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Effects of LPC on IK(ACh) and ICa


[Original Article]

Differential effects of lysophosphatidylcholine and ACh on muscarinic K⁺, non-selective cation and Ca²⁺ currents in guinea-pig atrial cells

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Abstract
We compared the effects of lysophosphatidylcholine (LPC) and acetylcholine (ACh) on I_K(ACh), I_Ca and a non-selective cation current (I_NSC) in guinea-pig atrial myocytes to clarify whether LPC and ACh activate similar G_i/o-coupled effector systems. I_K(ACh), I_Ca and I_NSC were analyzed in single atrial myocytes by the whole cell patch-clamp. LPC induced I_NSC in a concentration-dependent manner in atrial cells. ACh activated I_K(ACh), but failed to evoke I_NSC. LPC also activated I_K(ACh) but with significantly less potency than ACh. The effects of both ligands on I_K(ACh) were inhibited by intracellular loading of pre-activated PTX. This treatment also inhibited LPC-induced I_NSC, indicating that I_K(ACh) and I_NSC induced by LPC are both mediated by G_i/o. LPC and ACh had similar potencies in inhibiting I_Ca, which was pre-augmented by forskolin, indicating that LPC and ACh activate similar amounts of α-subunits of G_i/o. The different effects of LPC and ACh on I_K(ACh) and I_NSC may suggest that LPC and ACh activate G_i/o having different types of βγ subunits, and that LPC-induced I_NSC may be mediated by βγ subunits of G_i/o, which are less effective in inducing I_K(ACh).

Key words : lysophosphatidylcholine (LPC), Acetycholine-activated K⁺ current (I_K(ACh)), non-selective cation current (I_NSC), G_i/o protein, Ca²⁺ current

Introduction
Lysophosphatidylcholine (LPC) is an intracellular phospholipid metabolite that accumulates in the heart during ischemia and triggers lethal arrhythmia. LPC is also a major component of oxidized low-density lipoprotein and has been proposed to play a role in the development of atherosclerotic lesions by stimulating endothelial cells, smooth muscles and blood cells. LPC induces a non-selective cation current (I_NSC) in guinea-pig cardiac ventricular cells and in human vascular endothelial cells. Recently, we found that LPC-induced I_NSC was mediated by activation of the pertussis toxin (PTX)-sensitive heterotrimeric G protein (G_i/o) as well as HMG-CoA reductase-dependent activation of the small GTP-binding protein, Rho.

In atrial cells, the cholinergic M₂ receptor is coupled with G_i/o and stimulation of this receptor with acetylcholine (ACh) induces an inwardly rectifying K⁺ current (I_K(ACh)). This effect on I_K(ACh) is mediated by βγ subunits dissociated from α-subunits of G_i/o. On the other hand, α-subunits of G_i/o inhibit pre-activated adenylyl cyclase, thereby decreasing cyclic AMP-dependent responses. This effect is known as 'accentuated antagonism' and can be demonstrated by the ACh inhibition of L-type Ca²⁺ current (I_Ca) pre-augmented by β₁-receptor stimul-
Therefore, if LPC activates $G_{lo}$, it may also activate $I_k(ACh)$ and inhibit $I_{Ca}$ pre-activated by forskolin-induced cyclic AMP accumulation. In this study, using atrial cells, we compared the effects of LPC and ACh on $I_k(ACh)$ and $I_{Ca}$ as well as $I_{NSC}$ to examine whether the activation of $G_{lo}$-mediated pathways by LPC and ACh utilize similar effector systems.

