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Molecules and Cells

Ca^{2+} Signaling Induced by Sphingosine 1-Phosphate and Lysophosphatidic Acid in Mouse B Cells

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Lysophospholipids (LPLs) such as lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) are chemotactic for lymphocytes, and increases of in cytosolic [Ca^{2+}] signal the regulation of lymphocyte activation and migration. Here, the authors investigated the effects of LPA and S1P on $[Ca^{2+}]_c$ in mouse B cell lines (WEHI-231 and Bal-17) and primary B cells isolated from mouse spleen and bone marrow, and focused on the modulation of store-operated Ca^{2+} entry (SOCE) by LPLs. In Bal-17 (a mature B cell line) both LPA and S1P induced a transient $[Ca^{2+}]_c$ increase via a phospholipase C pathway. In addition, pretreatment with LPLs was found to augment thapsigargin-induced SOCE in Bal-17 cells. However, in WEHI-231 (an immature B cell line) LPLs had no significant effect on $[Ca^{2+}]_c$ or SOCE. Furthermore, in freshly isolated splenic B cells (SBCs) and bone marrow B cells (BMBCs), LPLs induced only a small increase in $[Ca^{2+}]_c$. Interestingly, however, pretreatment with LPLs markedly increased SOCE in primary B cells, and this augmentation was more prominent in BMBCs than SBCs. The unidirectional influx of Ca^{2+} was measured using Ba^{2+} as a surrogate ion. Similarly, Ba^{2+} influx was also found to be markedly increased by LPLs in SBCs and BMBCs. Summarizing, LPLs were found to strongly augment SOCE-mediated Ca^{2+}-signaling in mouse B cells. However, unlike the mature Bal-17 cell line, PLC-dependent Ca^{2+} release was insignificant in primary B cells and inWEHI-231.

INTRODUCTION

Lysophospholipids (LPLs) are metabolites of phospholipids and commonly lack a fatty acid from one of the two possible positions of acylation on the glycerol backbone. Of the wide variety of LPLs, lysoglycero-phospholipids, like lysophosphatidic acid (LPA), and lysosphingo-phospholipids, like sphingosine 1-pho-sphate (S1P), have attracted research attention because of the intriguing roles they play in cell signaling and immunomodulation (Goetzl and Rosen, 2004; Meyer Zu Heringdorf and Jakobs, 2007; Rosen et al., 2009; Spiegel and Milstien, 2003).

In vivo, LPA and S1P are metabolites of phosphatidylethanolamine and sphingomyelin, respectively. For example, LPA and S1P can be released by degranulating platelets or synthesized locally at the cell membrane by autotaxin/lysoPLD. Furthermore, LPA and S1P are present in high-nanomolar to low-micromolar concentrations in physiological fluids and have diverse effects on many cells (Aoki et al., 2002; Clair et al., 2003; Goetzl and Rosen, 2004; Spiegel and Milstien, 2003).

S1P and LPA are both extracellular mediators, and they stimulate two families of G-protein coupled receptors (GPCRs) composed of five subtypes (LPA_{1-5} and $S1P_{1-5}$), respectively. Their cellular effects are highly complex because each receptor subtype could signal via multiple G proteins. According to a recent review, G-protein signaling can be categorized as; (1) $G_{i/o}$ signaling for cellular survival, proliferation, and motility; (2) $G_{12/13}$ signaling for cytoskeletal remodeling and cellular shape change; and (3) G_q signaling for miscellaneous cellular effector functions (Goetzl and Rosen, 2004; Noguchi et al., 2009; Rosen et al., 2009). Interestingly, in addition, to their roles as extracellular mediators, S1P and LPA are also intracellular messengers. For example, intracellular S1P induces Ca^{2+} mobilization and stimulates Ca^{2+} influx (Itagaki and Hauser, 2003; Payne et al., 2002), and LPA has been suggested to be an intracellular signaling agonist of PPARγ (McIntyre et al., 2003).

S1P and LPA are also chemotactic for immune cells, such as, T- and B lymphocytes. In particular, S1P is regarded as the predominant signaling molecule responsible for the triggering the lymphocyte egress from lymphoid organs (Matloubian et al., 2004; Rosen et al., 2003; Schwab and Cyster, 2007). The expressions of GPCRs for S1P and LPA in thymus are restricted to the last phases of T cell development. This maturation-dependent receptor expression suggests that S1P and LPA signaling contributes to the emigration and to the terminal dif-

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ferentiation of T cells (Goetzl and Rosen, 2004; Schwab and Cyster, 2007; Takabe et al., 2008). Furthermore, it has been suggested that the distribution of B cells in secondary lymphoid organs and their recirculation into efferent lymph and then into blood is regulated by S1P1 (Cinamon et al., 2004; Kunisawa et al., 2007).

