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Lysophosphatidylethanolamine increases intracellular Ca^{2+} through LPA_1 in PC-12 neuronal cells

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ABSTRACT

G protein-coupled receptors (GPCRs) have been implicated in lysophosphatidylethanolamine (LPE)-induced increases in intracellular Ca^{2+} ($[Ca^{2+}]_i$), but in different cell types, this response may be dependent or independent of lysophosphatidic acid (LPA) GPCR. The effects of LPEs from *Grifola frondosa* on the neuronal differentiation and apoptosis of PC-12 neuronal cells have been previously reported. In the present study, the authors sought to identify the mechanism responsible for the effects of LPEs in PC-12 neuronal cells. LPE increase $[Ca^{2+}]_i$ concentration-dependently in PC-12 neuronal cells, but this LPE-induced $[Ca^{2+}]_i$ increase was less than that elicited by LPA. Studies using specific inhibitors showed that LPE-induced Ca^{2+} response was mediated via pertussis toxin-sensitive $G_{i/o}$ proteins, edelfosine-sensitive phospholipase C, and 2-APB-sensitive IP_3 receptor and by Ca^{2+} influx across the cell membrane, and that this did not involve the conversion of LPE to LPA. Furthermore, LPE- and LPA-induced responses were found to show homologous and heterologous desensitization in PC-12 cells. VPC32183 and Ki16425 (antagonists of LPA_1 and LPA_3) inhibited LPE-induced $[Ca^{2+}]_i$ increases. Furthermore, AM-095 (a specific inhibitor of LPA_1) inhibited LPE-induced Ca^{2+} response completely in PC-12 cells. These findings indicate LPE increases $[Ca^{2+}]_i$ via a $LPA_1/G_{i/o}$ proteins/phospholipase C/IP_3/Ca^{2+} rise/Ca^{2+} influx pathway in PC-12 neuronal cells.

1. Introduction

Lysophosphatidic acid (LPA) is a representative lyso-type intercellular mediator that acts through G protein-coupled receptors (GPCRs; $LPA_{1\text{-}6}$) [1]. Another lysolipid, lysophosphatidylethanolamine (LPE) has been detected in human serum at concentrations of several hundreds of ng/ml [2,3]. However, its action has not been much studied. In SK-OV3 and OVCAR-3 ovarian cancer cells, the effect of LPE on intracellular Ca^{2+} ($[Ca^{2+}]_i$) was suggested to be mediated through GPCRs, but not through GPCRs for LPA [4]. On the other hand, in MDA-MB-231 cells, LPE-induced $[Ca^{2+}]_i$ increases were observed to be mediated via the GPCRs of LPA_1 and CD97 [5,6]. These findings show increases in $[Ca^{2+}]_i$ by LPE may be either dependent or independent of LPA_1 in different cells.

In a previous study, several types of LPEs isolated from *Grifola frondosa* were found to induce neuronal differentiation and suppress serum-deprivation induced apoptosis via MAPK activation in PC12 neuronal cells [7]. However, the involvements of GPCRs in these processes and the action mechanisms involved have not been elucidated. Therefore, in the present study, we investigated the signaling of LPE in PC-12 in neuronal cells and the mechanism involved.

2. Materials and methods

2.1. 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-myristoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (14:0 LPE), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate (sodium salt), and VPC32183 were purchased

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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). Ki16425 and edelfosine were obtained from Cayman Chemical (Michigan) and HA130 was purchased from Tocris (Missouri, USA). AM-095 was obtained from Chemscene (New Jersey), and WST from Daeil lab service (Seoul).

2.2. Cell culture

Rat PC-12 cells were kindly provided from Jae-Won Lee (Pusan National University, Busan, Republic of Korea). Cells were cultured at 37 °C in a 5% CO_2 humidified incubator, and maintained in high glucose RPMI1640, containing 5% (v/v) heat-inactivated horse serum, 100 units/mL penicillin, 50 μg/mL streptomycin, 2 mM glutamine, and 1 mM sodium pyruvate.

