Lysophospholipids Attenuate Acetylcholine-evoked Ca^{2+} Responses in Bovine Chromaffin Cells

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ABSTRACT: Adrenal medulla chromaffin cells are stimulated by acetylcholine released from sympathetic preganglionic neurons and secret catecholamines into the blood to accommodate short-term stress. Acetylcholine activates nicotinic and muscarinic receptors to elevate the intracellular Ca^{2+} concentration and causes the secretion of catecholamines. Lysophospholipids in the serum, including sphingosine 1-phosphate (S1P) and lysophosphatidic acid (LPA), are released primarily from immune cells during blood clotting and infection to modulate various physiological activities. To characterize the effects of lysophospholipids on Ca^{2+} responses in bovine chromaffin cells, cells were loaded with the Ca^{2+} indicator fura-2, and fluorescence intensity was used to monitor changes in intracellular Ca^{2+} concentration. Cells treated with 1 μM S1P or LPA for 0, 10, 20, 30, 40, 50, or 60 min were stimulated with acetylcholine (100 μM), muscarine (100 μM), DMPP (10 μM), or high K^+ buffer (50 mM KCl) for 5 sec. The results showed that the Ca^{2+} responses evoked by acetylcholine were significantly inhibited after incubation in LPA or S1P for 20 min. The Ca^{2+} response evoked by high K^+ buffer was not inhibited by S1P pretreatment and was significantly facilitated by LPA pretreatment. Muscarine-evoked Ca^{2+} responses were slightly attenuated by LPA and S1P pretreatments. Nicotine-evoked responses were inhibited in both LPA- and S1P-pretreated cells. Our findings demonstrate that nicotinic receptors may be the main targets of lysophospholipids in inhibiting acetylcholine-evoked Ca^{2+} responses.

KEY WORDS: Ca^{2+} channel, G-protein-coupled receptor, lysophosphatidic acid, muscarinic receptor, nicotinic receptor, sphingosine 1-phosphate.

Abbreviations: Ach: acetylcholine; AchR: acetylcholine receptor; $[Ca^{2+}]_i$: intracellular Ca^{2+} concentration; DMPP: dimethylphenylpiperazinium; LPA: lysophosphatidic acid; mAchR: muscarinic acetylcholine receptor; nAchR: nicotinic acetylcholine receptor; S1P: sphingosine 1-phosphate; LPL: lysophospholipid; VGCC: voltage-gated Ca^{2+} channel.

INTRODUCTION

Adrenal chromaffin cells are the neuroendocrine cells that respond to acetylcholine (Ach) released from presynaptic splanchnic nerves. Stimulated chromaffin cells release catecholamines into the blood stream and play an important role in urgent conditions such as the fight-or-flight response (Boarder et al., 1987; Douglas and Poisner, 1962; Douglas and Rubin, 1961). According to pharmacological studies, Ach receptors (AchRs) can be classified as ionotropic nicotinic AchRs (nAchRs) or metabotropic muscarinic AchRs (mAchRs)(Albuquerque et al., 2009; Olivos and Artalejo, 2008).

The activation of nAchRs opens non-selective cationic channels and depolarizes the membrane potential (Sala et al., 2008). This depolarization activates voltage-gated Ca^{2+} channels (VGCCs) to raise the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). On the other hand, mAchRs are G-protein-coupled receptors that induce the release of Ca^{2+} from intracellular Ca^{2+} stores to elevate $[Ca^{2+}]_i$ (Olivos and Artalejo, 2008). Therefore, the activation of both receptor types by Ach leads to an elevation in $[Ca^{2+}]_i$ and triggers exocytosis to release catecholamines into the milieu.

In rat and human adrenal glands, a population of macrophages is distributed throughout the cortex and medulla (Schober et al., 1998). This suggests that chromaffin cells can be modulated by immune cells in a paracrine signaling pathway. It has also been shown that a factor with a molecular weight less than 3,000 released by mononuclear cells can stimulate adrenal medulla cells to secret epinephrine at a level comparable to the maximal cholinergic response (Roberts et al., 1996). The release of this factor by macrophages can be feedback-inhibited by the released epinephrine. These results suggest interactions between the immune and neuroendocrine systems.

