The Journal of Applied Pharmacology, 14, 25-29(2006)

Distinct Effects of Lysophospholipids on Membrane Potential in C6 Glioma Cells

Yun-Kyung Lee and Dong-Soon Im*

Laboratory of Pharmacology, College of Pharmacy and Research Institute for Drug Development, Pusan National University, Busan 609-735, Republic of Korea

(Received January 20, 2006; Accepted March 16, 2006)

Abstract – We tested effects of bioactive lysophospholipids including lysophosphatidic acid (LPA), lysophosphatidylcholine (LPC), sphingosylphosphorylcholine (SPC), and sphingosine 1-phosphate (S1P) on membrane potential in C6 glioma cells to understand action mechanism of the lysophospholipids. Membrane potential was estimated by measuring fluorescence change of DiBAC-loaded glioma cells. LPA largely increased membrane potential and the increase was gradually diminished. LPC also increased the membrane potential, however, the increase sustained. SPC induced smaller increase of membrane potential than LPC. S1P was not able to change the membrane potential. We tested effects of suramin and pertussis toxin on lysophospholipid-induced membrane potential increase. However, there wasn't any effect. The membrane potential increase was partially diminished in Na^+-free media, suggesting Na^+ influx as a component of membrane potential changes. Thus, involvement of Na^+ influx in the increase of membrane potential by lysophospholipids and independence of suramin-sensitive GPCRs and pertussis toxin-sensitive G proteins are found in this study.

Keywords □ lysophosphatidic acid, sphingosine-1-phosphate; membrane potential, lysophosphatidylcholine, sphingosylphosphorylcholine, sphingosine, glioma, lysolipid, G-protein-coupled receptor

INTRODUCTION

Lysophospholipids including lysophosphatidic acid (LPA), lysophosphatidylcholine (LPC), sphingosylphosphorylcholine (SPC), and sphingosine 1-phosphate (S1P) are bioactive lipid mediators with diverse biological functions(Hla, T. *et al.* 2001; Im, D. S. 2004; Lynch, K. R. and Im, D. S. 1999). Eight members of a subfamily of G protein-coupled receptors (GPCR) for S1P and LPA, formerly named Edg (*e*ndothelial *d*ifferentiation gene), have been identified and characterized(Hla, T. *et al.* 2001; Im, D. S. 2004; Lynch, K. R. and Im, D. S. 1999). Additionally, GPR23 for LPA and GPR3, GPR6, and GPR12 for S1P have been reported(Im, D. S. 2004; Noguchi, K. *et al.* 2003; Uhlenbrock, K. *et al.* 2002). Selective agonists and antagonists are under development for medical applications such as OMPT, SEW2871, Ki16424, JTE-013, and VPC32183 (Hasegawa, Y. *et al.* 2003; Ikeda, H. *et al.* 2003; Im, D. S. 2003; Ohta, H. *et al.* 2003; Park, K. S. *et al.* 2005; Wei, S. H. *et al.* 2005). Three GPCRs including OGR1, GPR4, and GPR12 have been reported as SPC receptors(Ignatov, A. *et al.* 2003; Xu, Y. *et al.* 2000; Zhu, K. *et al.* 2001), and GPR4 and GPR119 were identified as LPC receptors(Soga, T. *et al.* 2005; Zhu, K. *et al.* 2001). However, pathophysiological significance of lysophospholipids in each cell type and tissue are still poorly studied(Hla, T. *et al.* 2001).