Changes in intracellular Ca^{2+} levels ($\Delta[Ca^{2+}]_c$) regulate a variety of intracellular processes, and play critical roles in immunological responses, such as, cell adhesion and migration (Feske, 2007; Scharenberg et al., 2007). Many reports have demonstrated receptor-mediated Ca^{2+} mobilization after stimulation with S1P or LPA in different cell types (Goetzl and Rosen, 2004; Meyer Zu Heringdorf, 2004; Payne et al., 2002; Spiegel and Milstien, 2003). The mechanism of this Ca^{2+} signaling has been suggested to involve the activation of phospholipase C (PLC) and the subsequent activation of IP_3 receptor.

Despite of extensive investigation of the immunological roles of LPLs, such as, of S1P, the Ca^{2+} signals induced by LPLs have been only rarely investigated in lymphocytes. In T cells, Wang et al. (2004) demonstrated that LPA stimulation induced Ca^{2+} influx in T_h cells, whereas S1P stimulation had no significant effect. In B cells, LPA-induced Ca^{2+} signaling has been studied in B lymphoblast cell lines (Roedding et al., 2006; Rosskopf et al., 1998), but as yet no investigation has been conducted on fresh isolated, primary B cells. In view of the fact that LPLs signals are responsible for the differential regulations of the egress and migration of lymphocytes, including B cells (Cinamon et al., 2004; Rosen et al., 2003), it was suggested that the inductions of differential Ca^{2+} responses might be dependent on the maturation of lymphocytes. Although the differential responses of mature and immature B cells to immunological signaling are well recognized (Harnett et al., 2005; King and Monroe, 2000; Kurosaki, 2002), no investigation has been performed to compare the effects of LPLs on Ca^{2+} signals in mature and immature B cells.

Based on the above, we investigated the effects of two representative LPLs (LPA and S1P) on $[Ca^{2+}]_c$ in the Bal-17 and WEHI-231 cell lines, which are commonly used cell models of mature and immature B cells, respectively (Igarashi et al., 1994). In addition, we compared Ca^{2+} responses to LPLs in freshly isolated B cells from mouse spleen (mature B cells) and from bone marrow (a mixture of pre-, pro-, and immature B cells). Our study reveals intriguing differences in Ca^{2+} signaling between lymphoma cell line and primary B cells as well as the difference between WEHI-231 and Bal-17 cell lines.

MATERIALS AND METHODS

Cell culture and mouse B cell isolation

Mouse B lymphocytes with the properties of immature B cells (WEHI-231) and mature B cells (Bal-17) were grown as previously described (Yoo et al., 2008). Briefly, WEHI-231 were grown in Dulbecco's modified Eagle's medium (DMEM) media (Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (Hyclone, USA), 50 μM 2-mercaptoethanol (Sigma, USA), and 1% penicillin/streptomycin (Gibco). Bal 17 were grown in 25 mM HEPES RPMI 1640 media (Gibco) supplemented with 10% (v/v) fetal bovine serum (Hyclone), and 1% penicillin/streptomycin (Gibco).

Primary B cells were isolated from the bone marrow and spleens of 8-week-old mice. Bone marrow and splenic B cells were isolated using a Spin-Sep™ B cell enrichment kit (Stem Cell Technologies, Canada), and isolated B cells were kept in AIM-V medium (Gibco, USA) until used within 8 h of isolation. All procedures involving animals were approved by our Institutional Review Board (IRB) committee.

Fura-2 fluorimetry and $[Ca^{2+}]_c$ measurements

Cells were harvested HEPES-buffered physiological salt solution (PSS), loaded with fura-2 acetoxymethyl ester (5 μM, 30 min, 25°C), and washed twice with fresh PSS. Fluorescence was monitored in a stirred quartz-microcuvette (1 ml) in the thermostated cell holder of a fluorescence spectrophotometer (Photon Technology Instruments, USA) at 340 and 380 nm (excitation) and 510 nm (emission). At the end of each experiment 5 μM ionomycin and 5 mM $CaCl_2$ were applied to produced a maximum fluorescence ratio of fura-2 (340/380 nm, R_{max}). Then, 10 mM EGTA was applied to confirm a minimum value of fluorescence ratio of fura-2 (R_{min}). The $[Ca^{2+}]_c$ values were calculated using the equation $[Ca^{2+}] = K_d \times b \times (R - R_{min})/(R_{max} - R)$ where K_d is the dissociation constant 224 nM for Fura-2, and b is the ratio of the fluorescence excitation intensities of fura-2 at 380 nm under Ca^{2+}-free and Ca^{2+}-saturated conditions. For the Ba^{2+} influx study, 0.5 M $BaCl_2$ was added to Ca^{2+} free bath solution, which yielded a final $[Ba^{2+}]_c$ of 2 mM.

Experimental solutions and chemicals

$[Ca^{2+}]_c$ measurements were performed in HEPES-buffered PSS containing 145 mM NaCl, 3.6 mM KCl, 10 mM HEPES, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM D-glucose (pH 7.4 adjusted with NaOH). The chemicals and drugs used in this study were purchased from Sigma (USA), with the exception of fura-2 AM (Molecular Probes, USA). LPA and S1P were initially dissolved in ethanol to produce stock solutions and then divided into small aliquots (2-10 μl). Thapsigargin, U73122 and U73343 were dissolved in dimethylsulfoxide (DMSO) and diluted in PSS just before experiments. DMSO concentrations in final experimental solutions were < 0.1%. For $[Ca^{2+}]_c$ measurements, drugs in stock solutions were directly applied into the cuvette that is continuously stirred by a magnet.

