Lysophospholipids elevate $[Ca^{2+}]_i$ and trigger exocytosis in bovine chromaffin cells

Chien-Yuan Pan^{a,b,*}, Hsinyu Lee^{a,b}, Chia-Lin Chen^b

^a Institute of Zoology, National Taiwan University, Taipei, Taiwan, ROC

^b Department of Life Science, National Taiwan University, Taipei, Taiwan, ROC

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Abstract

Sphingosine 1-phosphate (S1P) and lysophosphatidic acid (LPA) are responsible for many physiological functions, including angiogenesis, neuronal survival, and immunity. However, little is known about their effects in modulating the stimulus-secretion coupling in bovine chromaffin cells. The result of PCR showed that at least two receptors ($S1P_3$ and LPA_1) were expressed in bovine chromaffin cells. The elevation of $[Ca^{2+}]_i$ by S1P was fast and sustaining; but the elevation by LPA was slow and transient. The EC_{50} for S1P and LPA in elevating the $[Ca^{2+}]_i$ were 0.55 ± 0.01 and 0.54 ± 0.40 μ M, respectively. This elevation could be totally blocked by thapsigargin, 2-APB, and U73122. Pertussis toxin pretreatment inhibited about half of the elevation in $[Ca^{2+}]_i$ suggesting the involvement of G_i and other G-proteins. Repetitive $[Ca^{2+}]_i$ elevations elicited by S1P, but not LPA, were inhibited by ryanodine. S1P was more effective than LPA in triggering exocytosis as measured by the changes in membrane capacitance. The whole-cell Ca^{2+} current was inhibited by both lysophospholipids but Na^+ current was inhibited by S1P only. These results suggest the differential effects of LPA and S1P in releasing Ca^{2+} from the intracellular Ca^{2+} stores and modulating the stimulus-secretion coupling in bovine chromaffin cells.

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1. Introduction

The calcium ion is one of the most important second messengers in modulating many cellular responses, like neurotransmitter release, muscle contraction, and gene expression. Therefore, how a cell attenuates the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is an important issue for cell activities. The main sources responsible for $[Ca^{2+}]_i$ elevation are from either extracellular solution or intracellular Ca^{2+} stores. A voltage-gated Ca^{2+} channel on the plasma membrane is the main

pathway to allow Ca^{2+} influx into the cytosol; IP_3 and ryanodine receptors are responsible for Ca^{2+} release from intracellular Ca^{2+} stores. These pathways contribute differentially to the $[Ca^{2+}]_i$ elevation depending on the characteristics of stimulation (Cuchillo-Ibanez et al., 2002; Schulz and Krause, 2004; Shuttleworth and Mignen, 2003).

In rat and human adrenal glands, a population of macrophages is distributed throughout the cortex and medulla (Schober et al., 1998). This hints the possible paracrine interaction between chromaffin and immune cells. A factor, molecular weight less than 3000, released by mononuclear cell can stimulate adrenal medulla cells to secrete epinephrine to a level comparable to the maximal cholinergic response. However, the release of this factor can be feedback inhibited by the released epinephrine (Roberts et al., 1996). Some of the peptides stored in the secretory granules of chromaffin cells can display antimicrobial activities (Metz-Boutigue et al., 2003). These support the important linkages between immune and endocrine systems.

Abbreviations: CICR, calcium-induced calcium release; LPA, lysophosphatidic acid; LPL, lysophospholipid; PLC, phospholipase C; Ptx, pertussis toxin; S1P, sphingosine 1-phosphate; Tg, thapsigargin.

* Corresponding author. Institute of Zoology, National Taiwan University, Rm730, Life Science Building, No. 1 Roosevelt Road, Sec. 4, Taipei 106, Taiwan, ROC. Tel.: +886 2 33662452; fax: +886 2 23636837.

E-mail address: cypan@ntu.edu.tw (C.-Y. Pan).

The bioactive lysophospholipids (LPLs), including lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P), are released by many immune cells during blood clotting and can reach micrometer level in the plasma (Kishimoto et al., 2003; Okajima, 2001; Xiao et al., 2000). The LPLs induce the proliferation of cells around the wound to heal the tissue or work as a signal to other cells by circulation to evoke many other physiological responses. However, most of the studies about the functions of LPA and S1P are focused on cell survival, atherosclerosis and wound healing (Panetti, 2002; Xu et al., 2003). Their roles in modulating the excitable endocrine cells are not clear.