Gliomas represent about half of all brain tumors, and among them, glioblastoma multiformes is thought to be the most malignant and common intracranial tumor(VandenBerg, S. R. 1992). Although generally not metastatic, glioblastoma cells exhibit highly migratory and invasive behavior(Ishiuchi, S. *et al.* 2002). LPA and S1P have been reported to evoke migratory response in glioma cells(Malchinkhuu, E. *et al.* 2005; Manning, T. J., Jr. *et al.* 2000; Steiner, M. R. *et al.* 2002). In rat C6 glioma cells, LPA-induced migratory response was mediated through G_i-protein-coupled LPA_1 receptor in a PI3K/Cdc42/p38MAPK- and PI3K/Rac/JNK-dependent manner (Malchinkhuu, E. *et al.* 2005). Furthermore, LPA induces glioma proliferation in an NHE1 and Rho kinase dependent manner(Cechin, S. R. *et al.* 2005). And LPA also increases intracellular Ca^{2+} concentration and activity of Erk1/2(Manning, T. J., Jr. *et al.* 2000). LPA

*Corresponding author
Tel: +82-51-510-2817, Fax: +82-51-513-6754
E-mail: imds@pusan.ac.kr

and S1P also increase mRNA expression of c-fos in a pertussis toxin-dependent manner(Segura, B. J. *et al.* 2005).

S1P via $S1P_1$ receptor induces expression of fibroblast growth factor-2 and via $S1P_2$ stimulates PLC-Ca^{2+} system and PLD activation in C6 glioma cells(Sato, K. *et al.* 2000). S1P stimulates human glioma cell proliferation through G_i-coupled receptor and PI3K(Van Brocklyn, J. *et al.* 2002). S1P also increases intracellular Ca^{2+} concentration and reverses morphologic response by β-adrenaline in C6 glioma cells(Tas, P. W. and Koschel, K. 1998). SPC increases mRNA expression of early response gene c-fos in a pertusis toxin-independent manner(Segura, B. J. *et al.* 2005). LPC has been shown to enhance IL-1β-induced secretion of IL-6, an inflammatory cytokine(Zumwalt, J. W. *et al.* 1999).

In this study, we tested effects of lysophospholipids on membrane potential to understand action mechanism of the lysophospholipids in C6 glioma cells and found distinct responses by the lysophospholipids.

MATERIALS AND METHODS

Materials

1-oleoyl-*sn*-2-lysophosphatidic acid, 1-palmitoyl-*sn*-2-lysophosphatidylcholine, D-*erythro*-sphingosine 1-phosphate, D-*erythro*-sphingosylphosphorylcholine were purchased from Avanti Polar Lipids (Alsbaster, AL, USA). $DiBAC_4(3)$ was acquired from Biotium (Hayway, CA, USA). All other materials were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

Rat C6 glioma cells were maintained in high glucose DMEM containing 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 50 μg/ml streptomycin, 2 mM of glutamine, and 1 mM of sodium pyruvate at 37°C in a humidified 5% CO_2 incubator.

Measurement of membrane potential

The cells were sedimented, resuspended with a Hepes-buffered medium consisting of 20 mM of 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 $NaHCO_3$, 15 mM glucose and 0.1% bovine serum albumin (fatty acid free), and then incubated for 30 min with 5 μM of $DiBAC_4(3)$. Fluorescence emission at 530 nm wavelength from excitation wavelength (488 nm) were measured every 0.1 sec by F4500 fluorescence spectrophotometer (Hitachi, Japan). Membrane potential was estimated by measuring fluorescence change of DiBAC-loaded cells.

Data presentation

Representative traces for membrane potential were chosen out of 3 separate experiments and shown in Fig 1-5.

RESULTS

Lysophospholipids induce increases of membrane potential distinctly in C6 glioma cells.

LPA largely increased membrane potential and the increase was gradually diminished (Fig. 1). LPC also increased the membrane potential, however, the increase sustained. SPC induced smaller increase of membrane potential than LPC. S1P was not able to change the membrane potential. Increases of membrane potential by lysophospholipids were observed in a dose-dependent manner (Fig. 2). Significant increases were observed by LPA, LPC and SPC in higher concentrations than 10 μM (Fig. 2).