Materials and methods

Cell isolation

All experiments were performed in accordance with the regulations of the Animal Research Committee of Fukushima Medical University. Male guinea pigs weighing 250 to 400 g were anesthetized by intraperitoneal injection of 250 mg/kg sodium pentobarbital with 0.5 U/g heparin. The heart was removed and mounted in a Langendorff apparatus while perfusing with Tyrode solution and then with Ca$^{2+}$-free Tyrode solution. After the heart stopped beating, Ca$^{2+}$-free Tyrode solution containing 5~8 mg/50 ml collagenase (Wako, Tokyo, Japan) and protease (0.01 mg/50 ml, Nagase, Tokyo, Japan) was perfused for about 15 min. Then the solution was changed to a low Cl$^-$/high K$^+$ (KB or Kraftsbrühe) solution for 3 minutes. The atria were removed and shaken in KB solution to dissociate the cells. The cells were kept in KB solution at 4ºC. Solutions

Tyrode solution contained 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl$_2$, 1 mM MgCl$_2$, 0.33 mM NaH$_2$PO$_4$, 5.5 mM glucose and 5 mM HEPES (pH 7.4 with NaOH). The low Cl$^-$/high K$^+$ solution (KB solution) contained 70 mM KOH, 50 mM l-glutamic acid, 40 mM KCl, 20 mM KH$_2$PO$_4$, 3 mM MgCl$_2$, 0.2 mM EGTA, 20 mM taurine, 10 mM glucose and 10 mM HEPES (pH 7.2 with KOH). The pipette solution for measuring $K^+$ currents contained 20 mM KCl, 120 mM KOH, 60 mM aspartic acid, 5 mM MgATP, 3 mM MgCl$_2$, 20 mM BAPTA and 10 mM HEPES (pH 7.2 with aspartic acid). To measure $K^+$ current, 5 µM nifedipine was added to the Tyrode solution to block $I_{Ca}$. The pipette solution for measuring $I_{Ca}$ contained 130 mM CsCl, 20 mM TEA-CI, 5 mM EGTA, 5 mM MgATP, 0.1 mM TrisGTP and 10 mM HEPES (pH 7.2). The external solution for measuring $I_{Ca}$ contained 5.4 mM CsCl instead of KCl in the Tyrode solution to inhibit $I_{Ks}$.

Current recording

The whole-cell voltage clamp method was employed using pCLAMP8 software (Axon Instruments, Foster City, CA, USA). Patch pipettes were fabricated with a two-stage microelectrode puller (pp83, Narishige, Tokyo, Japan) to form a tip diameter less than 1.5 µm, which had a resistance of 2~3 MΩ when filled with the pipette solution. The patch–clamp amplifier was Model TM-1000 (Act ME, Tokyo). Current signals were filtered by low-pass 2.5 kHz. The temperature of the bath solution was kept constant at 35~36°C.

To record $K^+$ current, ramp pulses of 480 msec duration were given at 10 sec intervals. The ramp pulse was initially depolarized from the holding potential of −40 mV to 60 mV, then hyperpolarized to −120 mV and depolarized back to the holding potential at a speed of 750 mV sec$^{-1}$. The $I_{Ca}$ was recorded by a step pulse of 200 msec duration from the holding potential of −40 mV to 10 mV every 10 sec.

Drugs

Acetylcholine (Daichi pharmaceutical co., Ltd., Tokyo, Japan) was dissolved in distilled water to make a 1 mM stock solution and was stored at −20°C. LPC (L-1-palmitoyl-lysophosphatidylcholine, C16 : 0) (Sigma, St. Louis, Mo., USA) was dissolved in methanol to make a 10 mM stock solution which was stored at −20°C. Fluvastatin was a kind gift from Novartis (Basel, Switzerland). Forskolin (Sigma, St. Louis, USA) was dissolved in DMSO to make a 10 mM stock solution and was stored at −20°C. Pertussis toxin (PTX) (Sigma, St. Louis, USA) (50 µg) was pre-activated by mixing with 5 mM dithiothreitol and incubating at 37°C for 15~20 min in 5 ml of the pipette solution$^{12)}$. The solution was diluted to 50 ml with the pipette solution to make a final concentration of pre-activated pertussis toxin, A-proteme, of 1 µg/ml. Anti-G protein β subunit (Gβ) antibody (SA-125) was obtained from BIOMOL (Plymouth Meeting, PA, USA). For intracellular loading of the anti-Gβ antibody, the patch pipettes were filled with a pipette solution containing 1 : 1,000 diluted anti-Gβ antibody. All the chemicals used were the highest grade available.