The physiological functions of LPA and S1P are mediated through specific G-protein coupled receptors (Goetzl et al., 2002; Taha et al., 2004). Some of these receptors can activate the phospholipase C (PLC)-dependent pathway to release Ca^{2+} from intracellular Ca^{2+} store and possibly elicit calcium-induced calcium release (CICR) (Meyer Zu Heringdorf, 2004). LPA can elevate $[\text{Ca}^{2+}]_i$ and cause dopamine secretion in PC12 cells by increasing the amount of IP_3 inside the cell (Shiono et al., 1993). Therefore, these LPLs can work as Ca^{2+} mobilization agents to modulate the stimulus-secretion coupling in bovine chromaffin cells.

Using RT-PCR, we confirmed the expression of two LPL receptors, S1P_3 and LPA_1 , in bovine chromaffin cells. The elevations in $[\text{Ca}^{2+}]_i$ elicited by S1P and LPA were dose-dependent and blocked by thapsigargin (Tg). The S1P-elicited elevation in $[\text{Ca}^{2+}]_i$ was fast and sustaining; but LPA-elicited elevation was transient. This elevation in $[\text{Ca}^{2+}]_i$ could be blocked by inhibitors against PLC and IP_3 receptor. Repetitive S1P-, but not LPA-, induced elevations in $[\text{Ca}^{2+}]_i$ were suppressed by ryanodine. The elevation in $[\text{Ca}^{2+}]_i$ elicited by either S1P or LPA was high enough to evoke catecholamine release as measured by the changes in membrane capacitance. These LPLs also inhibited the Ca^{2+} currents but only S1P could inhibit Na^+ current. These suggest that S1P and LPA work differentially in modulating the $[\text{Ca}^{2+}]_i$ response and stimulus-secretion coupling in bovine chromaffin cells.

2. Material and methods

2.1. Chemicals

Oleoyl-L- α -lysophosphatidic acid (LPA, C18:1, 1-oleoyl-*sn*-glycerol-3-phosphate), D-erythro-sphingosine-1-phosphate (S1P), *N,N*-dimethylsphingosine (DMS), and ryanodine were purchased from Sigma (St. Louis, USA). Thapsigargin (Tg), U73122, and 2-APB, were from CalBiochem. Fura-2 acetomethoxymethyl ester (Fura-2 AM) was from TefLab (Austin, TX, USA). All other chemicals were purchased from Sigma unless otherwise indicated.

2.2. Isolation and culture of bovine chromaffin cells

Bovine adrenal glands were supplied by a local slaughterhouse. The adrenal glands were removed immediately after the animals were killed and kept on ice. Chromaffin cells were isolated as described by Pan et al. (2002). Cells were plated at a density of 2×10^5 cells/ml. All experiments were carried out between 3 and 10 days after cells were isolated. Each experiment was repeated using cells from different dishes and from different batches.

2.3. RT-PCR

Specific primer sets against LPL receptors were designed according to the published sequences of human LPL receptors. The forward and reverse primers for LPA_1 are 5'-cggagactgactgtcagcac-3' and 5'-ggccagaactatgccgaga-3'; LPA_2 are 5'-abctgcacagccgctgccccgt-3' and 5'-tgctgtccatgccagacctgtc-3'; LPA_3 are 5'-ttagctgctgcccgtattctt-3' and 5'-atgatgaggaggccatgag-3'; S1P_1 are 5'-gactctgctggcaaatcaagcgac-3' and 5'-accctcccagtcattgttcacag-3'; S1P_2 are 5'-ctctctacgccaaagcattatgtgt-3' and 5'-tcagaccacgtgttgcctc-3'; S1P_3 are 5'-cgtgctcttctgtcactgtcag-3' and 5'-cgatgaacagctcaccacaatc-3'; S1P_4 are 5'-agcctcttgcctctactc-3' and 5'-gtagatgatgggttgaccg-3'; S1P_5 are 5'-ggagtagtcccgaaggacc-3' and 5'-tctagaatccacgggtgtc-3'. Total bovine chromaffin cell RNA was collected using Trizol reagent (Life Technology, USA) according to the manufacturer's manual. Single strand cDNA was synthesized from the total RNA using oligo-dT as the primer by Superscriptase III (Life Technology, USA). The PCR product was subcloned to pGEMT vector (Life Technology, USA) for sequencing.