Involvement of GPCR and G proteins in lysophospholipids-induced membrane potential

Pertussis toxin has been used to elucidate involvement of $G_{i/o}$-type G proteins(Im, D. S. *et al.* 1997). Since GPCRs for lysophospholipids have been found, we treated C6 glioma cells with pertusis toxin (100 ng/ml, 24 hr). However, LPA, LPC, and SPC-induced changes of membrane potential were not

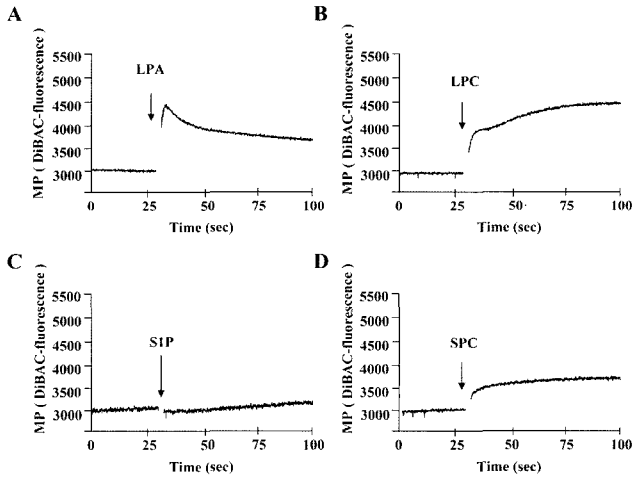

Fig. 1. Effects of lysophospholipids on membrane potential in C6 glioma cells. Representative traces of membrane potential with 20 μM of LPA (A), LPC (B), S1P (C), or SPC (D) in DiBAC-loaded C6 glioma cells were shown. Each lysophospholipid was added at the arrow (30 sec).

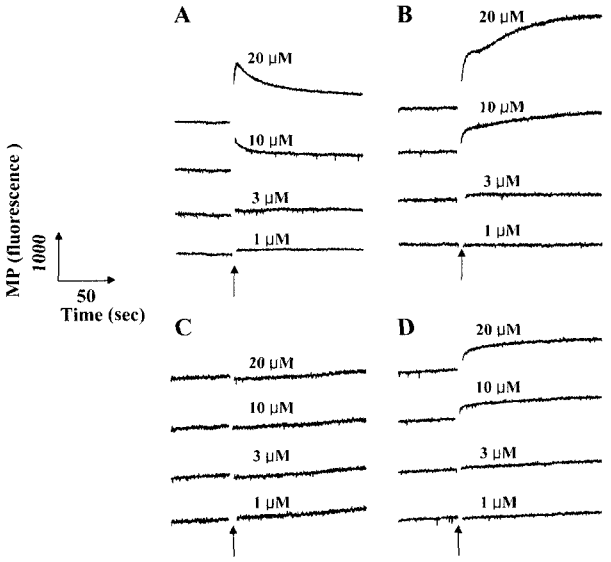

Fig. 2. Concentration-dependence of lysophospholipids-induced increases of membrane potential. Representative traces of membrane potential with different concentrations of LPA (A), LPC (B), S1P (C), or SPC (D) in DiBAC-loaded C6 glioma cells were shown.

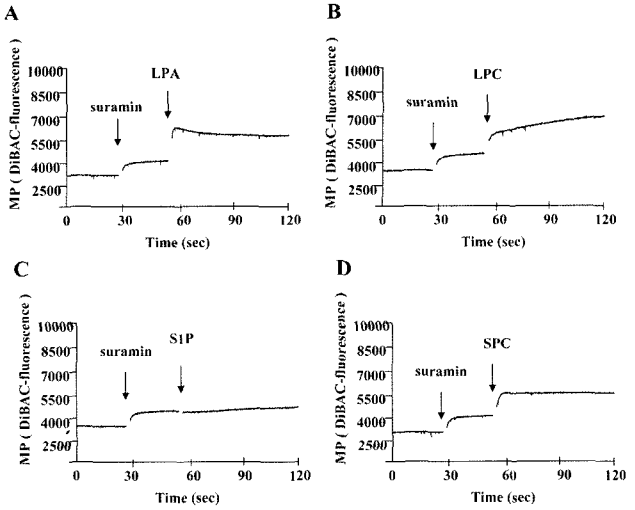

Fig. 4. Effect of suramin treatment on lysophospholipids-induced increases of membrane potential. Representative traces of membrane potential with 20 μM of LPA (A), LPC (B), S1P (C), or SPC (D) in DiBAC-loaded C6 glioma cells treated with suramin (100 mM, lines) were shown.