Two bioactive lysophospholipids (LPLs), lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P), are released by many immune cells during blood clotting and can reach micromolar levels in the plasma (Kishimoto et al., 2003; Xiao et al., 2000). These lipids work as signals to other cells in circulation to evoke many physiological responses. However, most of the studies on the functions of LPA and S1P focus on

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atherosclerosis and wound healing (Milstien et al., 2007; Panetti, 2002). Their roles in modulating excitable endocrine cells are unclear.

The activation of both nAchRs and mAchRs leads to elevations in $[Ca^{2+}]_i$. The aim of this study was to characterize how S1P and LPA modulate the Ca^{2+} response evoked by Ach. Our results show that the Ca^{2+} responses evoked by Ach were inhibited by LPL treatment and that nAchRs are likely the main targets responsible for this inhibition.

MATERIALS AND METHODS

Chemicals

Oleoyl-L-lysophosphatidic acid (LPA, C18:1, 1-oleoyl-*sn*-glycerol-3-phosphate), D-erythro-sphingosine-1-phosphate (S1P), acetylcholine, muscarine, and dimethylphenylpiperazinium iodide (DMPP) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium and all other reagents for cell culture were from Invitrogen Inc. (Carlsbad, CA, USA). Fura-2 acetomethoxylmethyl ester (Fura-2 AM) was from TefLab (Austin, TX, USA). All other chemicals were reagent grade from Sigma-Aldrich Inc. (St. Louis, MO, USA), unless otherwise indicated.

Cell preparation

Chromaffin cells were prepared by digesting bovine adrenal glands obtained from local slaughterhouses with collagenase (0.5 mg/mL), and purification was accomplished through density gradient centrifugation, as described previously (Pan et al., 2002). The cells were plated at a density of 2 x 10^5 on one 22-mm or three 10-mm poly-L-lysine-coated coverslips in a 35-mm culture dish and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The medium was replaced every two days. All experiments were carried out between days 3 and 10 after cell isolation.

Solutions

The bath buffer used for calcium imaging 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. High K^+ buffer contained (in mM): 145 KCl, 5 glucose, 10 Na-HEPES, 1 $MgCl_2$, 5 KCl, and 2.2 $CaCl_2$, pH 7.3 with NaOH. S1P and LPA were dissolved in chloroform:methanol/1:19 solution to a concentration of 1 mM. The solution was then air-dried and redissolved in ethanol to generate a 1 mM stock, which was stored at -20℃.

Calcium imaging

For $[Ca^{2+}]_i$ measurement, cells were incubated in bath buffer with 5 μM fura-2 AM for 1 hour at 37 °C. Cells were then washed three times with bath buffer and used for measurements. For fura-2 excitation, a 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 using 10-nm-wide filters centered at 340 and 380 nm (Semrock Inc., Rochester, NY, USA), capturing the emitted light (510-540 nm) at each excitation wavelength for 300 ms through a 20 x objective (Axiovert 200, Zeiss Inc., Germany) and directing it to a cooled CCD camera (CoolsnapFx; Roper Scientific, Tucson, AZ, USA). The ratio within each cell was computed from images obtained at 340 and 380 nm excitation wavelengths, subtracting the background fluorescence at each wavelength. Ratios were acquired every second.

To stimulate the cells, a glass micropipette with 2-μm opening was filled with buffer containing different stimulants and positioned 20 μm from the cell. The stimulants were 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).

Data analysis. To calculate the changes in ratios, the ratios before stimulation were averaged as the basal level and subtracted from the maximal ratio value after stimulation in each single cell. The results were analyzed by one-way ANOVA and considered statistically different when the p value was less than 0.05. To plot the traces of time-dependent changes in fura-2 ratios, the ratios from each cell of the same group were averaged. Please note that the maximal elevation in fura-2 ratios in each cell did not occur at the same time after stimulation; therefore, the changes in ratios calculated are higher than the differences observed in the averaged traces.

