Alterations in Ca\(^{2+}\) cycling by lysoplasmenylcholine in adult rabbit ventricular myocytes

Shi J. Liu, Richard H. Kennedy, Michael H. Creer, and Jane McHowat.

Alterations in Ca\(^{2+}\) cycling by lysoplasmenylcholine (LPlasC) resulted in an increase in a transient inward current (31) but lacked effects on \(I_{\text{K1}}\) and transient outward \(K^+\) currents (32). Nevertheless, these LPC- and palmitoylcarnitine-induced electrophysiological changes could not satisfactorily account for the observed concomitant positive inotropic effect (1, 25) and the increased intracellular Ca\(^{2+}\) (Ca\(_i\); see Ref. 33). In contrast to LPC and palmitoylcarnitine, little is known about the cellular mechanism underlying LPlasC-induced changes in cardiac contractile and electrical function.

Disturbance of Ca\(^{2+}\) handling such as that elicited by increasing Ca\(^{2+}\) influx or Ca\(^{2+}\) release from sarcoplasmic reticulum (SR), and/or by decreasing Ca\(^{2+}\) efflux, leads to Ca\(_i\) overload that is often associated with cardiac arrhythmias (for review, see Ref. 7). Thus, alterations in Ca\(^{2+}\) cycling by lysoplasmenylcholine in adult rabbit ventricular myocytes.

Address for reprint requests and other correspondence: S. J. Liu, Dept. of Pharmaceutical Sciences, Univ. of Arkansas for Medical Sciences, 4301 West Markham St., MS 522-3, Little Rock, AR 72205 (E-mail: sliu@uams.edu).

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in this study, we investigated the effects of LPlasC on contraction, Ca\(^{2+}\) transients, and membrane currents to assess its actions on excitation-contraction coupling in adult rabbit ventricular myocytes. We found that LPlasC exerted potent, distinct effects on contractile and electrical functions in ventricular myocytes. These effects could be accounted for by LPlasC-induced alterations in Ca\(^{2+}\) handling.

**MATERIALS AND METHODS**

**Myocyte isolation.** The protocol for the use of animals in this study conformed with the National Institutes of Health approved Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee, University of Arkansas for Medical Sciences.

Single adult ventricular myocytes were isolated from the hearts of adult rabbits (either sex, 2–3 kg), as described previously (20). Isolated ventricular myocytes were harvested and plated in 60-mm culture dishes (Falcon) for 2 h or overnight in culture medium composed of 60% medium-199 (GIBCO, Grand Island, NY) and 36% Earle’s balanced salt solution containing (in mM) 116 NaCl, 4.7 KCl, 0.9 NaHPO\(_4\), 0.8 MgSO\(_4\), 26 NaHCO\(_3\), 5.6 glucose, and 4% FBS (pH 7.40 in 5% CO\(_2\)-95% air at 37°C; GIBCO), as described previously (17). Rod-shaped cells with clear striations were used for experiments, and there was no significant difference in the response to lipid metabolites between freshly isolated and primary cultured myocytes (within 24 h). All experiments were carried out at 35–37°C.

**Measurement of cell shortening.** Unloaded cell shortening (CS) or contraction of myocytes was elicited in normal Tyrode solution containing (in mM) 140 NaCl, 5.4 KCl, 1 CaCl\(_2\), 0.8 MgCl\(_2\), 10 HEPES/Tris, and 5.6 glucose (pH 7.40 at 37°C; 290 mosmol/kgH\(_2\)O) through field stimulation with bipolar platinum electrodes at a frequency of 0.5 Hz with 1- to 2-ms voltage pulses. Cells were then superfused with normal Tyrode solution containing ethanamine, followed by solutions with different concentrations of LPlasC. The stimulating electrode was placed near the suction pipette in the perfusion chamber to minimize its damage to cells during long periods of stimulation. The video signal was fed into the video motion detector (Crescent Electronics, Sandy, UT) connected to a video monitor through a charge-coupled device video camera (Axon Instruments) mounted to a microscope. The analog voltage output was video monitored through a charge-coupled device video camera detector (Crescent Electronics, Sandy, UT) connected to a personal computer for later calibration and analysis. After subtraction of the background signal, fluorescent signals were recorded as the ratio (R or fls00/fls00) of the fluorescent intensity when excited at 340 nm (fls00) to that when excited at 380 nm (fls00). Because of difficulties with the in vivo calibration procedure, many results were represented as fls00/fls00.