Data analysis

The data were expressed as means±S.E.M. (number of cells) and analyzed by the Student’s t test. Differences with $p<0.05$ were considered significant.
Results

Effects of LPC on membrane current in atrial myocytes

Figure 1A shows representative current traces in response to 5 μM LPC added to the external solution. Soon after the application of LPC, the current increased slightly, and a marked increase appeared after about 2 min. Fig. 1B shows I-V curves obtained at the corresponding labels in Fig. 1A. The LPC–induced initial increase of the current crossed with the control current at about −70 mV (Fig. 1B a and b), indicating that the increased component was K⁺ current. The subsequent large LPC–induced current (c) crossed with the control (a) near 0 mV, suggesting that it was an I_{NSC}. Fig. 1C shows the concentration–response relationship between LPC and the I_{NSC}. The EC₅₀ of LPC was 1.8 μM. There was a lag between the LPC application and the I_{NSC} development and the lag was shorter at higher concentrations of LPC (Fig. 1D). These results indicate that LPC induces I_{NSC} in atrial cells in a manner similar to that we reported previously in ventricular myocytes.

Effect of LPC on I_{K(ACh)}

In the above experiment, a small inwardly rectifying K⁺ current was activated by LPC (Fig. 1 A-b, B-b). This would be an I_{K(ACh)} because LPC activates G_i/o in ventricular cells and the I_{K(ACh)} is activated by the βγ subunit of G_i/o in atrial cells. To test this, we compared the effects of ACh and LPC on I_{K(ACh)}. In this set of experiments, LPC was applied for a short period of time of 30–60 sec to avoid the development of I_{NSC}. When 1 μM ACh was added to the bath solution, I_{K(ACh)} was activated immediately (Fig. 2A). After washing out the ACh, application of 5 μM LPC induced a small inward current. I-V curves obtained with the control (a) and in the presence of 1 μM ACh (b) are superimposed in the left panel of Fig. 2B and those from another control (c) and in the presence of LPC (d) in the right panel.

Fig. 1. Effects of LPC on membrane current of an atrial cell.

A, current recording versus time. Ramp pulse was given every 10 sec. LPC at 5 μM increased current slightly soon after application and significantly after a delay. B, I-V curves obtained at the corresponding labels in A. C, the concentration–response curve of a nonselective cation current (I_{NSC}) induced by LPC. The net I_{NSC} was obtained by subtraction (c−b) and the current density was calculated at −100 mV. The EC₅₀ value of LPC was 1.8 μM. D, the relation between LPC concentrations and delay time before LPC began to induce I_{NSC}.
of Fig. 2B. The net ACh-induced current (b-a) and the LPC-induced current (d-c) are superimposed in Fig. 2C. The average reversal potential of ACh-induced $I_{K(ACh)}$ was $-70 \pm 3.5$ mV ($n=4$) and that of LPC-induced current was $-73 \pm 5.5$ mV ($n=4$), values which were almost identical. The I-V curves of the LPC-induced current showed an inwardly rectifying property, supporting the view that LPC activated $I_{K(ACh)}$.

Fig. 2D shows the relations between the current magnitude and the concentrations of ACh and LPC. The current density was measured at $-100$ mV. The $K^+$ current induced by LPC was six times smaller than that induced by ACh, both at a concentration of $1 \mu$M. This difference was not affected by reversing the order of application of ACh and LPC. Unlike LPC, ACh alone did not induce

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Fig. 2. Effect of LPC on $I_{K(ACh)}$.  
A, Chart recording of currents. Applications of ACh and LPC are indicated by horizontal bars at the top of the figure. B, I-V curves obtained at the currents indicated by a~d in A. C, Difference I-V curves between control and peak with ACh (b-a), and in the absence and presence of LPC (d-c). The shape of the two I-V curves and the reversal potentials of b-a and d-c are similar. D, Concentration-response curves of ACh- (triangles)- and LPC- (circles) induced $I_{K(ACh)}$. The $I_{K(ACh)}$ densities were measured at $-100$ mV.
Effects of LPC on \(I_{K(ACh)}\) and \(I_{Ca}\)

I\(_{NSC}\) during a prolonged application for 10 min (data not shown).

