Mechanisms of Lysophosphatidylcholine-Induced Increase in Intracellular Calcium in Rat Cardiomyocytes

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ABSTRACT

Previous reports have demonstrated that lysophosphatidylcholine (LPC) increases the intracellular concentration of calcium ([Ca$^{2+}$]) in the heart; however, the mechanisms responsible for this increase are not clear. We examined the effect of exogenous LPC on [Ca$^{2+}$], in freshly isolated cardiomyocytes from adult rats. Our results showed that LPC elevated the [Ca$^{2+}$] in a dose-dependent (2.5–10$^{-6}$ M) manner. The LPC (10$^{-5}$ M)-induced increase in [Ca$^{2+}$], was augmented upon increasing the concentration of extracellular Ca$^{2+}$ and was abolished by the removal of Ca$^{2+}$ from the medium. Preincubation of cardiomyocytes with sarcolemmal L-type Ca$^{2+}$ channel blocker, verapamil, did not affect the LPC-evoked increase in [Ca$^{2+}$], significantly. On the other hand, ouabain, a Na$^{+}$-K$^{+}$-ATPase inhibitor, and low concentrations of extracellular Na$^{+}$ enhanced the LPC response. The LPC-induced increase in [Ca$^{2+}$], was attenuated significantly by the inhibitors of Na$^{+}$-Ca$^{2+}$ exchanger such as Ni$^{2+}$ and amiloride. Depletion of the sarcoplasmic reticulum (SR) Ca$^{2+}$ stores by low micromolar concentrations of ryanodine (a SR Ca$^{2+}$ release channel activator) or by thapsigargin (a SR Ca$^{2+}$-pump ATPase inhibitor) depressed the LPC-mediated increase in [Ca$^{2+}$]. Combined blockade of Na$^{+}$-Ca$^{2+}$ exchanger and inhibition of SR Ca$^{2+}$-pump or ryanodine receptor had an additive effect on the LPC response. These observations suggest that the increase in [Ca$^{2+}$], induced by LPC depends on both Ca$^{2+}$ influx from the extracellular space and Ca$^{2+}$ release from the SR stores. Furthermore, Na$^{+}$-Ca$^{2+}$ exchange plays a critical role in the LPC-mediated entry of Ca$^{2+}$ into cardiomyocytes.

Lysophosphatidylcholine is the major lysophospholipid in mammalian tissues (Man et al., 1990), and is formed from phosphatidylcholine by the action of phospholipase A$_2$. An increase in the level of cardiac LPC because of ischemia is thought to be one of the mechanisms underlying the pathogenesis of ischemic injury (Sedlis et al., 1988, 1993). Recently we demonstrated that the accumulation of this lysophospholipid in the SL membrane under ischemic conditions may diminish the availability of phosphatidylinositol 4,5-biphosphate for the production of second messengers upon the activation of phospholipase C (Liu et al., 1997). It should be pointed out that LPC has toxic effects on the myocardium and these include electrophysiological disturbances like shortened refractory period, decreased membrane potential, increased membrane resistance as well as arrhythmogenesis (Clarkson and Ten Eick, 1983; Corr et al., 1982; Pogwizd et al., 1986), and mechanical alterations such as decreased contractility as well as contracture in cardiac myocytes and perfused hearts (Woodley et al., 1991; Ver Donck et al., 1992; Hoque et al., 1995, 1997). Although LPC has been shown to increase the [Ca$^{2+}$], in the heart (Corr et al., 1982; Sedlis et al., 1983; Karli et al., 1979), the mechanisms proposed for this effect are not conclusive. In this regard, some investigators have suggested increased SL permeability (Corr et al., 1982) and increased Ca$^{2+}$ influx through the L-type voltage-gated Ca$^{2+}$ channels (Sedlis et al., 1983), whereas others have indicated inhibition of the Na$^{+}$-K$^{+}$-ATPase activity (Karli et al., 1979) to explain the LPC-induced increase in [Ca$^{2+}$]. In the present study we investigated the effect of exogenous LPC on rat adult cardiomyocytes and probed the mechanisms responsible for the LPC-induced increase in [Ca$^{2+}$], by use of some inhibitors of the SL and SR membrane proteins which are involved in the regulation of [Ca$^{2+}$]. Our results indicate that LPC increases [Ca$^{2+}$], in a dose-dependent manner, and this increase depends on the entry of extracellular Ca$^{2+}$ via the Na$^{+}$-Ca$^{2+}$ exchanger in the SL membrane as well as on the status of intracellular SR Ca$^{2+}$ stores.