RESULTS

Initially, the effects of LPLs on $[Ca^{2+}]_c$ were tested in the mouse B cell lines (Bal-17 and WEHI-231). In Bal-17, both LPA (0.1-3 μM) and S1P (0.1-3 μM) increased $[Ca^{2+}]_c$, and the changes reversed spontaneously to basal levels (Figs. 1A and 1C). LPA appeared more potent than S1P, and increases in $[Ca^{2+}]_c$ were saturated by micromolar concentrations of LPA and S1P. In contrast to Bal-17, neither LPA nor S1P induced a significant $[Ca^{2+}]_c$ increase in WEHI-231 (Figs. 1B and 1D).

In Bal-17, the LPL-induced $[Ca^{2+}]_c$ changes were completely suppressed by pretreatment with U73122 (PLC inhibitor), while not by U73343 (negative analogue of U73122) (Figs. 2A and 2B). The additions of 2 μM of LPA after confirming the S1P-induced $[Ca^{2+}]_c$ increase showed an additional transient increase in $[Ca^{2+}]_c$, and *vice versa* (Figs. 2C and 2D). Such results suggested that the Ca^{2+} release from intracellular stores induced by S1P and LPA was incomplete.

An emptying of endoplasmic reticulum (ER) Ca^{2+} stores triggers Ca^{2+} influx via the plasma membrane, a well known phenomenon described as store-operated Ca^{2+} entry (SOCE) (Feske, 2007; Kurosaki, 2002). In a widely used protocol used to for monitor SOCE, Ca^{2+} release agonists are applied in the absence of extracellular Ca^{2+} to induce ER depletion. Thapsigargin, a potent inhibitor of sarco-/endoplasmic Ca^{2+} pump (SERCA), is the agent most widely used to trigger almost complete depletion of the ER. Under thapsigargin-pretreated conditions, supplementing extracellular Ca^{2+} (Ca^{2+} add-back procedure) induced an increase in $[Ca^{2+}]_c$, reflecting SOCE (Fig. 3A). We examined whether the partial depletion of Ca^{2+} stores by LPA or by S1P instead of thapsigargin could evoke SOCE.

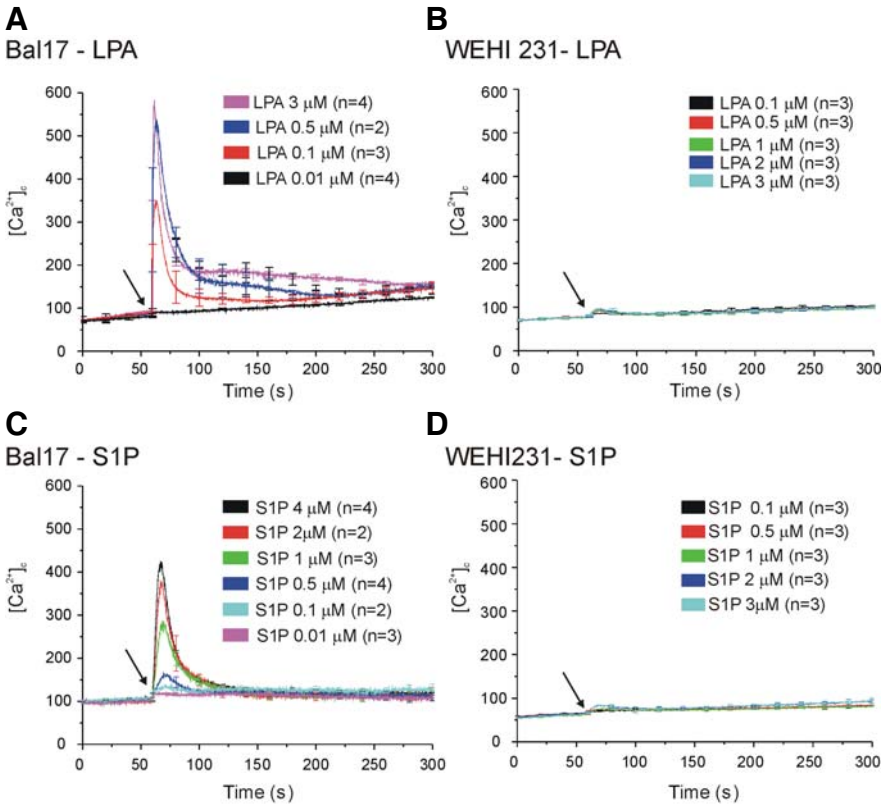

Fig. 1. Changes in $[Ca^{2+}]_c$ induced by lysophospholipids in mouse B cell line cells. $[Ca^{2+}]_c$ values measured in B cells were averaged and plotted with error bars at 20 s intervals. Numbers of tested cell groups are indicated in the figure. Various concentrations of LPA (A, B) or S1P (C, D) were administered at the times indicated by the arrows. In Bal-17 cells, LPA or S1P caused a transient increase in $[Ca^{2+}]_c$ (A, C), whereas WEHI-231 cells showed only a slight increase in $[Ca^{2+}]_c$ (B, D).