blunted, suggesting no involvement of GPCR coupling to $G_{i/o}$-type G proteins (Fig. 3). Suramin is a pharmacological tool to test involvement of proteins in the plasma membrane such as GPCRs(Kimura, T. *et al.* 2000). We treated C6 glioma cells with suramin and found increase of membrane potential by the treatment with suramin itself. Furthermore, significant change on the increases of membrane potential by each lysophospholipid was not observed in suramin-treated C6 glioma cells (Fig. 4)

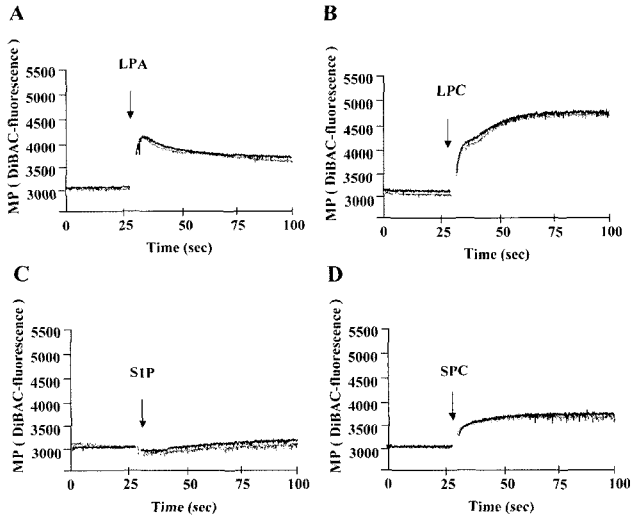

Fig. 3. Effect of pertussis toxin treatment on lysophospholipids-induced increases of membrane potential. Representative traces of membrane potential with 20 μM of LPA (A), LPC (B), S1P (C), or SPC (D) in DiBAC-loaded C6 glioma cells treated with pertusiss toxin (100 ng/ml, 24 hr, lines) or without pertussis toxin treatment (dotted lines) were shown.

Effect of Na^+-free media on lysophospholipids-induced membrane potential change

Next, we tested effect of Na^+-free media on lysophospholipids-induced membrane potential changes. In Na^+-free media, LPA-induced increase of membrane potential was diminished; the peak of transient increase became a half and the maintained increase became to the level of resting membrane potential (Fig. 5). LPC and SPC-induced increases of membrane potential were also partially inhibited (Fig. 5). Thus, Na^+ influx from the extracellular media to cytosol participates in the changes of membrane potential by lysophospholipids in C6 glioma cells.

DISCUSSION

In C6 rat glioma cells, we for the first time observed changes of membrane potential by bioactive lysophospholipids, that is LPA, LPC, and SPC by using $DiBAC_4(3)$ fluorescence dye. Previously, decrease of membrane potential and increase of intracellular Ca^{2+} concentration by bradykinin was reported in C6 glioma cells(Reetz, G. and Reiser, G. 1996). Increases of