2.3. 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 of 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 s using a F4500 fluorescence spectrophotometer (Hitachi, Japan). The ratios of fluorescence intensities ($\lambda_{340}/\lambda_{380}$) at these two wavelengths were used as a surrogate of $[Ca^{2+}]_i$, as previously described [8,9].

2.4. Reverse transcriptase-PCR

To detect the expression of LPA receptors in PC-12 cells by RT-PCR, first strand cDNA was synthesized using total RNA isolated using Trizol reagent (Invitrogen, USA). Synthesized cDNA products and primers for $LPA_{1\text{-}6}$ were used for PCR, which was conducted with Promega Go-Taq DNA polymerase (Madison, WI, USA). The primers used to amplify 317, 317, 321, 341, 308, 247, and 340 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 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′- AAC GAC CCC TTC ATT GAC -3′, antisense 5′- TCC ACG ACA TAC TCA GCA C -3′). PCR reactions were performed using 30 cycles of 95 °C for 30 s (denaturing) and 57 °C for 30 s (annealing) for $LPA_{1\text{-}6}$ or using 27 cycles of 52 °C for 30 s for (annealing) GAPDH followed by elongation at 72 °C for 30 s in all cases in an Eppendorf Mastcycler gradient unit (Hamburg, Germany) [10]. Aliquots of the PCR products (7 μl) so obtained were electrophoresed in 1.2% agarose gels and stained with ethidium bromide.

2.5. Statistics

The results are expressed as means±SEs for the number of indicated determinations. The statistical significances of differences were determined by student *t* test, and statistical significance was accepted for p values of <0.05.

3. Results

3.1. LPE increased $[Ca^{2+}]_i$ in PC-12 neuronal cells

Previously, we observed LPE-induced increases of $[Ca^{2+}]_i$ in MDA-MB-231 breast cancer cells and SK-OV3 ovarian cancer cells [4,5]. In the present study, we treated cells with structurally different LPEs, that is oleoyl LPE (18:1 LPE), stearoyl LPE (18:0 LPE), octadecanyl LPE (ether-linked 18:0 LPE), palmitoyl LPE (16:0 LPE), and myristoyl LPE (14:0 LPE). As shown in Fig. 1-A and B, synthetic LPEs, such as, 18:1 LPE, 18:0 LPE, 18:0 ether-linked LPE, and 14:0 LPE, induced transient $[Ca^{2+}]_i$ increases, whereas 16:0 LPE did not in PC-12 neuronal cells (Fig. 1-A and B). This type of response pattern to LPE is similar to responses observed in SK-OV3 cells, but different to responses in MDA-MB-231 cells [4–6]. For example, 18:0 LPE and ether-linked 18:0 LPE did not induce Ca^{2+} response in MDA-MB-231 cells, but induced responses in SK-OV3 cells and PC-12 cells. Concentration-dependent response to 18:1 LPE was found as like 18:1 LPA in PC-12 neuronal cells (Fig. 1- C and D). LPE was less potent than LPA in this respect, and its efficacy was also slightly less than that of LPA (Fig. 1-C and 1-D). In subsequent studies, 18:1 LPE was mainly used and we refer to it LPE hereafter, because 18:1 LPA was used to compare LPE-induced responses.