Under Ca^{2+}-free conditions (EGTA 2 mM), the application of LPA or S1P to Bal-17 induced a transient $[Ca^{2+}]_c$ increase, and the subsequent addition of Ca^{2+} (5 mM) revealed only weak SOCE activation. (Fig. 3B, arrowhead). Although smaller than the thapsigargin-induced responses, LPA induced a larger transient increase in $[Ca^{2+}]_c$ and in SOCE than S1P (black and gray lines in Fig. 3B, respectively). In WEHI-231 cells, as was expected from the results shown in Fig. 1, LPA- and S1P-induced SOCE responses were negligible (Supplementary Fig. 1).

As mentioned above, treatment with thapsigargin under Ca^{2+}-free conditions is generally regarded as a standard protocol for the full activation of SOCE. However, to our surprise pretreatment with LPA or S1P significantly augmented thapsigargin-induced SOCE in Bal-17 cells (Figs. 3C and 3D), although S1P appeared to be more effective than LPA in terms of SOCE augmentation in Bal-17 cells. However, no similar augmentation of SOCE by LPL was observed in WEHI-231 cells (Figs. 3E and 3F). It was also noticed that the SOCE was smaller in WEHI-231 cells than Bal-17 cells.

Next, we investigated the effects of LPA and S1P on $[Ca^{2+}]_c$ in fresh isolated B cells from spleen or bone marrow. Splenic B cells (SBCs) are mainly composed of mature B cells whereas bone marrow B cells (BMBCs) are composed of pre-, pro-, and the immature stages of B cells (Abbas, 2006). Both LPA and S1P induced only a slight increase in $[Ca^{2+}]_c$ in SBCs, and a slow but continuous increase of $[Ca^{2+}]_c$ in BMBCs (Figs. 4A and 4B). The effects of LPLs on $[Ca^{2+}]_c$ were generally larger for BMBCs than for SBCs. On the other hand, the effect of LPA was larger than that of S1P in both SBCs and BMBCs (Figs. 4A and 4B). Summarizing, the LPL-induced $[Ca^{2+}]_c$ increase in SBCs was smaller than in Bal-17 whereas the LPL-induced $[Ca^{2+}]_c$ increase in BMBC was larger than in WEHI-231.

In the absence of extracellular Ca^{2+}, both LPA and S1P (both at 2 μM) induced only slight $[Ca^{2+}]_c$ increases in SBCs and BMBCs (Figs. 4C and 4D, see inset). The $[Ca^{2+}]_c$ increase induced by thapsigargin under Ca^{2+}-free conditions was also only slight in BMBCs and SBCs, and SOCE in response to the Ca^{2+} add-back procedure was also relatively small (ca. 300 nM, Figs. 4C and 4D). However, despite the small $[Ca^{2+}]_c$ increase induced by LPA or S1P, pretreatment with these agents dramatically augmented thapsigargin-induced SOCE in both SBCs and BMBCs. Furthermore, $[Ca^{2+}]_c$ increases after Ca^{2+} add-back were more sustained in BMBCs than in SBCs, and the augmentation of SOCE was much larger in BMBCs than SBCs (Fig. 4D).

The $[Ca^{2+}]_c$ under conditions of SOCE activation would be determined by Ca^{2+}-influx and removal to extracellular space: the augmentation of SOCE could be due to increased Ca^{2+} influx or the inhibition of Ca^{2+} removal. Therefore, we investigated the effects of LPA and of S1P on unidirectional Ca^{2+} influx in B cells. To selectively measure the Ca^{2+} influx after the Ca^{2+} add-back procedure, we used Ba^{2+} as surrogate divalent cation and fura-2, because Ba^{2+} is not removed by plasmalemmal Ca^{2+} ATPase. Accordingly, 2 mM $BaCl_2$ was used instead of $CaCl_2$, a continuous increase in $F_{340/380}$ was observed (refer to the gray trace in Fig. 5A).

In Bal-17 cells, Ba^{2+}-induced increase in $F_{340/380}$ was rapid after LPA (2 μM) pretreatment, but to our surprise, S1P did not affect the Ba^{2+}-induced increase in $F_{340/380}$ in these cells (Fig. 5A). On the other hand, in SBCs and BMBCs, both LPA and S1P increased the speed of Ba^{2+} uptake. Interestingly, the effect of LPA was greater than that of S1P, and the LPA effect reached an earlier steady-state (Figs. 5B and 5C).