**Effect of PTX on LPC-induced \(I_{K(ACh)}\)**

Activation of \(I_{K(ACh)}\) by ACh is mediated by G\(_{i/o}\). We demonstrated previously that LPC-induced I\(_{NSC}\) is also mediated by G\(_{i/o}\) in cardiac ventricular cells\(^9\). Therefore, we tested whether the effect of LPC on the induction of \(I_{K(ACh)}\) is also mediated by G\(_{i/o}\). We added pre-activated PTX (1 \(\mu\)g/ml) in the pipette solution and allowed it to diffuse into the cell for 8~10 min. After confirming that PTX inhibited ACh-induced \(I_{K(ACh)}\), LPC was added to the bath solution. As shown in Fig. 3, PTX completely abolished the LPC-induced \(I_{K(ACh)}\). This result confirmed that the LPC-induced \(I_{K(ACh)}\) was mediated by activation of G\(_{i/o}\). PTX also abolished LPC-induced I\(_{NSC}\).

**Effects of LPC and ACh on L-type Ca\(^{2+}\) current enhanced by forskolin**

The above experiments demonstrated that both LPC and ACh induced \(I_{K(ACh)}\) by activation of G\(_{i/o}\) via \(\beta\gamma\) subunits. We next investigated the functions of \(\alpha\) subunits released from G\(_{i/o}\) proteins activated by LPC or ACh by measuring \(I_{Ca}\) pre-stimulated by forskolin, because \(I_{Ca}\) augmented by forskolin could be inhibited by \(G_{\alpha}\) subunits via inhibition of adenyl cyclase (AC) which was pre-stimulated directly by forskolin (FK)\(^{15,16}\). As shown in Fig. 4Aa and 4Ab, \(I_{Ca}\) was increased to about 200~300\% of the control by 3 \(\mu\)M FK. Subsequent application of 1 \(\mu\)M ACh reduced the FK-enhanced \(I_{Ca}\) (Fig. 4Ac). Finally, 5 \(\mu\)M nifedipine was added to inhibit \(I_{Ca}\) completely (Fig. 4Ad). The average value with standard deviation of the peak current amplitude of nifedipine-sensitive component of control \(I_{Ca}\) was 0.37±0.19 nA \((n=27)\), which was increased significantly by 3 \(\mu\)M FK to 0.71±0.55 nA \((n=27)\). The \(p\)-value of \(I_{Ca}\) obtained by the paired \(T\) test was 0.0000002.

Using the same protocol, 1 \(\mu\)M LPC was added instead of ACh (Fig. 4B). Fig. 4C shows concentration-inhibition curves for LPC and ACh, where 3 \(\mu\)M FK-augmented \(I_{Ca}\) was calculated as the maximum of 100\%. It is interesting to note that the inhibitory effects of LPC and ACh on FK-augmented \(I_{Ca}\) were similar over the concentration range from 0.1 \(\mu\)M to 3 \(\mu\)M. This result suggests that LPC and ACh activated similar amounts of \(\alpha\) subunits of G\(_{i/o}\). When the atrial myocytes were pre-treated with 2 \(\mu\)g/ml PTX for at least 3 h at 37ºC, the inhibitory effects of

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**Fig. 3.** Effects of PTX on ACh- and LPC-induced \(I_{K(ACh)}\).

A, Chart recording of currents. PTX was included in the pipette solution. After the response to ACh almost disappeared, LPC was added to the bath solution. B, Difference I-V curves from applications of ACh twice (b-a and d-c) and the application of LPC (f-e).
1 µM ACh and 1 µM LPC on I_{Ca} were attenuated (data not shown), suggesting that the effects of ACh and LPC on I_{Ca} were both mediated by G_{i/o}.