RESULTS

LPLs inhibit Ach-evoked Ca^{2+} responses

To measure changes in $[Ca^{2+}]_i$, cells were loaded with the Ca^{2+} indicator fluorescent dye fura-2. When bound with Ca^{2+}, fluorescence emissions excited at 340 and 380 nm increase and decrease, respectively. Therefore, the ratios of emissions excited at 340 and 380 nm reflect changes in Ca^{2+} concentration (Tsien et al., 1985). To characterize the effects of LPLs on Ach-induced $[Ca^{2+}]_i$ responses, cells were stimulated with Ach (100 μM) after being incubated in 1 μM LPLs for varying amounts of time. Figs. 1A & B show the averaged Ca^{2+} response curves of cells treated with S1P and LPA, respectively, for 0, 10, 20, and 40 min. $[Ca^{2+}]_i$

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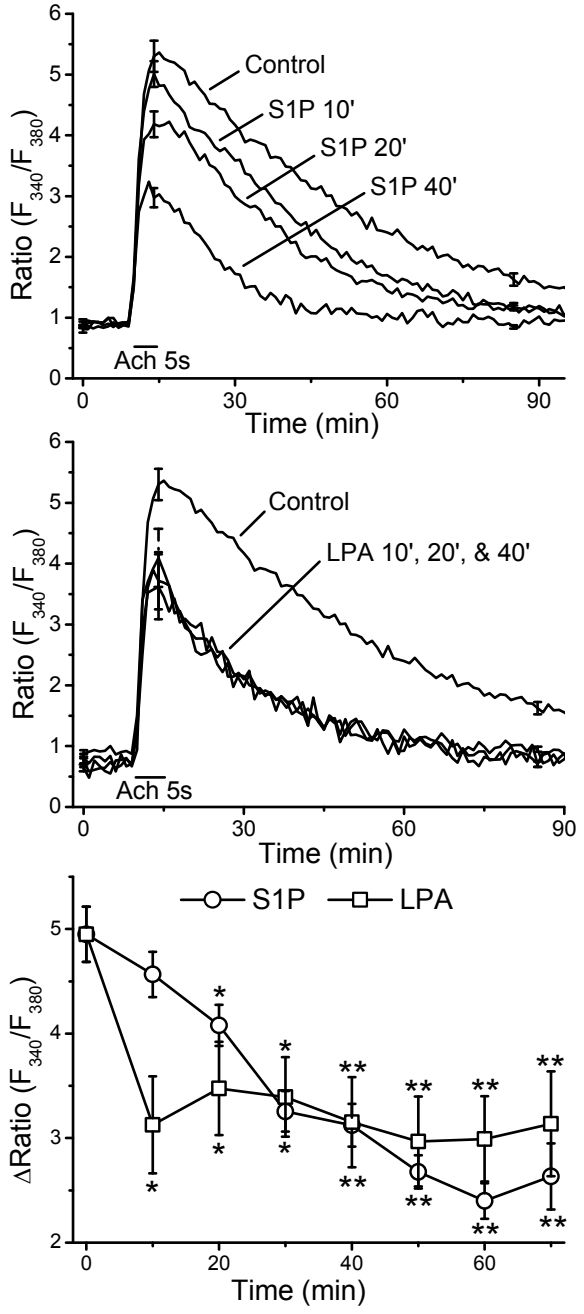


Fig. 1. Ach-evoked Ca^{2+} responses. Cultured bovine chromaffin cells were loaded with fura-2 and fluorescence ratios excited at 340 and 380 nm were recorded. Cells treated with 1 M S1P or LPA for varying amounts of time were stimulated with Ach for 5 sec as indicated. A. & B. plot the average Ca^{2+} response curves from cells treated with S1P and LPA, respectively, for 0, 10, 20, and 40 min. C. The average maximal changes in Ca^{2+} responses after subtracting the basal level before stimulation. The sample numbers for 0, 10, 20, 30, 40, 50, 60, and 70 min were 46, 43, 40, 37, 43, 44, 20, and 10, respectively, for S1P treatment; and 46, 5, 5, 5, 5, 5, 5, and 5, respectively, for LPA treatment. One-way ANOVA test * $p < 0.05$ and ** $p < 0.01$.