DISCUSSION

In this study, we found noticeable differences among the B cell

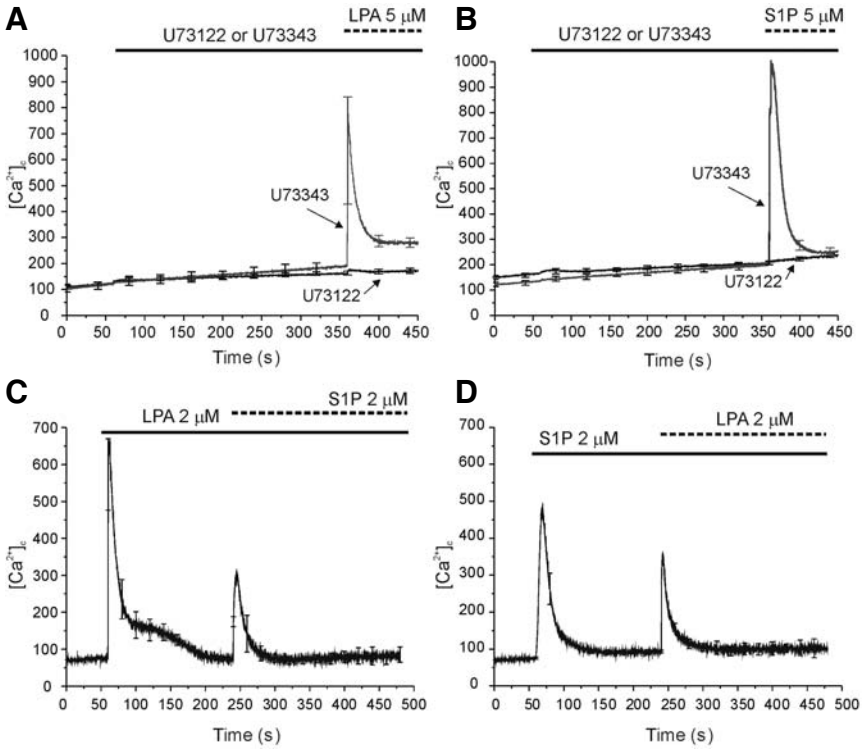

Fig. 2. PLC-mediated release of stored Ca^{2+} triggered by LPLs in Bal-17 cells. (A, B) The transient $[Ca^{2+}]_c$ in-crease induced by 5 μM LPA or S1P was completely blocked by pretreatment with U73122 (2 μM, n = 5), but no such inhibition was observed after with U73343 pretreatment (5 μM, gray trace, n = 3). (C, D) Pre-treatment with LPA did not interfere with Ca^{2+} response to S1P receptor stimulation [(C), n = 3], and pre-treatment with S1P did not interfere with Ca^{2+} response to LPA [(D), n = 3].

lines (WEHI-231 and Bal-17) and fresh isolated B cells (SBCs and BMBCs); 1) PLC-dependent transient Ca^{2+} release was obvious in Bal-17 cells, but not in SBCs, BMBCs, or WEHI-231 cells, 2) LPLs strongly augmented Ca^{2+} influx after SOCE activation in Bal-17, SBCs and BMBC while not in WEHI-231.

In general, classic Ca^{2+}-release activated Ca^{2+} channels (CRAC) are believed to be a fundamental component of the underlying Ca^{2+} influx pathway in lymphocytes. Furthermore, recently, the mechanism of CRAC activation was found to involve a heteromeric interaction between STIM (an ER membrane protein sensing Ca^{2+} depletion) and Orai (a plasma membrane protein as Ca^{2+} permeable pore) (Feske, 2007). However, we could not simply conclude that LPLs positively modulated the Orai/STIM complex in the B cells because our findings were highly cell type and agonist dependent (see below).

Augmentation of SOCE by LPLs

Our findings raise a question about the mechanism underlying the marked augmentation of Ca^{2+} influx by LPLs as opposed to the much smaller $[Ca^{2+}]_c$ increase induced by S1P or LPA alone. Although SOCE augmentation by LPLs was commonly observed in Bal-17 cells, SBCs, and BMBCs, augmentation patterns varied in these cells. For example, the SOCE of Bal-17 was augmented by S1P, whereas Ba^{2+} influx induced by store depletion was not changed by S1P. Also, in SBCs, the relative potency of SOCE augmentation by LPA and S1P was opposite to their augmentative effects on Ba^{2+} influx (Fig. 4C vs. Fig. 5B). In contrast, the effects of S1P and of LPA on SOCE and Ba^{2+} influx were same in BMBCs (Fig. 4D vs. Fig. 5C).

Since the above effects of LPA and S1P were observed at low micromolar ranges, it is suggested that biochemical signaling from the GPCRs for LPA and S1P positively modulated CRAC activity. However, we could not exclude that Ca^{2+} permeable channels other than CRAC had been activated by LPLs. The above-mentioned cell type and agonist dependence indicates that the Ca^{2+}-influx pathways recruited or facilitated by LPA and S1P are highly variable among different cell types. For example, the strong augmentation of Ba^{2+} influx by LPA indicated that a non-CRAC pathway might be activated in Bal-17 and in primary B cells (Fig. 6) because the classic CRAC (Orai) revealed far less Ba^{2+} permeability than Ca^{2+} (Lis et al., 2007). In contrast, the insignificant effect of S1P on the Ba^{2+} flux into Bal-17 cells suggested that SOCE augmentation by S1P might be mainly due to the facilitation of CRAC activity.