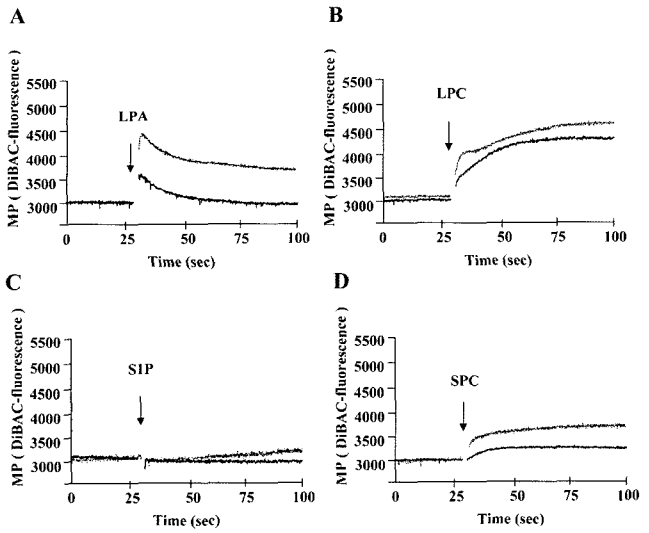

Fig. 5. Effect of Na^+-free media on lysophospholipids-induced increases of membrane potential. Representative traces of membrane potential with 20 μM of LPA (A), LPC (B), S1P (C), or SPC (D) in DiBAC-loaded C6 glioma cells in Na^+-free media (lines) or in Na^+-containing media (dotted lines) were shown.

intracellular Ca^{2+} concentration by LPA and S1P have been reported and $S1P_2$ receptor is supposed to be responsible for S1P Ca^{2+} increase in C6 glioma cells(Sato, K. *et al.* 2000). However, in our study, S1P did not evoke change of membrane potential. LPA, LPC and SPC induced increases of membrane potential, which have distinct magnitudes and different shapes.

There are four GPCRs for LPA, three GPCRs for SPC, and two GPCRs for LPC so far reported(Hla, T. *et al.* 2001; Ignatov, A. *et al.* 2003; Im, D. S. 2004; Lynch, K. R. and Im, D. S. 1999; Noguchi, K. *et al.* 2003; Uhlenbrock, K. *et al.* 2002; Xu, Y. *et al.* 2000; Zhu, K. *et al.* 2001; Soga, T. *et al.* 2005; Zhu, K. *et al.* 2001). However, changes of membrane potential by LPA, LPC, and SPC were not influenced by treatments with suramin or pertussis toxin, suggesting that the effects are not mediated through suramin-sensitive GPCRs or pertussis-toxin-sensitive G proteins. The experiment in Na^+-free media suggests that LPA, LPC, and SPC increase membrane potential partly through Na^+ influx.

In the present study, we showed increases of membrane potential by LPA, LPC, and SPC in C6 glioma cells. Although the precise mechanism for the increases was not elucidated, we found involvement of Na^+ influx in the process and independence of suramin-sensitive GPCRs and pertussis toxin-sensitive G proteins. Although further investigation is necessary to elucidate mechanism of Na^+ influx by each lysophospholipid, involvement of suramin-insensitive GPCRs and pertussis toxin-insensitive G proteins for the increase of membrane potential by lysophopsholipids have to be considered.

ACKNOWLEDGMENT

This work was supported by the Korea Science and Engineering Foundation Grant. (R01-2005-000-10011-02005)

REFERENCES

Cechin, S. R., Dunkley, P. R. and Rodnight, R. (2005). Signal transduction mechanisms involved in the proliferation of C6 glioma cells induced by lysophosphatidic acid. *Neurochem Res* **30**, 603-11.

Hasegawa, Y., Erickson, J. R., Goddard, G. J., Yu, S., Liu, S., Cheng, K. W., Eder, A., Bandoh, K., Aoki, J., Jarosz, R., Schrier, A. D., Lynch, K. R., Mills, G. B. and Fang, X. (2003). Identification of a phosphothionate analogue of lysophosphatidic acid (LPA) as a selective agonist of the LPA3 receptor. *J Biol Chem* **278**, 11962-9.

Hla, T., Lee, M. J., Ancellin, N., Paik, J. H. and Kluk, M. J. (2001). Lysophospholipids--receptor revelations. *Science* **294**, 1875-8.