2.4. Solutions

The bath Loading Buffer (LB) used for calcium imaging and electrophysiological recording contained (in mM): 145 NaCl, 5 glucose, 10 Na-HEPES, 1 MgCl_2 , 5 KCl, and 2.2 CaCl_2 , pH 7.3 with NaOH. The whole-cell patch pipette solution contained (in mM): 120 Cs-aspartate, 40 Na-HEPES, 5 MgCl_2 , 0.1 EGTA, 2 ATP, 0.3 GTP, pH 7.3 with CsOH. S1P and LPA was dissolved in Chloroform:Methanol/1:19 solution to make a concentration of 1 mM; it was then air dried and redissolved in ethanol to make a stock of 1 mM and stored in -20°C .

To stimulate the cell, a glass micropipette with 2- μm opening was filled with LB buffer containing S1P, LPA, or other blockers and positioned at 20 μm from the cell. The stimulant was puffed onto a single chromaffin cell for the time indicated at 5 psi under the control of Picospritzer III (Parker Instrument, Parker Hannifin, Fairfield, NJ, USA). To deplete the intracellular Ca^{2+} stores, cells were pretreated with 1 μM of Tg for 30 min before recording. 2-APB (10 μM) and U73122 (100 μM) were added 10 min before the recording; pertussis toxin (Ptx, 0.1 $\mu\text{g}/\text{mL}$) was added into the culture medium overnight. Ryanodine (20 μM) was added into the bath right before the experiment.

2.5. Calcium imaging

For $[\text{Ca}^{2+}]_i$ measurement, cells were incubated in LB with 5 μM fura-2 AM for 1 h at 37°C . Cells were then washed 3 times with LB and used for measurements. For fura-2 excitation, a Lambda DG4 system (Sutter Inc., Novato, CA, USA) was used, which was controlled by Metafluor software from Universal Imaging (Downingtown, PA, USA). Ratiometric calcium estimates were made by using 10-nm wide filters centered on 340 and 380 nm (Chroma Technology, Brattleboro, VT, USA), capturing the emitted light (510–540 nm) at each excitation wavelength for 300 ms through a 20 $\times$ objective (Zeiss Axiovert 200, Germany) and directing it to a cooled CCD camera (CoolsnapFx; Roper Scientific, Tucson, AZ). The ratio within each cell was computed from images obtained at 340 and 380 nm excitation wavelengths and subtracting the appropriate background fluorescence at each wavelength. Ratios were acquired every second. The calcium calibration buffer kit (Molecular Probes, Carlsbad, California) was used to transform the ratio into Ca^{2+} concentration according to manufacturer's manual.

2.6. Electrophysiological recording

The membrane capacitance and whole-cell current were recorded by EPC10 amplifier (HEKA GmbH, Germany). To monitor the membrane capacitance, the glass pipette was coated with sylgard (Dow Corning Co., Midland, MI, USA) and the cell was whole-cell voltage clamped at -70 mV. A sine wave of 1000 Hz was applied to monitor the membrane capacitance by the Lock-in amplifier in the Pulse software. To monitor the whole-cell inward currents, the cell was voltage clamped at -70 mV and repetitively depolarized

to +10 mV for 20 ms every 15 s. The inward maximal during the first 5 ms was calculated as the Na^+ current and the current between 12th and 18th ms was averaged as the Ca^{2+} current.

3. Results

The sequences for bovine S1P_1 and LPA_1 receptors have been published. To verify the expression profile of LPL receptors in bovine chromaffin cell, specific primers were designed according to published bovine and human LPL receptor cDNA sequences. The result showed that only two PCR products of expected size against S1P_3 and LPA_1 were obtained and sequenced (Fig. 1). The sequences of S1P_3 and LPA_1 were identical to the predicted XM_584989.1, similar to bovine endothelial differentiation receptor 3; and cloned NM147047.2, bovine endothelial differentiation receptor 2, respectively. This indicates that there are at least two LPL receptors expressed in bovine chromaffin cells and hints the effects of LPL on chromaffin cell.