**Methods**

*Chemicals.* L-α-Lysophosphatidylcholine, amiloride, ouabain, ryanodine, thapsigargin, EGTA, and BSA were purchased from the

**ABBREVIATIONS:** LPC, lysophosphatidylcholine; [Ca$^{2+}$], intracellular concentration of calcium; SL, sarcolemma; SR, sarcoplasmic reticulum; BSA, bovine serum albumin; EGTA, ethylene glycol-bis (β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid; Fura-2/AM, Fura-2 acetoxymethyl ester.

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Sigma Chemical Company (St. Louis, MO). Collagenase (type II, 310 U/mg) was from the Worthington Biochemicals (Freehold, NJ). Fura-2/AM was obtained from the Molecular Probes (Eugene, OR). All other substances were of analytical purity and purchased from Mallinkrodt (Montreal, Quebec, Canada).

Isolation of cardiomyocytes. Cardiomyocytes were isolated from rat hearts by a collagenase digestion procedure (Xu et al., 1996). Male Sprague-Dawley rats, weighing 300 to 350 g, were injected with heparin (1000 U/100 g b.wt.) and anesthetized with xylazine (10 mg/kg) combined with ketamine (60 mg/kg). After the rats were anesthetized, the heart was excised and cannulated on a Langendorff apparatus via the aorta and perfused in a noncirculatory manner with Ca²⁺-free buffer containing (mM): NaCl, 90; KCl, 10; KH₂PO₄, 1.2; MgSO₄, 5; NaHCO₃, 15; taurine, 30; glucose, 20 (pH 7.4); this medium was gassed with 95% O₂ and 5% CO₂ mixture. After 5 min of perfusion, the heart was switched to the same medium containing 0.04% collagenase and 50 μM CaCl₂. At the end of a 30-min recirculation period, the heart was taken off the cannula and the atria were excised. The ventricles were minced into small pieces and subjected to another 30 min of digestion in a fresh collagenase solution in the presence of 1% BSA gassed with 95% O₂ and 5% CO₂ on a shaking water bath at 37°C. Ventricular fragments were triturated gently with a glass Pasteur pipette (once every minute). The cell suspension was collected every 10 min and filtered through a 200 μM nylon mesh. The myocytes were resuspended in buffers, where the extracellular calcium concentration was increased in a gradual stepwise fashion (250 μM, 500 μM, 750 μM, 1000 μM); the cells were incubated in each calcium concentration for 15 min at room temperature. The rod-shaped myocytes comprised more than 80% of the final cell population.

Measurement of fluorescence. The technique for monitoring [Ca²⁺], with Fura-2/AM as the fluorescent dye has been described previously (Xu et al., 1996). Freshly isolated adult rat cardiomyocytes were incubated with 5 μM Fura-2/AM for 40 min in Krebs-Henseleit buffer containing (mM): NaCl, 90; KCl, 10; KH₂PO₄, 1.2; MgSO₄, 5; NaHCO₃, 15; glucose, 20 (pH 7.4), then washed three times to remove any extracellular dye. The cell number in the cuvette was adjusted to 0.3 million cells/ml. The alteration of the intensity of fluorescence proportionality coefficients obtained at 380 nm (excitation wavelength) under 340/380 nm, the emission wavelength was 510 nm. The integration time was 0.95 sec; the resolution time was 1.0 sec. The [Ca²⁺], levels were calculated according to the formula:

\[ [Ca^{2+}] = 224 \times [(R - R_{min})/(R_{max} - R)] \times Sf/Sb \]

The ratio (R) of 340/380 nm was calculated automatically. Rₘₐₓ and Rₐₘₚ values were determined by addition of 20 μl Triton X-100 (10%) and 40 μl EGTA (400 mM) respectively. Sf and Sb are the fluorescence cell proportionality coefficients obtained at 380 nm (excitation wavelength) under Rₘₐₓ and Rₐₘₚ conditions, respectively.