was elevated in response to Ach stimulation and then slowly declined to the basal level. For S1P treatment, the inhibition was enhanced as the incubation time increased. For LPA treatment, the maximal $[Ca^{2+}]_i$ responses were all inhibited to approximately the same level regardless of the length of the incubation time. The average changes in peak ratios after subtracting the basal level from each cell are plotted in Fig 1C. Without LPL treatment, the change in fura-2 ratio was 4.95 ± 0.26. The changes were reduced to 4.57 ± 0.22 and 3.12 ± 0.21 after incubations with S1P for 10 and 40 min, respectively. After 10 and 40 min of LPA treatment, the changes were reduced to 3.12 ± 0.46 and 3.15 ± 0.43, respectively. These results suggest that both S1P and LPA attenuate the Ach-evoked $[Ca^{2+}]_i$ response within 20 minutes, and this inhibition can persist for more than 70 minutes.

LPLs do not inhibit the Ca^{2+} responses evoked by membrane depolarization

It is known that Ach stimulation depolarizes chromaffin cells and opens VGCCs to elevate $[Ca^{2+}]_i$. To characterize the effects of LPLs on VGCC-evoked Ca^{2+} responses, cells were depolarized using a high K^+ solution to directly activate VGCCs. Figs. 2A & B shows representative high K^+-evoked Ca^{2+} response curves from cells incubated with S1P and LPA, respectively, for 0, 10, 20, and 40 min. The peak $[Ca^{2+}]_i$ levels were approximately the same in cells treated with S1P and control cells; however, the $[Ca^{2+}]_i$ levels were higher in cells treated with LPA than in control cells. Fig. 2C shows that the average maximal change in Ca^{2+} response was 5.63 ± 0.35 in cells without treatment. The figure further shows that 10 and 40 min after S1P treatment, the changes were reduced to 5.25 ± 0.21 and 4.91 ± 0.23, respectively; however, these decreases were not significant. In contrast, 10 min after LPA treatment, the change was significantly increased to 7.40 ± 0.47. This enhancement was progressively reduced to 6.50 ± 0.39 after incubation with LPA for 40 min. These results suggest that LPLs do not inhibit Ca^{2+} responses by directly activating VGCCs. On the contrary, $[Ca^{2+}]_i$ elevations are enhanced in LPA-treated cells.

Muscarine-induced $[Ca^{2+}]_i$ responses are inhibited by LPL treatment

Activation of mAchRs releases Ca^{2+} from intracellular Ca^{2+} stores and elevates $[Ca^{2+}]_i$. Figs. 3A & B shows the average Ca^{2+} response curves evoked by muscarine (100 μM) for cells treated with S1P and LPA, respectively, for 0, 10, 20, and 40 min. The peak Ca^{2+} responses evoked by muscarine were smaller than those evoked by Ach. Without treatment, the change in fura-2 ratio was 2.63 ± 0.25, which was reduced to 2.35