Ignatov, A., Lintzel, J., Hermans-Borgmeyer, I., Kreienkamp, H. J., Joost, P., Thomsen, S., Methner, A. and Schaller, H. C. (2003). Role of the G-protein-coupled receptor GPR12 as high-affinity receptor for sphingosylphosphorylcholine and its expression and function in brain development. *J Neurosci* **23**, 907-14.

Ikeda, H., Satoh, H., Yanase, M., Inoue, Y., Tomiya, T., Arai, M., Tejima, K., Nagashima, K., Maekawa, H., Yahagi, N., Yatomi, Y., Sakurada, S., Takuwa, Y., Ogata, I., Kimura, S. and Fujiwara, K. (2003). Antiproliferative property of sphingosine 1-phosphate in rat hepatocytes involves activation of Rho via Edg-5. *Gastroenterology* **124**, 459-69.

Im, D. S. (2003). Linking Chinese medicine and G-protein-coupled receptors. *Trends Pharmacol Sci* **24**, 2-4.

Im, D. S. (2004). Discovery of new G protein-coupled receptors for lipid mediators. *J Lipid Res* **45**, 410-8.

Im, D. S., Fujioka, T., Katada, T., Kondo, Y., Ui, M. and Okajima, F. (1997). Characterization of sphingosine 1-phosphate-induced actions and its signaling pathways in rat hepatocytes. *Am J Physiol* **272**, G1091-9.

Ishiuchi, S., Tsuzuki, K., Yoshida, Y., Yamada, N., Hagimura, N., Okado, H., Miwa, A., Kurihara, H., Nakazato, Y., Tamura, M., Sasaki, T. and Ozawa, S. (2002). Blockage of Ca(2+)-permeable AMPA receptors suppresses migration and induces apoptosis in human glioblastoma cells. *Nat Med* **8**, 971-8.

Kimura, T., Watanabe, T., Sato, K., Kon, J., Tomura, H., Tamama, K., Kuwabara, A., Kanda, T., Kobayashi, I., Ohta, H., Ui, M. and Okajima, F. (2000). Sphingosine 1-phosphate stimulates proliferation and migration of human endothelial cells possibly through the lipid receptors, Edg-1 and Edg-3. *Biochem J* **348 Pt 1**, 71-6.

Lynch, K. R. and Im, D. S. (1999). Life on the edg. *Trends Pharmacol Sci* **20**, 473-5.

Malchinkhuu, E., Sato, K., Horiuchi, Y., Mogi, C., Ohwada, S., Ishiuchi, S., Saito, N., Kurose, H., Tomura, H. and Okajima, F.

(2005). Role of p38 mitogen-activated kinase and c-Jun terminal kinase in migration response to lysophosphatidic acid and sphingosine-1-phosphate in glioma cells. *Oncogene* **24**, 6676-88.

Manning, T. J., Jr., Parker, J. C. and Sontheimer, H. (2000). Role of lysophosphatidic acid and rho in glioma cell motility. *Cell Motil Cytoskeleton* **45**, 185-99.

Noguchi, K., Ishii, S. and Shimizu, T. (2003). Identification of p2y9/GPR23 as a novel G protein-coupled receptor for Lysophosphatidic acid, structurally distant from the Edg family. *J Biol Chem* **278**, 25600-25606.

Ohta, H., Sato, K., Murata, N., Damirin, A., Malchinkhuu, E., Kon, J., Kimura, T., Tobo, M., Yamazaki, Y., Watanabe, T., Yagi, M., Sato, M., Suzuki, R., Murooka, H., Sakai, T., Nishitoba, T., Im, D. S., Nochi, H., Tamoto, K., Tomura, H. and Okajima, F. (2003). Ki16425, a subtype-selective antagonist for EDG-family lysophosphatidic acid receptors. *Mol Pharmacol* **64**, 994-1005.

Park, K. S., Lee, H. Y., Kim, M. K., Shin, E. H., Jo, S. H., Kim, S. D., Im, D. S. and Bae, Y. S. (2006). Lysophosphatidylserine stimulates L2071 mouse fibroblast chemotactic migration via a process involving pertussis toxin-sensitive trimeric G proteins. *Mol Pharmacol* **69**, 1066-1073.