Discussion

The present study demonstrated that LPC induces I_{NSC} in a concentration-dependent manner in guinea-pig atrial myocytes. Possible schema of signal transduction is illustrated in Fig. 5. The LPC-induced I_{NSC} in atrial cells appeared after a time lag and was inhibited by PTX, which is similar to what we reported previously in ventricular myocytes and in cultured human aortic endothelial cells. These results suggest that atrial cells possess LPC receptors, which are coupled to PTX-sensitive G protein. Recently, G-protein-coupled lipid receptors have been reported for LPC and other lipids, such as sphingosin-1-phosphate (S-1-P) and sphingosyl phosphorylcholine. G2A and GPR4 have been reported as LPC receptors that are coupled with PTX-sensitive G protein. The expression of G2A is restricted to lymphoid tissues, whereas GPR4 is expressed more ubiquitously, including in heart and aorta. Identification of receptor subtype that mediates LPC-induced responses in the atrial myocytes is remained to be determined.

The signal transduction mechanism underlying LPC-induced I_{NSC} is unique and complex, because it...
Effects of LPC on $I_{K(ACh)}$ and $I_{Ca}$

requirements an HMG-CoA reductase-dependent activation of the small GTP-binding protein, Rho$^{8,9}$. Although the underlying mechanism of Rho-INS C pathway is unknown, an activation of $G_\gamma$ plays an important role as the upstream regulator of Rho-dependent $I_{NSC}$ activation because of blockade of this pathway by PTX$^9$. In atrial cells, the activation of $G_\gamma$ stimulates $I_{K(ACh)}$ in a $\beta_\gamma$ subunit-dependent manner. Therefore, LPC might also activate $I_{K(ACh)}$. Indeed, a small inwardly rectifying $K^+$ current developed soon after the application of LPC in atrial cells. This current is most likely $I_{K(ACh)}$, because the IV-curve of the LPC-induced $K^+$ current was similar to that induced by ACh, and the current did not appear when PTX was in the pipette solution. However, LPC-induced $I_{K(ACh)}$ was significantly smaller than ACh-induced $I_{K(ACh)}$. In our result in Fig. 2, the outward component of LPC-induced current was minute, but this was not always the case (see Fig. 1B). We do not know the reason why there was a case that the outward component of LPC-induced $I_{K(ACh)}$ did not develop. Bümemann et al.$^{20}$ also described insignificant effects of LPC on $I_{K(ACh)}$ in atrial myocytes.

Intracellular loading of anti-$G_\beta$ antibody prepared from C-terminal of $G_\beta$ subunit have been shown to inhibit $\beta_\gamma$ subunits-mediated effects selectively$^{14}$. We attempted to investigate the effects of anti-$G_\beta$ antibody on LPC-induced $I_{NSC}$ and $I_{K(ACh)}$. Our preliminary data indicated that intracellular loading of anti-$G_\beta$ antibody inhibited ACh (1 $\mu$M)-induced $I_{K(ACh)}$ as well as LPC (5 $\mu$M)-induced $I_{NSC}$ or $I_{K(ACh)}$, suggesting that LPC-induced $I_{NSC}$ and $I_{K(ACh)}$ are both mediated by $\beta_\gamma$ subunits of $G_\gamma$ proteins.

The results with ACh and LPC clearly show that activations of two different $G_\gamma$-coupled receptors produce their effects through different effector systems, namely $I_{K(ACh)}$ and $I_{Ca}$. In cardiac myocytes, differential responses to activations of $G_\gamma$ family-coupled receptors were also demonstrated. Thus, $\beta_1$-adrenergic receptors fully stimulate cyclic AMP signaling, including cyclic AMP accumulation, phosphorylation of phospholamban, $Ca^{2+}$ channel ac-

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**Fig. 5.** Proposed mechanisms of LPC and ACh effects.