Experimental protocol. The Fura-2/AM-loaded cells were exposed to LPC in the range of 2.5 to 10 μM (final concentration), and the response was traced for 5 min. EGTA (1 mM) was used to remove the extracellular Ca²⁺ from the suspension containing the cells in a set of experiments. A group of experiments involved the incubation of myocytes for 10 min with inhibiting agents, namely: a) verapamil, a slow Ca²⁺ channel blocker; b) ouabain, an inhibitor of SL Na⁺-K⁺ ATPase; c) amiloride and Ni²⁺, inhibitors of SL Na⁺-Ca²⁺ exchanger; d) thapsigargin, a SR Ca²⁺-pump ATPase inhibitor; and e) ryanodine at low micromolar concentrations, a SR Ca²⁺-release channel stimulator. These treated cells subsequently were exposed to 10 μM LPC for 5 min. In another set of experiments, Fura-2/AM-loaded myocytes were incubated for 10 min with a combination of inhibitors, amiloride plus thapsigargin and amiloride plus ryanodine. These treated cells subsequently were exposed to 10 μM LPC.

Data analysis. All results are expressed as mean ± S.E. The increment of [Ca²⁺], was calculated on the basis of net increase above basal value in each experiment. Increase in [Ca²⁺], within the groups was assessed by calculating the peak and plateau [Ca²⁺]. Differences in basal values within groups also were calculated. Statistical analysis was performed by one-way analysis of variance. P values less than .05 reflected significant differences between control and experimental groups.

Results

The effects of varying concentrations of LPC on the [Ca²⁺], in cardiomyocytes were studied. Figure 1 shows a concentration-dependent increase in [Ca²⁺], by LPC; 10 μM LPC elicited the maximum increase in [Ca²⁺]. Under the experimental conditions used in this study, 10 μM LPC had no significant effect on cell viability. In fact, the percentage of rod-shaped cells in the absence or presence of 10 μM LPC was 87 ± 0.7 or 87.1 ± 1.1, respectively. Furthermore, the lactate dehydrogenase activity was not detectable in the culture medium under both the above-mentioned conditions. To test whether the increase in [Ca²⁺], induced by LPC depended on Ca²⁺-influx, the relationship between extracellular calcium concentration and LPC-induced increase in [Ca²⁺], was investigated. As shown in figure 2, the LPC-

![Fig. 1](https://example.com/fig1.png)

**Fig. 1.** Effect of different concentrations of LPC on [Ca²⁺], in adult rat cardiomyocytes. Freshly isolated adult rat cardiomyocytes were incubated with 10 μM Fura-2/AM for 40 min. The Fura-2/AM-loaded cells were exposed to varying LPC concentrations and the basal and LPC-evoked changes of peak and plateau [Ca²⁺], were monitored for a 5 min period by LM DMX-1100 spectrofluorometer (excitation: 340/380 nm; 510 nm). Panel A shows typical traces for different concentrations of LPC (2.5–10 μM) during a period of time. Panel B shows the bar graphs for the increase in peak [Ca²⁺], at different concentrations of LPC. Increase in [Ca²⁺], was the net increase in [Ca²⁺], above basal level in the absence of LPC which was 90.1 ± 6.1 nM. Values given in B are mean ± S.E. of six separate preparations.
The role of SL Na\(^{+}\)1 in the LPC action, a stimulator of SR Ca\(^{2+}\)-release, ryanodine (at submicromolar concentrations) (Hansford and Lakatta, 1987; Vigne et al., 1993) and a stimulator of SR Ca\(^{2+}\)-release, ryanodine (at submicromolar concentrations) (Hansford and Lakatta, 1987; Vigne et al., 1993) were used to deplete the SR Ca\(^{2+}\) stores. Treatment of the cells with 20 μM thapsigargin (fig. 8) or 5 μM ryanodine (fig. 9) significantly attenuated both peak and plateau [Ca\(^{2+}\)]\(_{i}\) in response to LPC.