**Electrophysiological measurements.** Ventricular myocytes were perfused with normal Tyrode solution and patch-clamped using perforated-patch (16) or conventional whole cell patch techniques (14) with a patch-clamp amplifier (Axopatch 200A; Axon Instruments; see Refs. 17 and 18). APs of myocytes were measured in normal Tyrode solution and in cell-permeable form of fura-PE3 that is a new analog of fura filter (640 nm; Chroma).

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**Measurement of intracellular free Ca\(^{2+}\) concentration.** Ventricular myocytes seeded on 25-mm coverslips in culture medium were loaded for 30 min in a culture incubator at 37°C with 2 μM fura-PE3-AM (TEFLABS, Austin, TX), a cell-permeable form of fura-PE3 that is a new analog of fura 2 and is retained inside the cell longer than fura 2. Myocytes were then transferred to a recording/perfusion chamber (Harvard Apparatus, Holliston, MA) on the stage of an inverted microscope (model TE300; Nikon, Irving, TX) and superfused with normal Tyrode solution. Fluorescent measurements were made through a ×40 long-working-distance ultraviolet (UV) objective (Nikon Fluor with numerical aperture of 1.3). Fura-PE3-loaded cells were alternately excited with UV light at 340- and 380-nm wavelengths via a filter wheel, controlled by a spectrophotometry unit (Cairn Research, search) at 60–75 Hz. The emitted fluorescence signal at 510 nm was collected through an adjustable diaphragm and a photomultiplier tube (Cairn) to the spectrophotometer control unit. The signals were sampled at 200–300 Hz using pClamp software (Axon Instruments) and stored in a personal computer for later calibration and analysis. After subtraction of the background signal, fluorescent signals were recorded as the ratio (R or fls00/fls00) of the fluorescent intensity when excited at 340 nm (fls00) to that when excited at 380 nm (fls00). Because of difficulties with the in vivo calibration procedure, many results were represented as fls00/fls00.

The measured parameters of the Ca\(^{2+}\) transient included peak magnitude, maximum rates of the rising phase (+dRI/dt\(_{max}\)) and the declining phase (−dRI/dt\(_{max}\)), and the rise and decay times between 10 and 90% of peak amplitude. In some experiments with successful in situ calibrations, cytosolic free Ca\(^{2+}\) concentrations were determined using the equation (13) \[ [Ca^{2+}]_{i} = K_{d} \times \beta \times (R - R_{min}/(R_{max} - R) \text{ where } K_{d} \text{ is the apparent dissociation constant of 224 nM at 37°C and } \beta \text{ is the ratio of fls00/free to fls00/free measured under R_{min} and R_{max} conditions, respectively. R_{min} is the minimum fluorescent intensity in Na\(^{+}\)-free solutions containing 3 μM ionomycin and 10 mM EGTA, and R_{max} is the maximum intensity ratio in perfusion buffer solution containing 3 μM ionomycin and 2 mM CaCl\(_{2}\). In some experiments, myocyte contraction was recorded simultaneously with Ca\(^{2+}\) transients when the cells were illuminated with a halogen lamp (Nikon) through a long-wavelength pass filter (640 nm; Chroma).

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column chromatography on a 2.5 × 60-cm column of silica using a stepwise gradient elution procedure. According to our previous study (20), the majority of experiments in this study used 1 μM LPlasC, which elicited apparent cardiac effects, unless otherwise indicated.

**Chemicals.** Most reagents were purchased from Sigma Chemical (St. Louis, MO) and were added directly when needed. Stock solutions of lipid metabolites (10–2 M) were prepared in 100% ethanol. The final concentration of ethanol in extracellular solutions was <0.01% and had no significant effect (<10%) on measured parameters.

**Statistics.** In all experiments, data in response to LPlasC were compared with the steady-state control before treatment in each individual cell and thus expressed as a ratio or percentage of each control value before combining for statistical analysis. Values are presented as means ± SE. Statistical significance was evaluated by the two-tailed Student’s paired t-test or, when more than two conditions were compared, by one- or two-way ANOVA with Duncan’s multiple-range test. Differences with P < 0.05 were considered significant.