S1P_3 and LPA_1 can couple to G_i and G_q to activate PLC to release IP_3 into the cytosol, therefore, the roles of the LPLs in releasing Ca^{2+} from the intracellular Ca^{2+} stores were characterized. Single chromaffin cells were puffed with LPLs and the changes in $[\text{Ca}^{2+}]_i$ were monitored by fura-2 calcium-sensitive fluorescent dye. The results showed that LPA and S1P raised the $[\text{Ca}^{2+}]_i$ in a different mode (Fig. 2). During the period of LPA application, the $[\text{Ca}^{2+}]_i$ was elevated slowly and reached maximum in about 25 s; after that, the $[\text{Ca}^{2+}]_i$ declined to basal even though the cell was still stimulated by LPA. However, the $[\text{Ca}^{2+}]_i$ was raised almost immediately by S1P application and kept at a plateau during the period of S1P application. The maximal changes in the LPL-elicited elevation in $[\text{Ca}^{2+}]_i$ were dose-dependent; the EC_{50} for LPA and S1P, calculated by sigmoidal fitting module in Origin software (OriginLab Corp., Northampton, MA), in elevating the $[\text{Ca}^{2+}]_i$ are 0.55 ± 0.01 and 0.54 ± 0.40 μM , respectively. This is comparable to the physiological concentrations that LPLs can reach in plasma. These observations suggest that LPA and S1P may raise the $[\text{Ca}^{2+}]_i$ through different pathways under physiological conditions.

To verify the sources responsible for LPL-elicited $[\text{Ca}^{2+}]_i$ elevation, chromaffin cells were treated with Tg to deplete the intracellular Ca^{2+} stores. Fig. 3 shows that S1P- and LPA-elicited elevations in $[\text{Ca}^{2+}]_i$ represented by fura-2 ratio were blocked by Tg from 0.53 ± 0.12 and 0.54 ± 0.04 to 0.13 ± 0.02 and 0.13 ± 0.02 , respectively. This suggests that the main source responsible for the elevation in $[\text{Ca}^{2+}]_i$ is the intracellular Ca^{2+} stores.

The IP_3 and ryanodine receptors are responsible for Ca^{2+} release from intracellular Ca^{2+} stores. Fig. 4 shows that when a cell was treated with different blockers against the PLC- IP_3 pathway, the elevation in $[\text{Ca}^{2+}]_i$ was significantly suppressed. For control cells, the maximal changes in $[\text{Ca}^{2+}]_i$ represented by fura-2 ratio were 0.70 ± 0.09 and 0.81 ± 0.07 for S1P and LPA, respectively. The S1P- and LPA-elicited elevations were significantly inhibited by U73122, a PLC blocker, to 0.12 ± 0.02 and 0.02 ± 0.00 ; and

2-APB, an IP_3 receptor blocker, to 0.08 ± 0.01 and 0.09 ± 0.02 , respectively. When cells were treated with Ptx overnight to inhibit G_i protein, the elevations in $[\text{Ca}^{2+}]_i$ represented by fura-2 ratio elicited by S1P and LPA were partially, but significantly, inhibited to 0.36 ± 0.03 and 0.40 ± 0.05 , respectively. These confirm that IP_3 receptor is the main pathway gating the Ca^{2+} release from the intracellular stores and both G_i and G_q proteins are activated by the LPL receptors in bovine chromaffin cells.

It has been reported that ryanodine receptor can be opened to release more Ca^{2+} into the cytosol when $[\text{Ca}^{2+}]_i$ is elevated, the so called CICR (Verkhratsky, 2002). To understand whether CICR was involved in LPL-elicited elevation in $[\text{Ca}^{2+}]_i$, a single chromaffin cell was stimulated consecutively by LPLs twice in the presence or absence of ryanodine (Fig. 5). The representative traces showed that the second S1P stimulation could bring the $[\text{Ca}^{2+}]_i$ slightly higher than that of the first stimulation in the absence of ryanodine. However, in the presence of ryanodine, the second stimulation was greatly suppressed. On the contrary, irrespective of the presence of ryanodine or not, the second LPA stimulation was slightly lower than the first one. After normalization of the second LPL-elicited maximal change in $[\text{Ca}^{2+}]_i$ to the first one, the averaged results showed that the repetitive LPA stimulations had no significant difference in the presence ($69.2 \pm 3.8\%$) or absence ($75.7 \pm 7.5\%$) of ryanodine. However, the second $[\text{Ca}^{2+}]_i$ response elicited by S1P was significantly suppressed to $56.3 \pm 13.7\%$ in the presence of ryanodine comparing to $120.0 \pm 32.2\%$ of control cells. These suggest that ryanodine receptor is involved in the $[\text{Ca}^{2+}]_i$ response elicited by S1P, but not LPA.