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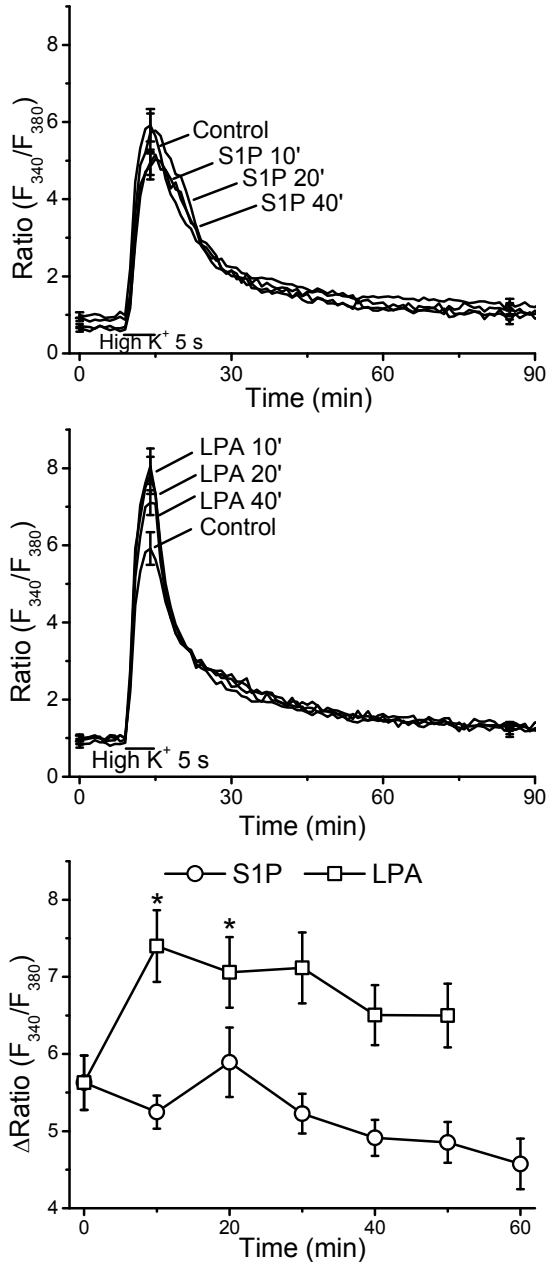


Fig. 2. Ca^{2+} responses stimulated with high K^+ buffer. Bovine chromaffin cells were loaded with fura-2 and the ratios of fluorescence intensities excited at 340 and 380 nm were recorded to represent the $[Ca^{2+}]_i$. Cells treated with 1 M S1P or LPA for varying amounts of time were stimulated with high K^+ buffer (50 mM) for 5 sec as indicated. A. & B. The average Ca^{2+} response curves from cells treated with S1P and LPA, respectively, for 0, 10, 20, and 40 min. C. The average maximal changes in Ca^{2+} responses after subtracting the basal level before stimulation. The sample numbers for 0, 10, 20, 30, 40, 50, and 60 min were 36, 22, 22, 21, 20, 20, and 13, respectively, for S1P treatment; and 36, 12, 12, 12, 12, 12, and 12, respectively, for LPA treatment. One-way ANOVA test * p < 0.05.