As for Ba^{2+} permeable store-operated Ca^{2+} entry mechanisms that are modulated by LPLs, one might suspect that TRPC channels are associated with STIM1-dependent regulation (Zeng et al., 2008). In a previous study on human peripheral B cells, LPA activated Ca^{2+} influx pathways that were also permeable to Ba^{2+} (Roedding et al., 2006). Based on ionic current activation by diacylglycerol and the expression of TRPC3 mRNAs, the authors concluded that TRPC3 was responsible for LPA-induced Ca^{2+} influx. However, in this previous study a much higher concentration of LPA (100 μM) was used than in the present study (2 μM). The previous studies in polymorphonuclear cells and erythrocytes have also suggested that S1P and LPA directly activate SOCE or nonselective cation channels (Itagaki and Hauser, 2003; Itagaki et al., 2005; Yang et al., 2000). Furthermore, in the study by Itagaki et al. (2005), higher LPL concentrations (10-200 μM) were used to examine the non-receptor effects of LPLs in polymorphonuclear cells. More rigorous investigations are still required to identify the Ca^{2+} influx pathways modulated by LPLs, and the STIM1-dependent TRPC activation might be a hypothetical mechanism in B cells.

LPA and S1P receptors in B cells

Although data from *in vitro* studies are difficult to compare with *in vivo* data, one could envisage that LPA is released at sites of local inflammation (in response to cell injury, during wound healing, or from growing tumors) or during generalized inflam-