The increase in $[\text{Ca}^{2+}]_i$ can usually trigger exocytosis to release catecholamine in bovine chromaffin cells. To verify whether LPLs can induce exocytosis or not, whole-cell patch clamp technique was applied to monitor the changes in membrane capacitance (Fig. 6). The representative traces showed that the capacitance was decreased during the period of the control buffer application. When stimulated with LPA and S1P, the membrane capacitances were slowly increased. The averaged membrane capacitance change from the cells stimulated with control buffer was -149.0 ± 75.5 fF; S1P stimulation could significantly increase the capacitance value to 169.6 ± 25.2 fF. However, for cells stimulated with LPA, 4 in 10 cells showed endocytosis (-131.9 ± 48.4 fF) similar to that of control cells; the rest showed significant increase in membrane capacitance (106.6 ± 18.7 fF). These indicate that the Ca^{2+} released from the intracellular Ca^{2+} stores elicited by LPLs can induce the occurrence of exocytosis in bovine chromaffin cells.

Many factors which activate the G-proteins may have an effect in modulating the ion channels. To understand the effect of these LPLs on inward Na^+ and Ca^{2+} currents, whole-cell patch clamp technique was used to measure electrophysiological properties of the chromaffin cell (Fig. 7). The representative traces showed that these inward currents were not significantly changed for cells puffed with control solution. After stimulating the cell with S1P for 2 min, both the Na^+ and Ca^{2+} inward

Fig. 1. S1P₃ and LPA₁ are expressed in bovine chromaffin cells. Specific primers against each LPL receptors were used to amplify the genes expressed in bovine chromaffin cells. (A) The agarose gel electrophoresis of the PCR products. Primers against each LPL receptors as indicated were used to amplify the cDNA synthesized from bovine chromaffin cells. M is 100 bp marker; N is negative control using primers against LPA₁ in the absence of cDNA. (B) and (C) The sequences of the subcloned LPA₁ and S1P₃ PCR products, respectively.

currents were inhibited; however, LPA stimulation inhibited only the Ca²⁺ current. After normalizing the currents after LPL treatment to those before stimulation, the averaged results of control cells were 99.7 ± 3.5 and $113.8 \pm 10.4\%$ for Na⁺ and Ca²⁺ currents, respectively. S1P treatment significantly inhibited the Na⁺ and Ca²⁺ currents to 59.5 ± 11.8 and $41.1 \pm 17.0\%$, respectively; however, LPA inhibited only the Ca²⁺ current ($71.0 \pm 3.8\%$) but not the Na⁺ current ($96.4 \pm 3.6\%$). These suggest that LPLs differentially modulate the channel activities in bovine chromaffin cells.

4. Discussion

This is the first report demonstrating how LPA and S1P modulate the [Ca²⁺]_i, inward currents, and exocytosis in

bovine chromaffin cells. Previous reports about the effects of these LPLs on bovine chromaffin cells were published by Oak's lab (Houchi et al., 1995; Tokumura et al., 1996; Tokumura et al., 1998). They focused their studies on how LPA enhances the Na⁺/Ca²⁺ exchange activity to extrude ⁴⁵Ca²⁺ out of the cell. Their results suggest that protein kinase C and protein tyrosine kinase are involved negatively and positively, respectively, in activating the Na⁺/Ca²⁺ exchanger. However, they did not investigate the roles of LPA in releasing Ca²⁺ from intracellular stores. As for S1P, there is no report published yet about its effects on chromaffin cells.

The physiological functions of LPLs are mediated by G-protein coupled receptors (Meyer Zu Heringdorf, 2004; Taha et al., 2004). So far, evidence for receptors of subtypes 5 and 3 for S1P and LPA, respectively, have been published. The signaling

Fig. 2. S1P and LPA elevates $[Ca^{2+}]_i$ in a dose-dependent mode. Single fura-2-loaded bovine chromaffin cells were stimulated with different concentrations of LPA and S1P puffed from a micropipette with an opening of 2 μ m positioned at a distance of 20 μ m from the recorded cell for 60 s. The $[Ca^{2+}]_i$ was presented as the ratio between the intensity of fluorescence emission excited by 340 and 380 nm. (A) Plotted are representative $[Ca^{2+}]_i$ recordings stimulated by S1P (5 μ M) and LPA (1 μ M) as indicated by the black bar. (B) Plotted are averaged maximal ratio changes stimulated by different concentrations of S1P and LPA. The curve was sigmoid fitted for IC_{50} . Data are mean $\pm$ SEM, $n = 7$ at least for each data point.