3.2. Effects of PTX, edelfosine, 2-APB, EGTA, and of HA130 on LPE-induced $[Ca^{2+}]_i$ response

In order to investigate $[Ca^{2+}]_i$ response to LPE, PC-12 cells were treated with specific inhibitors of; extracellular Ca^{2+} (EGTA), $G_{i/o}$-type G proteins (PTX), phospholipase C (edelfosine), inositol 1,4,5-trisphosphate receptor (IP_3R; 2-APB), or autotaxin (HA130) [4,11–13]. Treatment with EGTA inhibited LPE-induced $[Ca^{2+}]_i$ increases by ~50%, suggesting that Ca^{2+} influx across the cell membrane contributed in part to observed $[Ca^{2+}]_i$ increases in PC-12 cells (Fig. 2). PTX inhibited $[Ca^{2+}]_i$ response to LPE completely, suggesting the involvement of $G_{i/o}$ proteins in $[Ca^{2+}]_i$ response to LPE (Fig. 2). Edelfosine and 2-APB both inhibited response to LPE by ~50%, suggesting the involvements of phospholipase C and IP_3 (Fig. 2). These results suggest that LPE mobilizes $[Ca^{2+}]_i$ via PTX-sensitive $G_{i/o}$-type proteins, phospholipase C, IP_3R, Ca^{2+} release from intracellular Ca^{2+} stores, and by Ca^{2+} influx across the cell membrane in PC-12 cells, and imply the involvements of GPCRs. LPA-induced $[Ca^{2+}]_i$ increases were also inhibited by these pharmacological agents, although inhibition degrees by PTX and 2-APB were slightly larger (Fig. 2). In order to determine whether LPE was converted to LPA by autotaxin (also known as lysophospholipase D), and whether the produced LPA mediated the action of LPE in cells, we treated cells with HA130 [13]. However, LPE-induced $[Ca^{2+}]_i$ increase was not affected by HA130, indicating that LPA was not responsible for the action of LPE in PC-12 cells (Fig. 2).

3.3. Homologous and heterologous desensitizations of LPE- and LPA-induced $[Ca^{2+}]_i$ responses

Because LPA receptor has been implicated in effect of LPE on MDA-MB-231 cells, we studied the homologous and heterologous desensitizations of LPE- and LPA-induced $[Ca^{2+}]_i$ increases in PC-12 cells in order to test the possible involvements of endogenously expressed LPA receptors in LPE-induced $[Ca^{2+}]_i$ response. In desensitization experiments, cells were pretreated with LPE

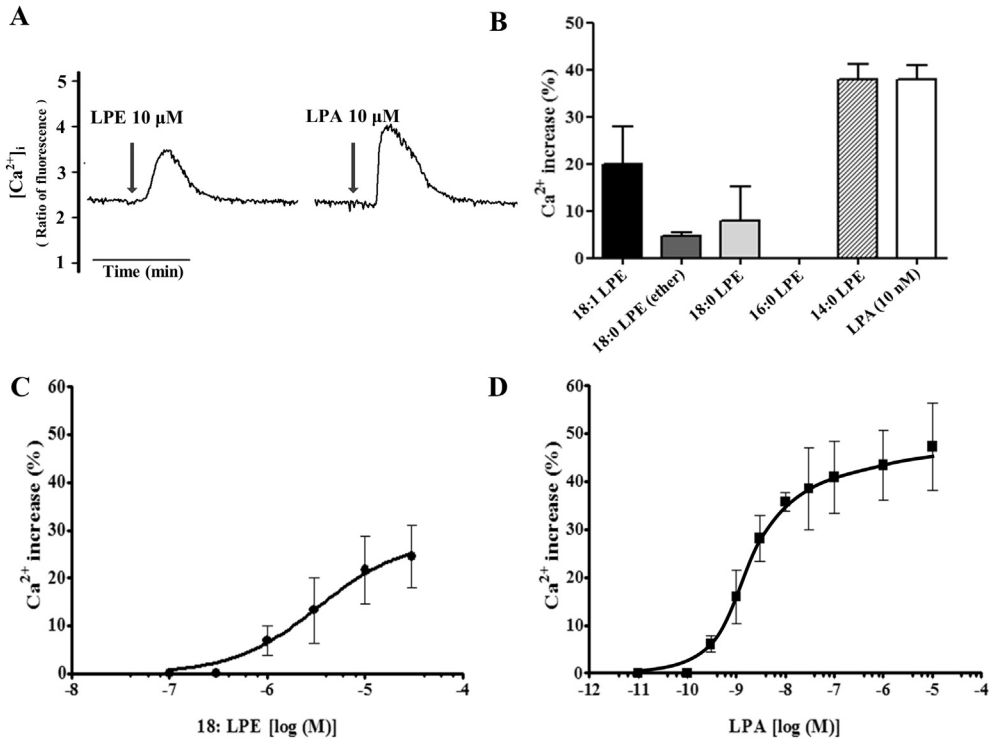

Fig. 1. Concentration-dependences of LPE- and of LPA-induced $[Ca^{2+}]_i$ increases in PC-12 neuronal cells. Representative $[Ca^{2+}]_i$ traces of PC-12 cells treated with 10 μM of 18:1 LPE and 18:1 LPA (A). Arrows indicate when lipids were added. The data shown are representative of more than three independent experiments. Ca^{2+} responses are presented as the means ± SEs of three independent experiments (B). 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. Efficacies of different chain-length LPE- and LPA-induced Ca^{2+} responses versus digitonin in PC-12 cells (B, C, and D). Results are the means ± SEs of three independent experiments.