Different $\beta_\gamma$ subunits are expressed with different shades. AC indicates adenylyl cyclase. FK indicates forskolin. LPCR indicates LPC receptor. Both ACh and LPC activate $G_\alpha$ protein with the same $G_\gamma$ subunits but different $\beta_\gamma$ subunits. $G_\beta$ subunits activated by ACh and LPC inhibit FK-activated $I_{Ca}$ by inhibiting AC. ACh-activated $\beta_\gamma$ subunit activates $I_{K(ACh)}$, while LPC-activated $\beta_\gamma$ subunit activates $I_{NSC}$. PTX inhibits $G_\gamma$-mediated effects. $G_\beta$ antibody inhibited ACh-induced $I_{K(ACh)}$ and LPC-induced $I_{NSC}$ by inhibiting $\beta_\gamma$ subunit function. M2: muscarinic receptor.
tivation and positive inotropic effects, whereas β2-adrenergic receptors failed to induce Ca\(^{2+}\) channel activation and positive inotropic effects. These differences are explained by a differential localization of receptors; β2-receptors are highly compartmentalized into the caveolae microdomain, but β1-receptors are more widely distributed throughout the plasma membrane. Different responses to LPC and ACh may be also explained by such receptor localizations. However, M\(_4\) receptor is demonstrated to be distributed widely in the cardiac cells similar to β1-receptor. Furthermore, unlike Gs-cyclic AMP signaling systems which are regulated by α subunit of Gs protein in the cardiac tissue, I\(_{K(ACh)}\) and I\(_{NSC}\) are activated by the βγ subunits of G\(_{i/o}\) which exist as varied isoform complexes. To date, five β-subunits (β\(_1\)~β\(_5\)) and twelve γ-subunits (γ\(_1\)~γ\(_{12}\)) have been cloned\(^{21}\). Wickman et al.\(^{22}\) tested 6 recombinant β- and γ-subunits and found that they activated I\(_{K(ACh)}\) with various k\(_{act}\) values ranging between 3 and 30 nM. The most potent combination was β\(_2\)γ\(_1\) at 3.9 nM and the least potent was β\(_1\)γ\(_1\) at 30 nM. Thus, it is the βγ subunits which determine the potency of the interaction with the effector, I\(_{K(ACh)}\). It is therefore more likely that LPC and ACh may activate G\(_{i/o}\) proteins having different types of βγ subunits in guinea pig atrial cells.

Even if the effects of βγ subunits released are different between LPC and ACh, the effects of α-subunits of G\(_{i/o}\) may be similar. In cardiac myocytes, L-type Ca\(^{2+}\) current is enhanced by forskolin, which directly activates adenylyl cyclase (AC), and promotes phosphorylation of L-type Ca\(^{2+}\) channels by cAMP-dependent protein kinase\(^{16,23}\). On the other hand, α-subunits released from G\(_{i/o}\) inhibit AC and decrease forskolin-elevated cAMP, thereby blocking cyclic AMP-dependent activation of I\(_{Ca}\), a phenomenon known as ‘accentuated antagonism’\(^{16,24}\). As shown in Fig. 4, I\(_{Ca}\) pre-stimulated by forskolin was inhibited to a similar extent by LPC and ACh over the concentration range between 0.1 ~3 μM. Since these effects were also inhibited by PTX, the inhibition of I\(_{Ca}\) by LPC and ACh were mediated by G\(_{i/o}\). This indicates that the α subunits of G\(_{i/o}\) activated by LPC and ACh function in a similar manner. The AC isoforms expressed most abundantly in the heart are types V and VI which are both inhibited with similar potency by three different G\(_{i/o}\) isoforms; G\(_{i/o-V}\), G\(_{i/o-VI}\) and G\(_{i/o-VII}\)\(^{20,26}\). In addition, the G\(_i\)-related proteins, G\(_{i3}\), G\(_{i4}\), G\(_{i5}\) and G\(_{i6}\) have been shown to inhibit AC in a PTX-sensitive manner\(^{27}\). These observations suggest that similar amounts of G\(_{i/o}\) are activated by LPC and ACh. Hashizume et al.\(^{28}\) reported that LPC at micromolar concentrations significantly decreased the heart rate and even stopped the beating of isolated perfused rat heart. This may be due to the effect of LPC on I\(_{Ca}\) and I\(_{K(ACh)}\), although the effect on the former was larger than on the latter.

In conclusion, as shown in Fig. 5, we propose that LPC and ACh activate G\(_{i/o}\) having different βγ-subunits, which determines the potency of activation of I\(_{K(ACh)}\) and I\(_{NSC}\). Further study is necessary to identify the types of heterotrimer subunits of G-proteins which are coupled with the receptors.

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Conflict of interest disclosure

We have no conflict of interest to disclose.

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