pathways for each receptor are different from each other; therefore, the expression profile of the LPL receptors can hint at the responses of these cells to LPLs. The result of RT-PCR suggests that there are at least two LPL receptors, LPA_1 and $S1P_3$, expressed in bovine chromaffin cells. Both receptors can

activate PLC to release IP_3 into the cytosol, it is possible that LPL may release Ca^{2+} from the intracellular Ca^{2+} stores to elevate the $[Ca^{2+}]_i$ (Schulz and Krause, 2004). Other subtypes of LPL receptors may be expressed but cannot be detected by

Fig. 3. Thapsigargin blocks the LPL-elicited elevation in $[Ca^{2+}]_i$. Chromaffin cells were pretreated with 1 μ M Tg for 30 min before the stimulation. The fura-2 fluorescence ratio were used to represent the $[Ca^{2+}]_i$. Plotted are the averaged maximal changes stimulated with either 5 μ M S1P or 1 μ M LPA in the presence (black bar) or absence (empty bar) of Tg for 60 s. (*) indicates Student's t -test $p < 0.05$. Data are presented as mean $\pm$ SEM.

Fig. 4. Blockers against IP_3 pathway inhibit the $[Ca^{2+}]_i$ elevation. Single chromaffin cells were stimulated with S1P (5 μ M, empty bar) and LPA (1 μ M, black bar) with or without blockers against IP_3 pathway. Plotted are the averaged maximal $[Ca^{2+}]_i$ changes in the presence or absence of blockers. (*) indicates Student's t -test $p < 0.05$ when comparing to control cells without blocker treatment. The digit in or beside each column is the number of cells tested. Data are presented as mean $\pm$ SEM.

Fig. 5. Ryanodine blocks repetitive S1P-elicited $[Ca^{2+}]_i$ elevation. A single bovine chromaffin cell was repetitively stimulated with LPLs for 60 s twice with an interval of 150 s in the presence or absence of ryanodine (20 μ M). The $[Ca^{2+}]_i$ was represented as the fluorescence ratio between the 340 and 380 nm excitation. (A) Plotted are traces from single chromaffin cells stimulated with S1P (5 μ M) or LPA (1 μ M) with or without ryanodine (Ry) as indicated by the black bar under each traces. (B) Plotted are the averages of the normalized ratio of the second LPL stimulation to the first stimulation in the presence (Ry, black bar) or absence (Control, empty bar) of ryanodine. (*) indicates Student's *t*-test $p < 0.05$; data are presented as mean $\pm$ SEM.

the primer sets we designed. These LPL receptors may modulate other important physiological activities in chromaffin cells.

Our results suggest that both S1P and LPA elevate the $[Ca^{2+}]_i$ in a dose-dependent mode with an EC_{50} of around 0.5 μ M. The elevation in $[Ca^{2+}]_i$ estimated by using standard solution (Life Technology, USA) for 10 μ M of LPA and 10 μ M of S1P were 166.4 ± 15.1 and 224.3 ± 40.0 μ M, respectively. It has been reported that the concentrations of LPLs in plasma can reach above micrometer level when platelet is activated (Kishimoto et al., 2003). Besides, the

Fig. 6. LPL stimulation elicits exocytosis. A single chromaffin cell was whole-cell voltage clamped at -70 mV and the membrane capacitance change during stimulation was recorded. (A) Plotted are representative capacitance traces from cells stimulated with a buffer containing only BSA (control), 5 μ M S1P, or 1 μ M LPA for 2 min as indicated by the black bar under each traces. (B) Plotted are the averaged maximal capacitance changes during the stimulation. Cells stimulated with LPA were divided into group1 (LPA1), and group2 (LPA2) showing decrease and increase in membrane capacitance, respectively. (*) indicates Student's *t*-test $p < 0.05$ when comparing to the control cells. Data are presented as mean $\pm$ SEM.

macrophage in the adrenal gland may release LPLs to stimulate the nearby chromaffin cells. Therefore, LPLs should be able to modulate the stimulus-secretion coupling in chromaffin cells under physiological conditions.