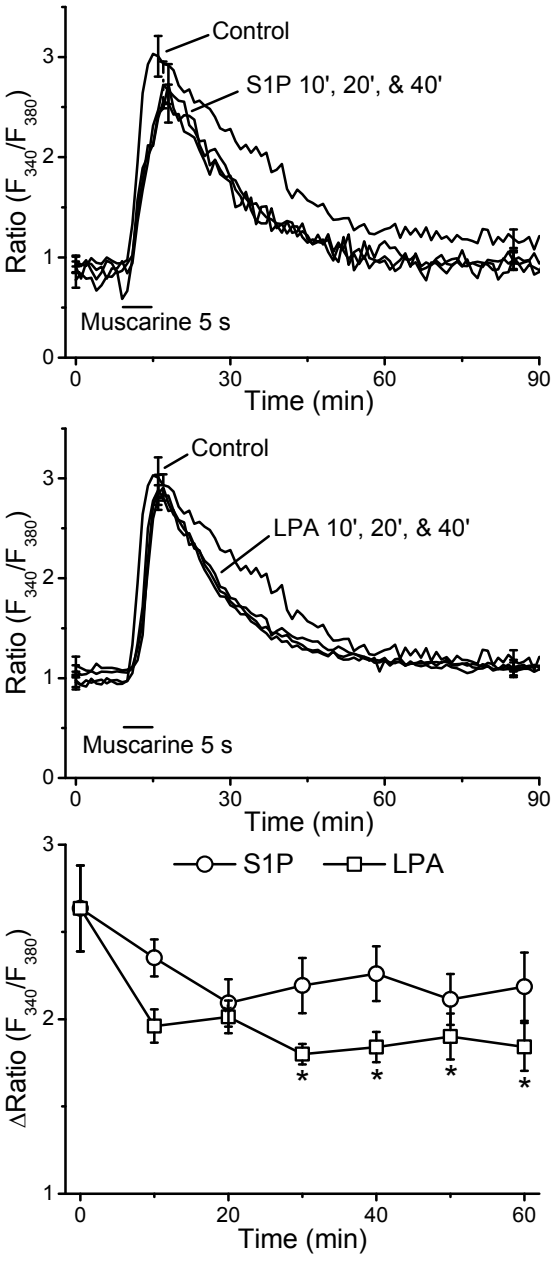


Fig. 3. Ca^{2+} responses stimulated by muscarine. Bovine chromaffin cells were loaded with fura-2 and the ratios of fluorescence intensities excited at 340 and 380 nm were recorded to represent the $[Ca^{2+}]_i$. Cells treated with 1 M S1P or LPA for varying amounts of time were stimulated with muscarine (100 M) for 5 sec as indicated. A. & B. plot the average Ca^{2+} response curves from cells treated with S1P and LPA, respectively, for 0, 10, 20, and 40 min. C. The average maximal changes in Ca^{2+} responses after subtracting the basal level before stimulation. The sample numbers for 0, 10, 20, 30, 40, 50, and 60 min were 36, 22, 22, 21, 20, 20, and 13, respectively, for S1P treatment; and 36, 12, 12, 12, 12, 12, and 12, respectively, for LPA treatment. One-way ANOVA test * p < 0.05.

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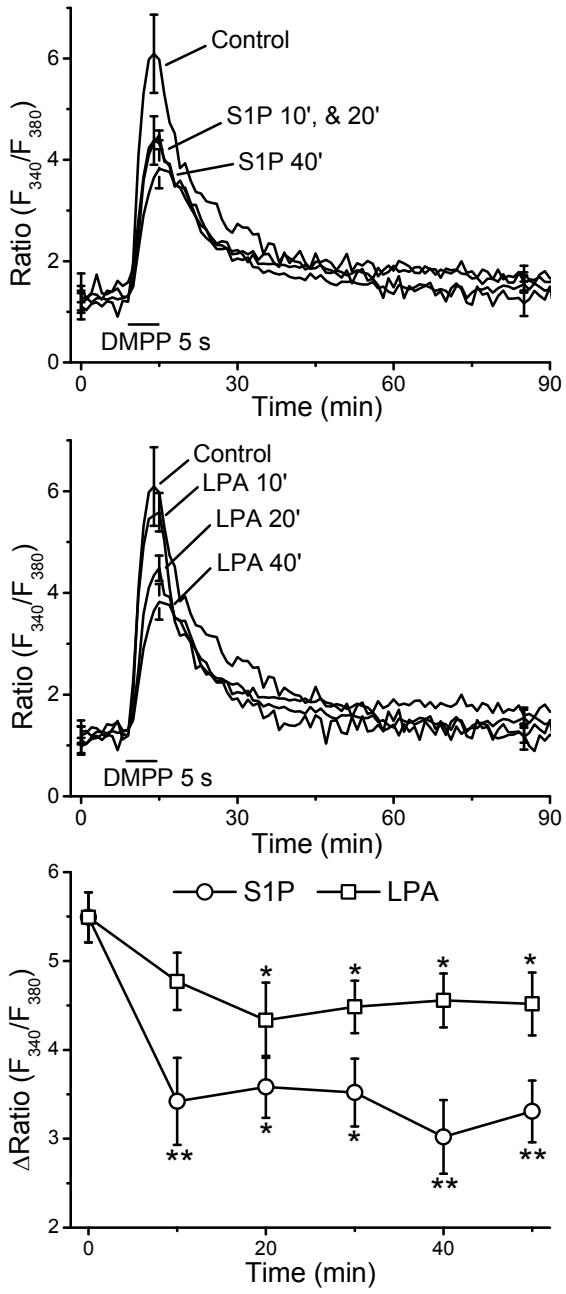