(10 μM) or LPA (10 nM) for 1 min before adding LPE (10 μM) or LPA (10 nM). LPE pre-treatment attenuated both LPE- and LPA-induced $[Ca^{2+}]_i$ responses by 83% and 97%, respectively (Fig. 3), and similarly, LPA pre-treatment attenuated these responses by 100% and 71%, respectively (Fig. 3). Thus, both LPE and LPA desensitized responses to themselves (homologous desensitization) and to the other (heterologous desensitization) (Fig. 3). These results imply that LPE may act on LPA receptors in PC-12 cells.

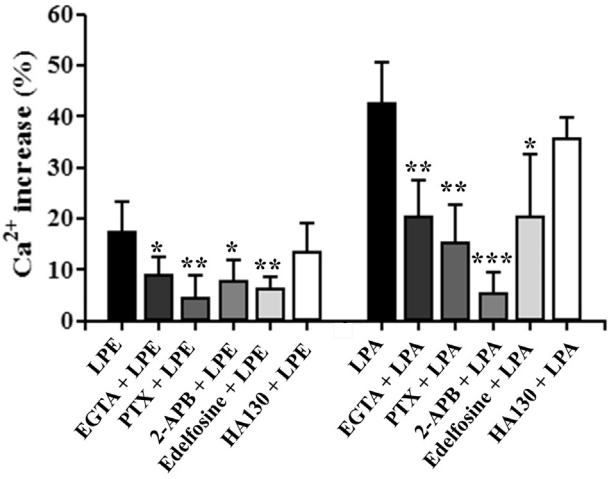

Fig. 2. Effects of EGTA, PTX, 2-APB, edelfosine, and HA130 on LPE- or LPA-induced $[Ca^{2+}]_i$ increases in PC-12 neuronal cells. $[Ca^{2+}]_i$ levels in cells pretreated with or without EGTA (5 mM, 1 min), PTX (100 ng/mL, 24 h), 2-APB (100 μM, 15 min), edelfosine (10 μM, 6 h), or the autotaxin inhibitor HA130 (5 μM, 5 min) were monitored after treatment with LPE (10 μM) or LPA (10 nM), respectively. Results are presented as the means±SEs of three independent experiments. Statistical significance: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. non-prtreated cells.

3.4. Effects of LPA_1 and LPA_3 antagonists on LPE-induced $[Ca^{2+}]_i$ response

Because the heterologous desensitizations of LPE- and LPA-induced $[Ca^{2+}]_i$ responses suggested LPE utilizes LPA receptors in PC-12 cells, we applied two structurally-different antagonists of LPA_1 and LPA_3 receptors, that is VPC32183 and Ki16425 [10,14,15]. VPC32183 (1 μM) and Ki16425 (10 μM) completely inhibited both LPA- and LPE-induced $[Ca^{2+}]_i$ responses in PC-12 cells (Fig. 4). This suggests, LPE increase $[Ca^{2+}]_i$ mainly via Ki16425-and VPC32183-sensitive LPA receptors coupled to PTX-sensitive G proteins in PC-12 cells. Because LPA_1 and LPA_3 are known to couple to $G_{i/o}$ and $G_{q/11}$ proteins, respectively, we considered LPA_1 might act as the GPCR for LPE in PC-12 cells. Therefore, we treated PC-12 cells with AM-095 (a specific LPA_1 antagonist) to verify the involvement of endogenously expressed LPA_1 receptors in LPE-induced $[Ca^{2+}]_i$ response in PC-12 cells [16,17]. AM-095 (500 nM) inhibited LPA-induced $[Ca^{2+}]_i$ response and completely blocked LPE-induced $[Ca^{2+}]_i$ response in PC-12 cells (Fig. 4). The results suggest that as is observed in MDA-MB-231 cells [5,6], LPE increases $[Ca^{2+}]_i$ mainly via LPA_1 receptors in PC-12 cells.