Ca\(^{2+}\)-release from SR is induced by a small amount of Ca\(^{2+}\) entering the cell by the Ca\(^{2+}\)-induced Ca\(^{2+}\)-release process (Fabiato, 1985). To assess the effect of LPC on the SR function, an inhibitor of the SR Ca\(^{2+}\)-pump ATPase, thapsigargin (Kirby et al., 1992; Chen and Van Breemen, 1993) and a stimulator of SR Ca\(^{2+}\)-release, ryanodine (at submicromolar concentrations) (Hansford and Lakatta, 1987; Vigne et al., 1993) were used to deplete the SR Ca\(^{2+}\) stores. Treatment of the cells with 20 μM thapsigargin (fig. 8) or 5 μM ryanodine (fig. 9) significantly attenuated both peak and plateau [Ca\(^{2+}\)]\(_{i}\) in response to LPC.

To further assess the contribution of Na\(^{+}\)-Ca\(^{2+}\) exchanger and SR Ca\(^{2+}\)-regulating mechanisms in the LPC action, amiloride was used in combination with thapsigargin or ryanodine. Treatment of the cells with 10 μM amiloride plus 15 μM thapsigargin or 10 μM amiloride plus 2.5 μM ryanodine produced an additive depressant effect of these inhibitors on [Ca\(^{2+}\)]\(_{i}\) (fig. 10A, B).

From the data in table 1, it can be seen that the basal induced increase of [Ca\(^{2+}\)]\(_{i}\) above the basal value was proportional to the concentration of Ca\(^{2+}\) in the extracellular medium. In addition, the increase in [Ca\(^{2+}\)]\(_{i}\) caused by LPC was abolished by chelating Ca\(^{2+}\) in the medium with 1 mM EGTA. To assess the role of L-type Ca\(^{2+}\) channel as a possible mechanism involved in the LPC-evoked entry of Ca\(^{2+}\), the effect of verapamil, a specific blocker of the L-type Ca\(^{2+}\) channel (Lee and Tsien, 1983) on the LPC-induced increase in [Ca\(^{2+}\)]\(_{i}\) was studied. Figure 3 shows that verapamil did not cause any significant depression of the [Ca\(^{2+}\)]\(_{i}\) response to LPC. The role of SL Na\(^{+}\)-Ca\(^{2+}\) exchanger in promoting the Ca\(^{2+}\)-induced increase, was studied. Figure 3 shows that verapamil did not cause any significant depression of the [Ca\(^{2+}\)]\(_{i}\) response to LPC. The role of SL Na\(^{+}\)-Ca\(^{2+}\) exchanger in promoting the Ca\(^{2+}\)-induced increase, was studied. Figure 3 shows that verapamil did not cause any significant depression of the [Ca\(^{2+}\)]\(_{i}\) response to LPC. The role of SL Na\(^{+}\)-Ca\(^{2+}\) exchanger in promoting the Ca\(^{2+}\)-induced increase, was studied. Figure 3 shows that verapamil did not cause any significant depression of the [Ca\(^{2+}\)]\(_{i}\) response to LPC.
[Ca\textsuperscript{2+}]i values in cardiomyocytes with several treatments under the experimental conditions used in this study were not different from those of the untreated cardiomyocytes.

**Discussion**

Although LPC is present in small quantities (0.5–3.5% of the total phospholipids) in the myocardium under physiological conditions (White, 1975), its concentration has been shown to increase because of ischemia. Such an increase in LPC concentration seems to depend on the species and tissue compartments: 120 \textmu M in the ischemic pig myocardium (Shaikh and Downar, 1981), 197 \textmu M in cardiac lymph and 200 \textmu M in interstitial fluid of the ischemic region in dogs with myocardial ischemia (Akita et al., 1986), and 178 \textmu M in the coronary sinus of humans with atrial pacing (Sedlis et al., 1990). From such observations, it appears that 100 to 200 \textmu M concentrations of LPC may be present in the ischemic heart.

However, under in vivo conditions, LPC may be bound nonspecifically to proteins in the myocardium and this may reduce the concentration of the free LPC probably by about 10 times (Corr et al., 1981; Man and Choy, 1982). Thus, 10 to 20 \mu M free LPC may be present in the ischemic tissue, which is compatible with the concentrations used in this study for demonstrating an increase in [Ca\textsuperscript{2+}]i in rat cardiomyocytes.