Reetz, G. and Reiser, G. (1996). [Ca2+]i oscillations induced by bradykinin in rat glioma cells associated with Ca2+ store-dependent Ca2+ influx are controlled by cell volume and by membrane potential. *Cell Calcium* **19**, 143-56.

Sato, K., Ui, M. and Okajima, F. (2000). Differential roles of Edg-1 and Edg-5, sphingosine 1-phosphate receptors, in the signaling pathways in C6 glioma cells. *Brain Res Mol Brain Res* **85**, 151-60.

Segura, B. J., Zhang, W., Xiao, L., Logsdon, C. D. and Mulholland, M. W. (2005). Sphingosine-1-phosphate induces early response gene expression in C6 glioma cells. *Brain Res Mol Brain Res* **133**, 325-8.

Soga, T., Ohishi, T., Matsui, T., Saito, T., Matsumoto, M., Takasaki, J., Matsumoto, S., Kamohara, M., Hiyama, H., Yoshida, S., Momose, K., Ueda, Y., Matsushime, H., Kobori, M. and Furuichi, K. (2005). Lysophosphatidylcholine enhances glucose-dependent insulin secretion via an orphan G-protein-coupled receptor. *Biochem Biophys Res Commun* **326**, 744-51.

Steiner, M. R., Urso, J. R., Klein, J. and Steiner, S. M. (2002). Multiple astrocyte responses to lysophosphatidic acids. *Biochim Biophys Acta* **1582**, 154-60.

Tas, P. W. and Koschel, K. (1998). Sphingosine-1-phosphate induces a Ca2+ signal in primary rat astrocytes and a Ca2+ signal and shape changes in C6 rat glioma cells. *J Neurosci Res* **52**, 427-34.

Uhlenbrock, K., Gassenhuber, H. and Kostenis, E. (2002). Sphingosine 1-phosphate is a ligand of the human gpr3, gpr6 and gpr12 family of constitutively active G protein-coupled receptors. *Cell Signal* **14**, 941-53.

Van Brocklyn, J., Letterle, C., Snyder, P. and Prior, T. (2002). Sphingosine-1-phosphate stimulates human glioma cell proliferation through Gi-coupled receptors: role of ERK MAP kinase and phosphatidylinositol 3-kinase beta. *Cancer Lett* **181**, 195-204.

VandenBerg, S. R. (1992). Current diagnostic concepts of astrocytic tumors. *J Neuropathol Exp Neurol* **51**, 644-57.

Wei, S. H., Rosen, H., Matheu, M. P., Sanna, M. G., Wang, S. K., Jo, E., Wong, C. H., Parker, I. and Cahalan, M. D. (2005). Sphingosine 1-phosphate type 1 receptor agonism inhibits transendothelial migration of medullary T cells to lymphatic sinuses. *Nat Immunol* **6**, 1228-35.

Xu, Y., Zhu, K., Hong, G., Wu, W., Baudhuin, L. M., Xiao, Y. and Damron, D. S. (2000). Sphingosylphosphorylcholine is a ligand for ovarian cancer G-protein-coupled receptor 1. *Nat Cell Biol* **2**, 261-7.

Zhu, K., Baudhuin, L. M., Hong, G., Williams, F. S., Cristina, K. L., Kabarowski, J. H., Witte, O. N. and Xu, Y. (2001). Sphingosylphosphorylcholine and lysophosphatidylcholine are ligands for the G protein-coupled receptor GPR4. *J Biol Chem* **276**, 41325-35.

Zumwalt, J. W., Thunstrom, B. J. and Spangelo, B. L. (1999). Interleukin-1beta and catecholamines synergistically stimulate interleukin-6 release from rat C6 glioma cells in vitro: a potential role for lysophosphatidylcholine. *Endocrinology* **140**, 888-96.