3.5. Expression of LPA receptors in PC-12 cells

RT-PCR was conducted to assess the expression levels of the six known LPA receptors in PC-12 cells [18–22]. It was found LPA_1, LPA_2, and LPA_3 were strongly expressed, LPA_6 was weakly

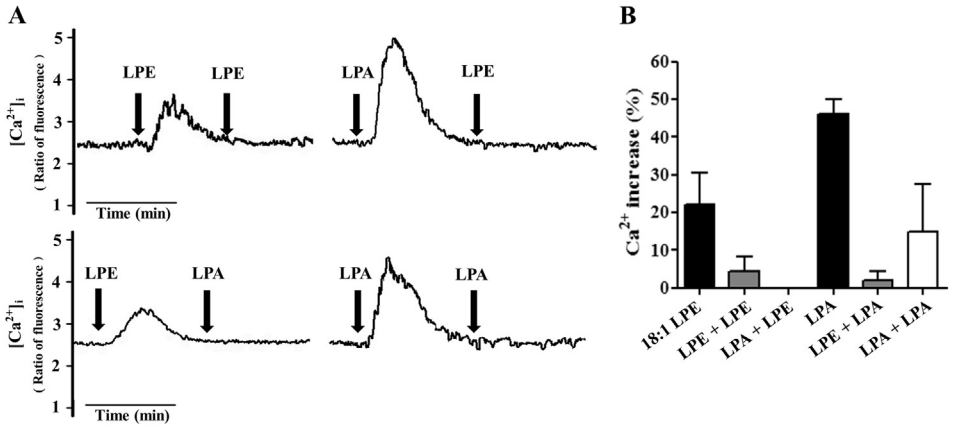

Fig. 3. Desensitization of LPE- or LPA-induced $[Ca^{2+}]_i$ increases by LPE or LPA in PC-12 neuronal cells. Representative $[Ca^{2+}]_i$ traces of PC-12 cells pretreated with 10 μM LPE or 10 nM LPA and then treated with 10 μM of LPE or 10 nM LPA (A). Arrows indicate when lipids were added. The data shown are representative of more than four independent experiments. B: $[Ca^{2+}]_i$ increases induced by 10 μM of LPE or 10 nM of LPA alone and $[Ca^{2+}]_i$ increases induced by 10 μM of LPE or 10 nM of LPA after pretreating cells with 10 μM LPE or 10 nM LPA. Results are the means±SEs of three independent experiments (B). Statistical significance: $^{***}P < 0.001$ vs. non-prtreated cells.