Several investigators have shown the deleterious effects of LPC on myocytes within the above-mentioned pathophysiological range of concentrations (Woodley et al., 1991; Ver Donck et al., 1992; Hashizume and Abiko, 1996). Because lysophospholipids are wedge-shaped structures, their accumulation inside the membranes may alter the membrane integrity and modify the membrane protein function (Corr et al., 1984; Weltzien, 1979). Accordingly, arrhythmogenesis and contractile dysfunction caused by myocardial ischemia have been linked with LPC accumulation in the ischemic heart (Clarkson and Ten Eick, 1983; Corr et al., 1982; Pogwizd et al., 1986; Woodley et al., 1991; Ver Donck et al., 1992; Hoque et al., 1995, 1997). Liu et al. (1991) have shown that 20 \mu M LPC significantly increased cell shortening, produced spontaneous contractile activity and caused Ca\textsuperscript{2+} overload resulting in contracture in guinea pig ventricular myocytes. Woodley et al. (1991) detected increases in [Ca\textsuperscript{2+}]i when chick embryonic myocytes were exposed to 1 to 100 \mu M LPC.
Thus, the results regarding the mechanisms of LPC-induced $[\text{Ca}^{2+}]_i$, described in the present study can be seen to address an important problem in the area of ischemic heart disease.

In this study we have shown that the increase in $[\text{Ca}^{2+}]_i$, in cardiomyocytes not only depends on the concentration of LPC but also is related to the concentration of $\text{Ca}^{2+}$ in the medium. These observations suggest that $\text{Ca}^{2+}$-influx from the extracellular space is required for the occurrence of LPC-induced increase in $[\text{Ca}^{2+}]_i$ in cardiomyocytes. The SL-Ltype channel may be responsible for the entry of extracellular $\text{Ca}^{2+}$, and its activation seems to be an attractive mechanism for the LPC-induced increase in $[\text{Ca}^{2+}]_i$. Five minutes exposure of myocytes to verapamil, a specific blocker of the L-type $\text{Ca}^{2+}$ channel, blocked the increase in $[\text{Ca}^{2+}]_i$, caused by 100 $\mu$M LPC (Sedlis et al., 1983). In contrast, verapamil did not affect the LPC-induced increase in $[\text{Ca}^{2+}]_i$ in spontaneously contracting chick embryo cell cultures (Woodley et al., 1991) and rat cardiomyocytes (Hashizume and Abiko, 1996). Our results with verapamil are in good agreement with the latter reports demonstrating that the L-type $\text{Ca}^{2+}$ channel may not be the site of action for LPC. On the other hand, $\text{Na}^+-\text{K}^+$ ATPase, which maintains the resting membrane potential and controls entry of $\text{Ca}^{2+}$ indirectly via $\text{Na}^+-\text{Ca}^{2+}$ exchange, has been shown to be inhibited by LPC in SL membrane preparations from dog and rabbit hearts (Karli et al., 1979; Owens et al., 1982). However, Pitts and Okhuyzen (1984) reported that the concentrations of LPC required to inhibit the $\text{Na}^+-\text{K}^+$ ATPase activity in the dog heart SL vesicles were higher than those causing changes in membrane permeability, which suggests that the inhibition of $\text{Na}^+-\text{K}^+$ ATPase by LPC may not occur to any significant extent under in vivo conditions. Our results with ouabain, a specific blocker for $\text{Na}^+-\text{K}^+$ ATPase, show an augmentation of LPC-induced increase in $[\text{Ca}^{2+}]_i$ in rat cardiomyocytes and support the view that $\text{Na}^+-\text{K}^+$ ATPase may be a site of action for LPC in the intact myocyte. Blockade of SL $\text{Na}^+-\text{K}^+$ ATPase would lead to an increase in the intracellular concentration of $\text{Na}^+$, which in turn would bring about the reversal of the $\text{Na}^+-\text{Ca}^{2+}$ exchanger activity resulting in the entry of extracellular $\text{Ca}^{2+}$ (Vemuri et al., 1989). The indirect involvement of SL $\text{Na}^+-\text{Ca}^{2+}$ exchange system also has been suggested from studies showing the LPC-induced stimulation of the $\text{Na}^+-\text{H}^+$ exchanger in intact beating rat hearts, which results in the accumulation of $\text{Na}^+$ in cardiomyocytes (Hoque et al., 1997) and then produces a secondary increase.
membranes and the stimulation of Na$^{+}$-pump ATPase was depressed by about 20%, an uncoupling of Na$^{+}$ entry re- sults. This may be because of different sites of action of LPC on the Na$^{+}$-pump and SR Ca$^{2+}$-release channels by thapsigargin (Kirby et al., 1992; Chen and Van Breemen, 1993) or by stimulation of SR Ca$^{2+}$-release channels by low concentrations of ryanodine (Hansford and Lakatta, 1987; Vigne et al., 1990), attenuated the LPC-induced increase in [Ca$^{2+}$] in cardiomyocytes. Thus it seems that the Ca$^{2+}$-induced Ca$^{2+}$-release from SR also may play an important role in eliciting the LPC-induced increase in [Ca$^{2+}$] in cardiomyocytes. In fact, the combined blockade of Na$^{+}$-Ca$^{2+}$ exchanger and SR Ca$^{2+}$-pump as well as ryanodine receptor, had an additive effect on the LPC response confirming the involvement of both mechanisms in the LPC-mediated increase in [Ca$^{2+}$]. Accordingly, it is suggested that the LPC-induced increase in [Ca$^{2+}$] in cardiomyocytes may be caused by its direct and indirect
Lysophosphatidylcholine and [Ca\textsuperscript{2+}]\textsuperscript{7}