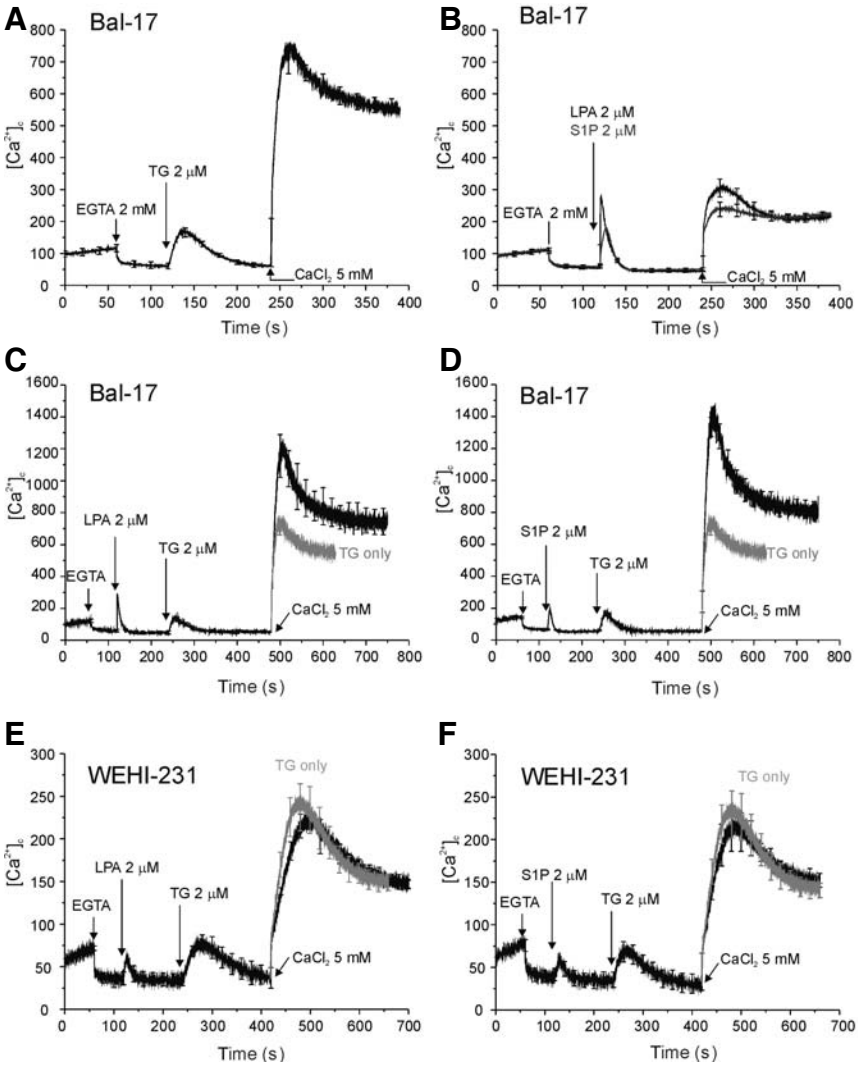

Fig. 3. LPA and S1P augmented thapsigargin-induced SOCE in Bal-17 cells, but not in WEHI-231 cells. (A) Averaged $[Ca^{2+}]_c$ traces of Bal-17. 2 mM EGTA was applied to chelate extracellular Ca^{2+}, and then thapsigargin (TG, 2 μM) was applied to deplete ER-stored Ca^{2+}. The addition of 5 mM of $CaCl_2$ evoked a maintained phasic increase in $[Ca^{2+}]_c$. Peak $[Ca^{2+}]_c$ on Ca^{2+} repletion reflected of SOCE. The mean values obtained from five experiments are shown with error bars (n = 5). (B) LPA (n = 9) or S1P (n = 11) were applied instead of thapsigargin during the Ca^{2+} add-back procedure. The averaged trace of the S1P treatment experiment is indicated as a gray line. (C-F) In the presence of 2 mM EGTA, LPA or S1P (2 μM) were applied prior to the addition of thapsigargin (2 μM). In each panel, the control SOCE responses without pretreatment by with lysophospholipids (thapsigargin-only, gray traces) are overlaid. (C, D) Averaged results obtained in Bal-17 cells (n = 3). (E, F) Averaged results obtained in WEHI-231 cells (n = 3).

mation (septic states or rheumatoid arthritis). High concentrations of LPA are also released from activated platelets and fibroblasts. Furthermore, it has been reported that inflammatory exudates contain high levels of sPLA2 generating LPAs (Graler and Goetzl, 2002; Lambeau and Gelb, 2008)

The GPCRs of S1P ($S1P_{1-5}$) and LPA (LPA_{1-5}) are expressed in almost every type of cell in mammals. Furthermore, S1P is known to be an evolutionary conserved Ca^{2+} signaling molecule in yeast, plants, and mammals (Spiegel and Milstien, 2003). According to the literature, T and B cells express predominantly $S1P_1$, $S1P_4$, and LPA_2 (Goetzl and Rosen, 2004). However, the identification of individual LPL receptors that mediate specific responses is difficult because the majority of cells express more than one S1P or LPA receptor, and receptor subtype-specific pharmacological tools are lacking. For such reasons, we did not attempt to identify the receptor subtypes linked with specific pathways of Ca^{2+} signal amplification in B cells.

Ca^{2+}-signaling by LPLs in mature and immature B cells

Our initial goal was to determine whether Ca^{2+} responses to LPLs differ for mature (Bal-17) and immature (WEHI-231) B cells (Ralph, 1979). At first, our Bal-17 and WEHI-231 results suggested that LPL receptors and Ca^{2+} signaling might be present exclusively in mature B cells (Bal-17). However, in primary B cells (BMBCs and SBCs), the sustained increase in $[Ca^{2+}]_c$ by LPLs and the augmentation of SOCE were larger in BMBCs than mature SBCs. Moreover, the relative potencies of LPA and S1P for the augmentation of Ca^{2+} influx were opposite sin SBCs and BMBCs (Figs. 4C and 4D), which suggests that the functional expressions of LPA and S1P receptors change during B cell development.

Then, what would be the physiological meaning of the larger LPL-induced Ca^{2+} signals in BMBCs than SBCs? In T cells, the expression of S1P receptors is known to be dynamically changed depending on the maturation stages, and the migration/egression of T cells from lymphoid organs are dynamically regulated by S1P (Schwab and Cyster, 2007). Similar roles might be suggested in B cells, and the differential levels of LPL-induced Ca^{2+} signals might control the migration of B cells. The $[Ca^{2+}]_c$ critically determine B cell activation as well as apoptosis (Scharenberg et al., 2007). The BCR-ligation induced proliferation further differentiation of mature B cells while apoptosis is induced in immature B cells. In this respect, the excessive augmentation of BCR-ligation induced Ca^{2+} signals by LPLs might accelerate the apoptosis of immature B cells. Further investigation is required to elucidate the physiological meaning of the Ca^{2+} signaling in mature vs. immature B cells.

