-induced $[Ca^{2+}]_i$ response in SH-SY5Y cells is similar to that in MDA-MB-231 cells and PC-12 cells in terms of LPA_1 involvement.

In the present study, LPE-induced Ca^{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 *et al*., 2007b, 2013, 2014a; Lee *et al*., 2015) (Table 1). It has been previously reported that 16:0 LPE does not induce $[Ca^{2+}]_i$ response in SK-OV3 cells, MDA-MB-231 cells, or PC-12 cells (Park *et al*., 2013, 2014a; Lee *et al*., 2015). However, in the present study, 16:0 LPE induced $[Ca^{2+}]_i$ response in SH-SY5Y cells (Table 1). In addition, in previous studies, 14:0 LPE induced similar $[Ca^{2+}]_i$ responses to LPA in all four cell types (Park *et al*., 2014a; Lee *et al*., 2015). However, 14:0 LPE-induced $[Ca^{2+}]_i$ response may be driven by a different mechanism to 18:1 LPE-induced $[Ca^{2+}]_i$ response, because 14:0 LPE induced Ca^{2+} response in cells non-responsive to 18:1 LPE. These findings show that LPE-induced $[Ca^{2+}]_i$ responses have similar and dissimilar features in these four cell types; that is, MDA-MB-231, PC-12 and SH-SY5Y cells all exhibit LPA_1 involvement in responses, whereas their responses to different LPE structural types differ (Park *et al*., 2007a, 2013, 2014a; Lee *et al*., 2015) (Table 1).

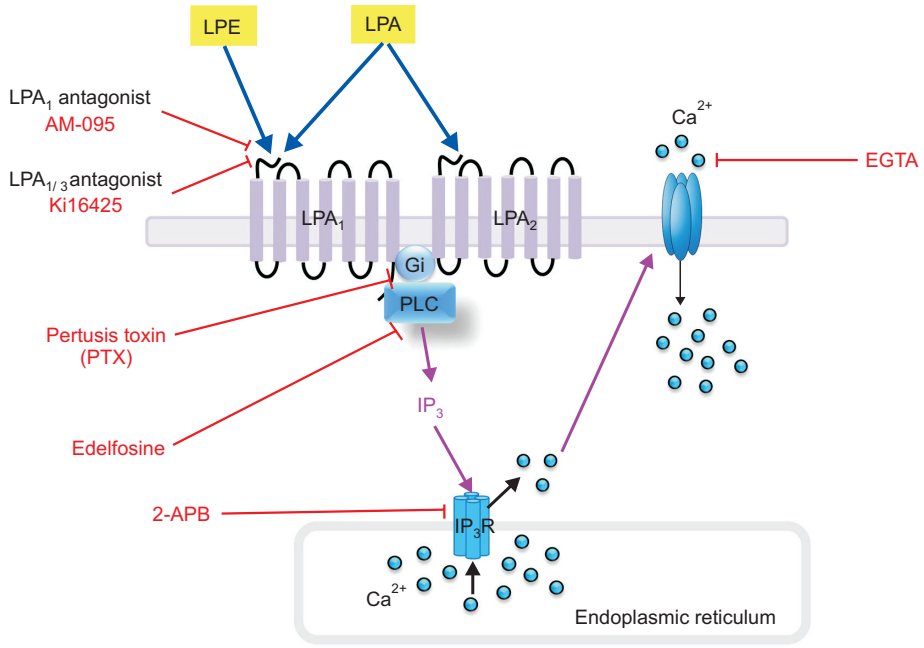

Fig. 7. Proposed methods of signaling by LPE and by LPA in SH-SY5Y cells.

Therefore, in SH-SY5Y cells, LPE was found to act on LPA_1 to induce $[Ca^{2+}]_i$ increase via $G_{i/o}$ proteins, phospholipase C, and IP_3R, and LPA was found to use LPA_1 and LPA_2 to mobilize Ca^{2+} (Fig. 7). Significance of this study is not only LPE action on LPA_1 in SH-SY5Y cells but also involvement of $G_{i/o}$ proteins and phospholipase C in LPA 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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