Fig. 10. Inhibition of LPC-induced increase in [Ca\textsuperscript{2+}], by amiloride plus thapsigargin or amiloride plus ryanodine. Freshly isolated adult rat cardiomyocytes were incubated with 10 \mu M Pum-2AM for 40 min. Aliquots of the Fura-2/AM- cells were incubated with the appropriate combination of drugs for 10 min, and then exposed to 10 \mu M LPC for 5 min. Panel A shows the effect of LPC on [Ca\textsuperscript{2+}] in the presence and absence of 10 \mu M amiloride plus 15 \mu M thapsigargin. Panel B shows the effect of LPC on [Ca\textsuperscript{2+}] in the presence and absence of 10 \mu M amiloride plus 2.5 \mu M ryanodine. Tracings are representative of three separate experiments.

**TABLE 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Basal [Ca\textsuperscript{2+}] values for cardiomyocytes in the absence (control) and presence (treated) of different interventions used in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without Ca\textsuperscript{2+}</td>
<td>92.5 ± 4.2</td>
</tr>
<tr>
<td>4 mM Ca\textsuperscript{2+}</td>
<td>94.3 ± 3.1</td>
</tr>
<tr>
<td>20 \mu M verapamil</td>
<td>93.2 ± 4.1</td>
</tr>
<tr>
<td>84 \mu M Na\textsuperscript{+}</td>
<td>88.7 ± 3.2</td>
</tr>
<tr>
<td>42 \mu M Na\textsuperscript{2+}</td>
<td>90.2 ± 4.1</td>
</tr>
<tr>
<td>1 mM ouabain</td>
<td>96.5 ± 5.0</td>
</tr>
<tr>
<td>2 mM ouabain</td>
<td>91.2 ± 4.6</td>
</tr>
<tr>
<td>2.5 mM Ni\textsuperscript{2+}</td>
<td>95.4 ± 3.7</td>
</tr>
<tr>
<td>5.0 mM Ni\textsuperscript{2+}</td>
<td>94.5 ± 4.5</td>
</tr>
<tr>
<td>10 \mu M amiloride</td>
<td>90.2 ± 2.7</td>
</tr>
<tr>
<td>20 \mu M thapsigargin</td>
<td>94.3 ± 2.7</td>
</tr>
<tr>
<td>1 \mu M ryanodine</td>
<td>91.1 ± 4.1</td>
</tr>
<tr>
<td>5 \mu M ryanodine</td>
<td>90.2 ± 3.7</td>
</tr>
</tbody>
</table>

Vemuri R, Longoni S and Philipson KD (1989) Ouabain treatment of cardiac...


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