The role of LPL in releasing Ca^{2+} from intracellular Ca^{2+} stores mediated by the activation of PLC and IP_3 receptor has

Fig. 7. LPLs inhibit the inward currents. A single chromaffin cell was whole-cell patched and repetitively step depolarized from the holding potential of -70 mV to $+10$ mV for 20 ms with an interval of 15 s. The inward maximal peak current was recorded as the Na^+ current and the current between 12th and 18th ms during depolarization was averaged as the Ca^{2+} current. The cell was puffed with solutions containing BSA (BSA) only, S1P (5 μM), or LPA (1 μM) from a micropipette positioned at 20 μm from the cell for 2 min. (A) Plotted are the representative current traces from cells stimulated with BSA (left), S1P (middle), and LPA (right). 1 and 2 indicate the traces before and after the LPL stimulation, respectively. (B) Plotted are the normalized inward Na^+ and Ca^{2+} currents. The current recorded 2 min after the stimulation was normalized to the current before drug application. (*) indicates Student's t -test p value < 0.05 when comparing to cells without LPL treatment. Data are presented as mean $\pm$ SEM.

been reported (Holtsberg et al., 1997). Our results also support this pathway. Tg, U73122, and 2-APB can all suppress the LPL-elicited elevation in $[\text{Ca}^{2+}]_i$ suggesting that LPLs release Ca^{2+} from IP_3 receptor-gated Ca^{2+} stores. However, Ptx treatment can only partially inhibit this elevation suggesting that G_i and other G-proteins as well are involved in activating PLC. These reveal the importance of the IP_3 in LPL-elicited elevation in $[\text{Ca}^{2+}]_i$ and the involvement of G-protein coupled receptors in mediating the LPL signaling pathways in bovine chromaffin cell.

However, when the extracellular Ca^{2+} and Mg^{2+} was removed by EGTA, neither S1P nor LPA can induce any significant $[\text{Ca}^{2+}]_i$ elevation (data not shown). A similar result has been reported in LPA-elicited $[\text{Ca}^{2+}]_i$ elevation in rat hippocampus neurons (Holtsberg et al., 1997). It is possible that an initial Ca^{2+} influx from extracellular bath is required to trigger the release of Ca^{2+} from intracellular stores; and this

influx of Ca^{2+} is too small to be detected by fura-2 fluorescence when the intracellular Ca^{2+} stores are depleted by Tg. However, we did not find any significant inward current from whole-cell voltage clamped cell during LPL applications (data not shown). Therefore, it is unlikely that Ca^{2+} influx from extracellular solution is required for the elevation of $[\text{Ca}^{2+}]_i$. However, we cannot exclude the possibility that this current may not be able to be detected by the condition used in this report.

Another possibility is that the binding of LPLs to the receptors needs Ca^{2+} or Mg^{2+} as a cofactor for this activation. It has been reported recently that the curvature of the LPA can be influenced by the salt condition (Kooijman et al., 2005). Therefore, it is possible that LPL may need some divalent cations to acquire a specific conformation for receptor binding. Another possibility is that the maximal elevations in $[\text{Ca}^{2+}]_i$ elicited by S1P and LPA are not huge at about 200 nM; application of EGTA extracellularly may somehow pull the cytosol Ca^{2+} out of the cell and mask the elevation in $[\text{Ca}^{2+}]_i$ detected by fura-2 fluorescence. Physiologically, both Ca^{2+} and Mg^{2+} are present in the extracellular medium, therefore, Ca^{2+} release from intracellular Ca^{2+} stores should be much more important in elevating the $[\text{Ca}^{2+}]_i$ and eliciting exocytosis. Overall, the binding of LPLs to their receptors will activate the G_i and other G-proteins to activate the IP_3 pathway to release Ca^{2+} from the intracellular Ca^{2+} store in bovine chromaffin cells.

Though both S1P- and LPA-elicited elevations in $[\text{Ca}^{2+}]_i$ are mediated by the same pathway, the elevation profiles are different. S1P evokes a fast and sustaining elevation in $[\text{Ca}^{2+}]_i$ during the period of drug application; on the contrary, LPA works slowly in elevating the $[\text{Ca}^{2+}]_i$ and the $[\text{Ca}^{2+}]_i$ usually declines during LPA application. Other than IP_3 , CICR, which involves the opening of ryanodine receptor, is the other pathway to release Ca^{2+} from the intracellular stores. Ryanodine can lock the activated ryanodine receptor at the open state to dump all of the store content into the cytosol. Our results show that ryanodine can suppress the elevation in $[\text{Ca}^{2+}]_i$ elicited by repetitive S1P stimulations but not LPA. The second consecutive S1P stimulation is significantly suppressed in the presence of ryanodine suggesting that ryanodine receptor is opened during the first stimulation and then kept at open state by ryanodine. Then, these ryanodine receptor-gated Ca^{2+} stores are emptied and cannot provide Ca^{2+} for next stimulation. When second stimulation arrives, there will not be enough ryanodine-sensitive Ca^{2+} stores to maintain the $[\text{Ca}^{2+}]_i$ elevation. Therefore, CICR is involved in sustaining the S1P-, but not LPA-, elicited elevation in $[\text{Ca}^{2+}]_i$. However, the kinetics of IP_3 production should still be checked to understand the differential effects of LPA and S1P in chromaffin cells.