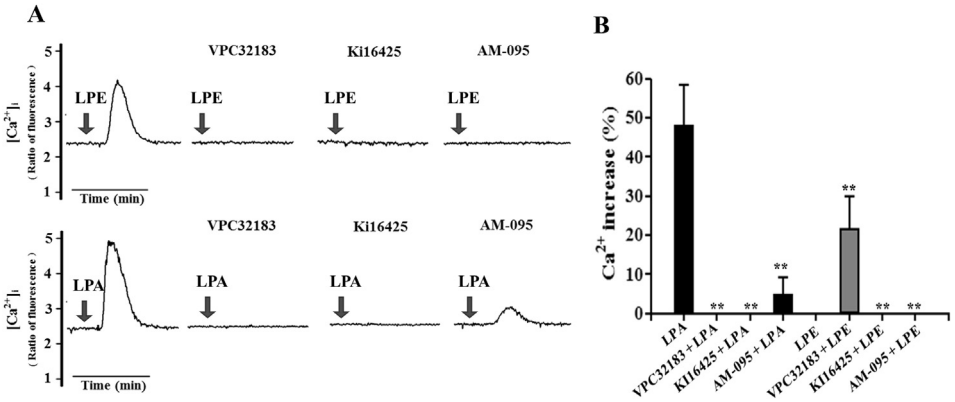

Fig. 4. Effects of Ki16425, VPC32183, and AM-095 on LPE- or LPA-induced $[Ca^{2+}]_i$ increases in PC-12 neuronal cells. Representative $[Ca^{2+}]_i$ traces of PC-12 cells treated with 10 μM of LPE or 10 nM LPA in the presence of Ki16425, VPC32183, AM-095, or vehicle (A). Arrows indicate when lipids were added. The results shown are representative of more than three independent experiments. B: Increases in $[Ca^{2+}]_i$ by 10 μM of LPE or 10 nM LPA were observed in cells pre-treated with or without VPC32183 (1 μM), Ki16425 (10 μM), or AM-095 (500 nM). Results are presented as the means ± SEs of three independent experiments (B). Statistical significance: $^{**}P < 0.01$, $^{***}P < 0.001$ vs. non-treated cells.

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

	Inhibition by PTX	Responses to different LPEs			Heterologous desensitization with LPA	Inhibition by LPA_1 antagonists
		18:0	16:0	14:0		
PC-12	yes	yes	no	yes	yes	yes
MDA- MB-231	yes	no	no	yes	yes	yes
SK-OV3	yes	yes	no	yes	no	no

expressed, and that LPA_4 and LPA_5 were not expressed (data not shown).

4. Discussion

In the present study, LPE-induced $[Ca^{2+}]_i$ increase was found to be mediated via LPA_1 in PC-12 neuronal cells. Five results support this finding: 1) the heterologous desensitization exhibited by LPE- and LPA-induced $[Ca^{2+}]_i$ increases, 2) the abrogation of LPE-induced response by the LPA_1 and LPA_3 antagonists, Ki16425 and VPC32183, 3) the $G_{i/o}$-coupling character of LPA_1 and the PTX-sensitivity of LPE-induced $[Ca^{2+}]_i$ increase observed in PC-12 cells, 4) the complete inhibition of LPE-induced response by the LPA_1 antagonist, AM-095, and 5) the observed expression of LPA_1 in PC-12 cells. LPE-induced $[Ca^{2+}]_i$ increases have been previously observed in ovarian and breast cancer cells [4–6]. Table 1 provides a summary of responses observed in PC-12 cells and in ovarian (SK-OV3) and breast cancer (MDA-MB-231) cells in the present study.

In ovarian cancer cells, LPE-induced $[Ca^{2+}]_i$ increases were not inhibited by Ki16425, VPC32183, or AM-095, but LPA-induced $[Ca^{2+}]_i$ increases were completely inhibited [4,6]. Furthermore, heterologous desensitization was not observed in ovarian cancer cells, although homologous desensitization was observed for LPE- and LPA-induced $[Ca^{2+}]_i$ increases [4]. These results indicate that the mechanism of LPE-induced response in PC-12 cells differs from that in SK-OV3 ovarian cancer cells (Table 1). On the other hand, in MDA-MB-231 breast cancer cells, LPE-induced $[Ca^{2+}]_i$ response was inhibited by Ki16425, VPC32183, or AM-095, and heterologous

desensitization was observed, which indicates the mediation of LPE-induced response in MDA-MB-231 cells by LPA_1. Accordingly, LPE-induced $[Ca^{2+}]_i$ response in PC-12 cells is similar to that in MDA-MB-231 cells in terms of LPA_1 involvement. However, our structural studies using different LPE analogs in the three cell types showed similarity between LPE-responses in SK-OV3 cells and PC-12 cells. In particular, ether-linked 18:0 LPE and 18:0 LPE elicited responses in SK-OV3 and PC-12 cells, but not in MDA-MB-231 cells. On the other hand, 16:0 LPE did not failed to induce $[Ca^{2+}]_i$ response in SK-OV3 cells, MDA-MB-231 cells and PC-12 cells, and 14:0 LPE induced similar $[Ca^{2+}]_i$ responses to LPA in all three cell types (Table 1). These findings show that LPE-induced $[Ca^{2+}]_i$ responses in these three cell types have both common and dissimilar characteristics; that is, LPA_1 involvement in response is common to PC-12 and MDA-MB-231 cells, but differs in SK-OV3 cells, whereas responses to different LPE structural types differed in the three cell lines.

Conflict of interest

None.

Acknowledgments

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