Fig. 4. DMPP-evoked Ca^{2+} responses. Bovine chromaffin cells were loaded with fura-2 and the ratios of fluorescence intensities excited at 340 and 380 nm were recorded to represent the $[Ca^{2+}]_i$. Cells treated with 1 M S1P or LPA for varying amounts of time were stimulated with DMPP (10 M) for 5 sec as indicated. A. & B. plotted are the average Ca^{2+} response curves from cells treated with S1P and LPA, respectively, for 0, 10, 20, and 40 min. C. The average maximal changes in Ca^{2+} responses after subtracting the basal level before stimulation. The sample numbers for 0, 10, 20, 30, 40, and 50 min were 32, 11, 10, 10, 10, and 9, respectively, for S1P treatment; and 32, 13, 15, 13, 12, and 13, respectively, for LPA treatment. One-way ANOVA test * $p < 0.05$ and ** $p < 0.01$.

± 0.11 and 2.26 ± 0.16 after being treated with S1P for 10 and 40 min, respectively. Similarly, muscarine-evoked Ca^{2+} responses were inhibited to 1.96± 0.09 and 1.83 ± 0.09 in cells treated with LPA for 10 and 40 min, respectively. These results indicate that mAchR-evoked Ca^{2+} responses are inhibited by LPLs.

LPLs inhibit DMPP-evoked Ca^{2+} responses

Activation of nAchRs leads to membrane depolarization and VGCC activation. Figs. 4A & B shows representative Ca^{2+} response curves evoked by DMPP (10 μM) for cells treated with S1P and LPA, respectively, for 0, 10, 20, and 40 min. The peak ratios were inhibited by S1P and LPA treatments. Fig. 4C shows the average changes in Ca^{2+} responses to DMPP stimulation. The change in fura-2 ratio was 5.49 ± 0.28 for control cells, and was reduced to 3.42 ± 0.49 and 3.02 ± 0.41 after being incubated with S1P for 10 and 40 min, respectively. LPA also inhibited the changes to 4.77 ± 0.32 and 4.56 ± 0.30 after incubation for 10 and 40 min, respectively. These data reveal that the Ca^{2+} responses mediated by nAchRs are attenuated by LPLs.

DISCUSSION

The adrenal medulla is stimulated by Ach released from preganglionic sympathetic neurons to accommodate short-term stress (Kvetnansky et al., 2009). Our previous results have shown that LPLs modulate Ca^{2+} currents, exocytosis and action potential firing patterns (Pan et al., 2006; Pan et al., 2007). In the current report, we showed that Ca^{2+} responses evoked by Ach were significantly inhibited by LPL treatments. Ach stimulation releases Ca^{2+} from intracellular Ca^{2+} stores and depolarizes cells to activate the VGCC. The high K^+- and muscarine-evoked Ca^{2+} responses were not inhibited in LPL-treated cells. In contrast, DMPP-evoked Ca^{2+} responses were inhibited by S1P and LPA at levels similar to those stimulated by Ach. These results suggest that the S1P- and LPA-induced inhibitions of Ach-evoked Ca^{2+} responses may be primarily mediated by nAchRs.

Both muscarine and LPLs elevate $[Ca^{2+}]_i$ via a similar mechanism, activating G-protein-coupled receptors to release Ca^{2+} from intracellular Ca^{2+} stores (Felder, 1995; Mutoh and Chun, 2008). Our results showed that the peak $[Ca^{2+}]_i$ responses evoked by muscarine are slightly inhibited in LPL-treated cells. Furthermore, the declining phases of LPL-treated cells were faster than those of control cells. It is possible that a portion of the intracellular Ca^{2+} stores was depleted by LPL pretreatments, diminishing the following Ca^{2+} responses evoked by muscarine.