It is well known that regulated exocytosis need the Ca^{2+} as the trigger whether the Ca^{2+} comes from extracellular buffer or intracellular Ca^{2+} stores (Cuchillo-Ibanez et al., 2002). The differential kinetics of S1P and LPA in elevating the $[\text{Ca}^{2+}]_i$ may explain the difference in exocytosis. S1P can elicit exocytosis in all of the cells recorded; however, only

60% of the cells stimulated with LPA showed significant exocytosis. Using carbon fiber amperometry technique, we could detect the catecholamine release from a single chromaffin cell stimulated by S1P and LPA (unpublished result). This suggests that the increase in membrane capacitance is not just due to the insertion of the lipids onto the plasma membrane. These indicate that LPL treatments can elicit not only the $[Ca^{2+}]_i$ elevation but the catecholamine release as well.

Many G-protein coupled receptors have been shown to modulate the ion channels through the $\beta\gamma$ subunits (Dolphin, 2003). To further investigate how LPLs modulate the stimulus-secretion coupling, we examine the inward Na^+ and Ca^{2+} current. Our results showed that S1P and LPA can both inhibit inward currents, especially the inhibition induced by S1P. This inhibition may not come from the direct interaction between the ion channels and $\beta\gamma$ subunits because a strong prepulse cannot rescue the inhibition (unpublished result). LPA inhibits the Ca^{2+} current but not the Na^+ current; this again verifies the differential effect of LPA and S1P in modulating the physiological activities of chromaffin cells.

It has been reported that Ca^{2+} channels can be modulated by kinases, it is possible that S1P and LPA inhibit the Ca^{2+} and/or Na^+ currents through protein phosphorylation (Strock and Diverse-Pierluissi, 2004). The elevation of $[Ca^{2+}]_i$ can activate many Ca^{2+} -dependent protein kinases and phosphatase. How the ionic currents and the specific type of channels are modulated by lysophospholipids need further investigations. Another possibility to decrease the inward current is the activation of an outward current. Because of the Cs^+ in the pipette solution, most of the K^+ channels should have been blocked. Some reports have suggested that LPLs can activate the chloride channels and the influx of Cl^- will be an outward current for electrophysiological recording (Kim et al., 2000; Wang et al., 2002). However, how the inward currents are inhibited need to be further characterized.

Some factors secreted by immune cells modulating the stimulus-secretion coupling of chromaffin cells have been extensively studied. It has been reported that prostaglandin E_2 , one of the eicosanoids released by activated macrophage, can inhibit the Ca^{2+} channels on bovine chromaffin cells mediated by Ptx-sensitive G-protein (Currie et al., 2000). Low concentration of angiotensin II has been found to inhibit the Ca^{2+} current and exocytosis, this inhibition can be blocked by Ptx suggests the involvement of $G_{i/o}$. However, at high concentration of angiotensin II, another G-protein is activated to enhance the PKC activity and exocytosis (Teschmacher and Seward, 2000). These suggest that G-proteins are important in the regulation of stimulus-secretion coupling of chromaffin cells and those factors released by immune cells may modulate the neuroendocrine cells through G-protein pathways. However, the sequence for the complete set of LPL receptors in bovines has not been cloned yet; we are now trying to use degenerate primers to identify the types of LPL receptors expressed in chromaffin cell.

The catecholamine release from the medulla of the adrenal gland is the major factor responsible for fight-or-flight physiological response. Therefore, the rising of blood LPL

concentration may reflect the activation of platelet, which is an indicator of body damage, a sign of danger. Our results demonstrate that the LPL can raise $[Ca^{2+}]_i$ and elicit catecholamine secretion from bovine chromaffin cells. This hints that when a body is hurt, the adrenal medulla can be activated by LPL to release more catecholamine into the circulation system to modulate the physiological response, possibly even when people are in a coma. On the other hand, the release of catecholamine is accompanied by many other proteins now known to have a function as antibiotics which could facilitate the immunity (Metz-Boutigue et al., 2003). S1P and LPA work differentially in elevating the $[Ca^{2+}]_i$, inducing exocytosis, and inhibiting the inward currents. These support the interaction between immune and endocrine systems and the importance of LPLs in modulating long-term physiological effects.

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