Summarizing, the above results demonstrate that S1P and

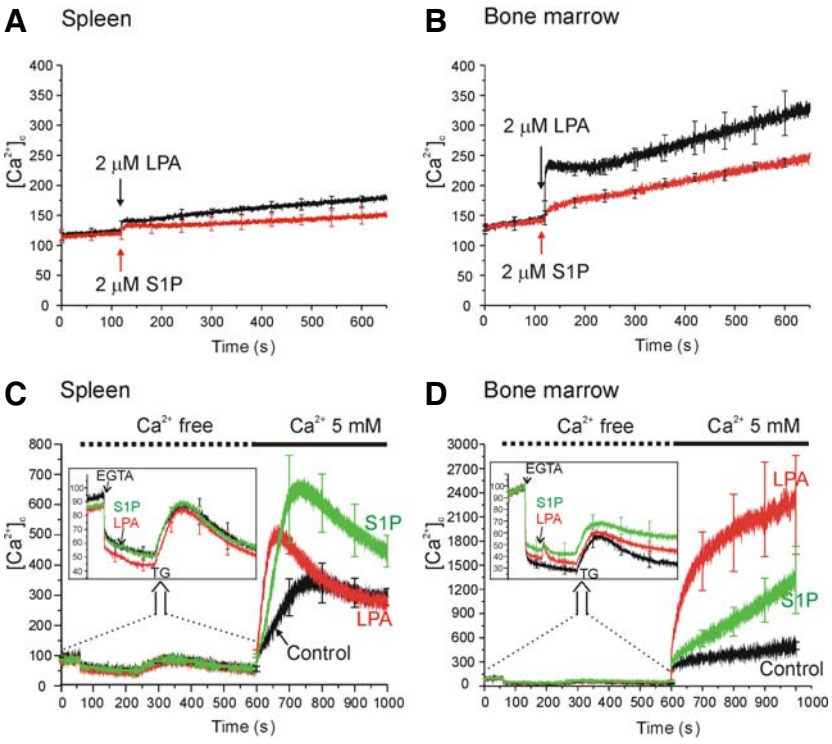

Fig. 4. Effects of LPA and S1P on $[Ca^{2+}]_c$ levels of freshly isolated splenic B cells (SBCs) and bone-marrow B cells (BMBCs). (A, B) The 2 μM of LPA or S1P induced sustained increases in $[Ca^{2+}]_c$ in SBCs (A) and BMBCs (B). The upper traces (black) are averaged responses to LPA (SBCs n = 6, BMBCs n = 3), and the lower traces are averaged responses to S1P (SBCs n = 5, BMBCs n = 4). (C, D) In the presence of 2 mM EGTA, LPA or S1P (2 μM) were applied prior to the addition of thapsigargin (2 μM). In each panel, control SOCE responses without pretreatment with lysophospholipids (thapsigargin-only) are overlaid (black lines). Averaged results for SBCs [n = 5, (C)] and BMBCs [n = 5, (D)] are overlaid using red and green colors for LPA and S1P, respectively. Note the different $[Ca^{2+}]_c$ scales used for SBCs and BMBCs. *Insets;* vertically expanded recordings of $[Ca^{2+}]_c$ increases induced by lysophospholipids and thapsigargin.

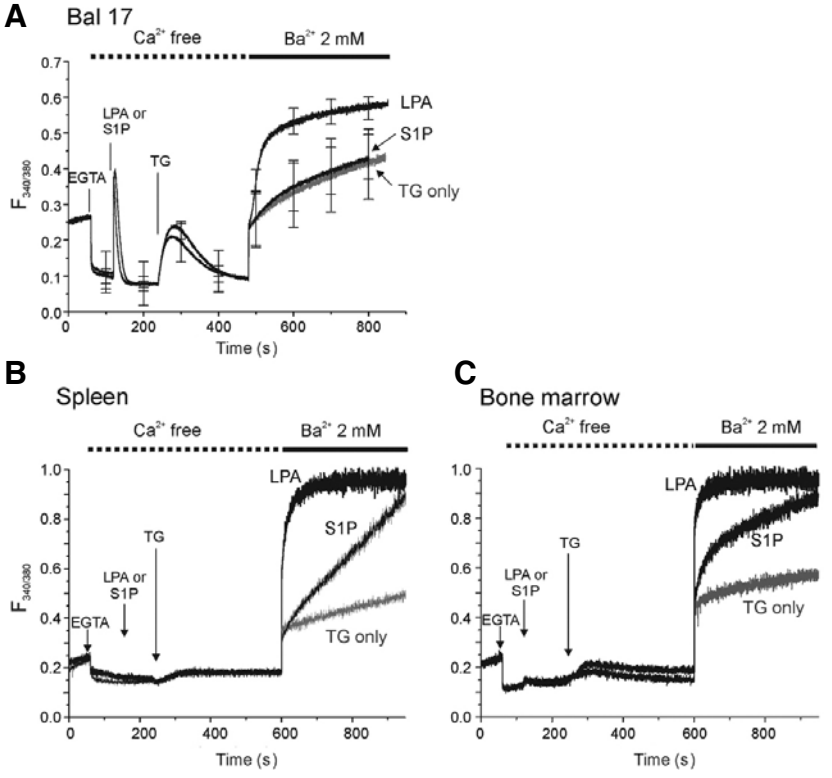

Fig. 5. Effect of LPA and of S1P on thapsigargin-induced Ba^{2+} influx in mouse B cells. Fura-2 fluorescence ratios ($F_{340/380}$) were measured in Bal-17 (A), splenic (B), and bone-marrow B cells (C). 2 mM $BaCl_2$ was added after the sequential applications of lysophospholipids (2 μM LPA or S1P) and 2 μM thapsigargin. Averaged control responses to thapsigargin are overlaid (gray traces) in each panel. In (A), the numbers of tested cases were; 3 (LPA), 3 (S1P), and 5 (control, TG only). (B, C) show the original traces of primary B cells from two mice.

LPA, at micromolar concentrations, augment Ca^{2+} signaling when combined with store-depleting stimuli in B lymphocytes. Furthermore, the augmentation of Ca^{2+} influx by LPL was marked in BMBCs, in which LPA was more effective than S1P. In view of the fact that sustained $[Ca^{2+}]_c$ increase by SOCE is critical for the activation and transcriptional regulation of lymphocytes (Feske, 2007; Scharenberg et al., 2007), our findings suggest that the augmentation of Ca^{2+} signaling contributes to the biological effects of LPLs *in vivo*.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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