Elevations in $[Ca^{2+}]_i$ resulting from activation of VGCCs are not inhibited by LPL treatments; they are

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actually significantly increased in LPA-treated cells. Our previous reports have shown that LPLs inhibit Ca^{2+} currents (Pan et al., 2006; Pan et al., 2007), suggesting that the Ca^{2+} responses evoked by high K^+ buffer should be inhibited. However, considering that the buffering capacity of intracellular Ca^{2+} stores is diminished by LPL, it is possible that the inability of the intracellular Ca^{2+} stores to sequester Ca^{2+} compensates for the attenuated Ca^{2+} currents and enhances the $[Ca^{2+}]_i$ measured. Furthermore, the $[Ca^{2+}]_i$ measured by fura-2 represents an averaged concentration throughout the entire cell, not the local concentration where Ca^{2+} flows into the cell. Due to its temporal and spatial resolution, fura-2 measurement cannot reflect changes in Ca^{2+} currents (Paredes et al., 2008). Therefore, the Ca^{2+} influx remains large enough to bring the $[Ca^{2+}]_i$ to a level similar to control cells even when Ca^{2+} currents are inhibited.

By stimulating LPL-treated cells with the nAchR agonist DMPP, Ca^{2+} responses are inhibited to a level similar to that stimulated by Ach. These results indicate that nAchRs may be responsible for the LPL-induced inhibition. The activation of nAchRs opens the linked non-selective cation channels and depolarizes the membrane potential (Cherdchu et al., 1987; Nooney and Feltz, 1995). In future research, we will investigate the effects of LPLs in modulating the nAchR-evoked cationic currents.

When immune cells are activated during blood clotting or infection, S1P and LPA in the serum are elevated to modulate many physiological activities. On the other hand, chromogranin A and catecholamines released from adrenal chromaffin cells have been suggested to modulate the development of immune cells (Perez et al., 2009; Zhang et al., 2009). Our results highlight the interaction between the immune and endocrine systems, which is vital to understanding how an organism responds to environmental stresses.

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摘要：交感神經節前神經分泌乙醯膽鹼，刺激腎上腺嗜鉻細胞將兒茶酚胺釋放到血液中，以反應短期壓力。乙醯膽鹼活化尼古丁及蕈毒鹼受器，提高胞內鈣離子濃度 ($[Ca^{2+}]_i$) 因而釋放兒茶酚胺。而在血液中，由免疫細胞所分泌的水解磷酸脂 (lysophospholipids, LPLs)，包括鞘胺醇 1-磷酸鹽 (sphingosine 1-phosphate, S1P) 及溶血磷脂酸 (lysophosphatidic acid, LPA)，則會引起許多不同的生理反應。為探討 LPLs 對嗜鉻細胞$[Ca^{2+}]_i$ 變化的效應，細胞先以鈣離子指示劑 Fura-2 標定，以觀察 $[Ca^{2+}]_i$ 的變化。再以 1 μM S1P 或 LPA 處理 0、10、20、30、40、50、或 60 分鐘後，以乙醯膽鹼 (100 μM)、蕈毒鹼 (100 μM)、DMPP (10 μM)、或高鉀溶液 (50 mM KCl) 刺激 5 秒。結果顯示在 LPA 或 S1P 處理 20 分鐘後，乙醯膽鹼所引起的 $[Ca^{2+}]_i$ 上升，受到明顯的抑制。然而高鉀溶液所引起的 $[Ca^{2+}]_i$ 上升，並不會受到 S1P 前處理的影響，但會被 LPA 所增加。蕈毒鹼刺激所引起的反應，僅會受 LPLs 稍微抑制；然而以 DMPP 活化尼苦丁受器所引起的 $[Ca^{2+}]_i$ 反應，則會受到 LPA 及 S1P 的顯著抑制。這些結果顯示，LPLs 可能主要是透過抑制尼古丁受器，而達到抑制乙醯膽鹼所引起的 $[Ca^{2+}]_i$ 上升。

關鍵詞：鈣離子通道、G 蛋白連結受器、水解磷酸脂、蕈毒鹼受器、尼古丁受器、鞘胺醇 1-磷酸鹽。

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