**RESULTS**

**Effects of LPlasC on contraction of intact ventricular myocytes.** We first examined the effect of LPlasC on the unloaded contractile function of intact ventricular myocytes under physiological conditions. Figure 1 shows that LPlasC increased contractility, which reached a plateau in 5 min and was followed by a small decrease. In combined data, LPlasC elicited a maximum positive inotropic effect (2.59 ± 0.44-fold increase, n = 25) in 3–8 min (varied from cell to cell), followed by a decline (83.9 ± 4.3% of the maximum, n = 18). After the baseline to the control level was offset, Fig. 1C shows superimposed CS before, during, and after exposure to 1 μM LPlasC, as shown in Fig. 1B, each of which was obtained by averaging five to six shortening traces. The first derivative of contraction traces (Fig. 1C, inset) shows that +dL/dt<sub>max</sub> and −dL/dt<sub>max</sub> were increased dramatically during exposure to LPlasC. Combined data in Fig. 1E show that LPlasC caused a 3-fold and 4.3-fold increase in +dL/dt<sub>max</sub> and −dL/dt<sub>max</sub>, respectively, concomitant with reductions in the rise time and decay time of contraction. Normalized traces (to each peak amplitude) as shown in Fig. 1D confirmed that LPlasC had a more profound effect on −dL/dt<sub>max</sub> than +dL/dt<sub>max</sub>, and combined data show 41 and 18% increases in −dL/dt<sub>max</sub> and +dL/dt<sub>max</sub>, respectively (Fig. 1E). Thus LPlasC elicited positive inotropic and lusitropic effects in adult rabbit ventricular myocytes. Figure 1 also shows that, after removal of LPlasC, a rebound stimulation of contraction was observed before returning to the control level. Nine of twenty-five tested myocytes showed the rebound activation of contraction (1.32 ± 0.11-fold of the amplitude before LPlasC removal), whereas nine cells did not (also see Fig. 2). The remaining cells did not survive during LPlasC exposure or washout because of arrhythmia and/or contracture.

**Effects of LPlasC on the Ca<sup>2+</sup> transient in fura-PE3-loaded ventricular myocytes.** The effect of LPlasC on Ca<sub>2+</sub> handling in fura-PE3-loaded myocytes was examined under conditions similar to those described for CS. Figure 2 shows results from simultaneous measurements of CS (Fig. 2A) and the Ca<sup>2+</sup> transient (Fig. 2B) in a myocyte. LPlasC caused rapid increases in systolic and diastolic Ca<sup>2+</sup> levels, and the magnitude of the Ca<sup>2+</sup> transient, accompanied by a fourfold increase in CS, an isotropic response similar to that observed in non-fura-PE3-loaded cells. The time course for the increase in Ca<sup>2+</sup> transients appears to be more rapid than that of the positive inotropy (τ: 63.8 vs. 91.2 s), suggesting that LPlasC increases Ca<sup>2+</sup> cycling and alters the Ca<sup>2+</sup> sensitivity of contractile machinery. Upon removal of LPlasC, Ca<sup>2+</sup> transients and contraction partially recovered without a rebound activation. Steady-state Ca<sup>2+</sup> transients and contraction before and during LPlasC exposure were superimposed and shown in Fig. 2, D and C, respectively. It is noticeable in Fig. 2C that an aftercontraction was developing 8 min after exposure to LPlasC compared with the control. In addition, Fig. 2D, inset, shows that LPlasC increased +dR/dt<sub>max</sub> and −dR/dt<sub>max</sub> of the Ca<sup>2+</sup> transient. The LPlasC-induced increases in systolic and diastolic f<sub>340/f<sub>380</sub></sub> were 0.041 ± 0.004 and 0.019 ± 0.002 (n = 29), respectively, which approximated 340 and 80 nM of free Ca<sub>2+</sub>, respectively. Combined data in Fig. 2E show that LPlasC increased the magnitude of the Ca<sup>2+</sup> transient approximately twofold and doubled +dR/dt<sub>max</sub> and −dR/dt<sub>max</sub>. It is also worth mentioning that the declining phase of the Ca<sup>2+</sup> transient in the presence of LPlasC was better fit by a bieponential function, whereas it was best fit by a single exponential in control conditions. The LPlasC-associated initial rapid phase (i.e., −dR/dt<sub>max</sub>) and later slow phase of the f<sub>340/f<sub>380</sub></sub> decline were more rapid and slower than the decay time constant in control, respectively. As a consequence, LPlasC increased the area under the Ca<sup>2+</sup> transient and the decay time (Fig. 2E). A phase-plane plot of contraction as a function of the simultaneously measured f<sub>340/f<sub>380</sub></sub> (using data in Fig. 2, C and D) shows a hysteresis relationship between CS and free Ca<sub>2+</sub> (or fluorescence ratio). Figure 3 shows that LPlasC shifted the hysteresis relationship upward and to the right. When this relationship was replotted using relative changes in CS vs. f<sub>340/f<sub>380</sub></sub> (normalized to each peak magnitude), a left shift in the contraction-CS trajectory during the early phase of relaxation is revealed, suggesting an increase in the myofilament Ca<sub>2+</sub> sensitivity (Fig. 3, inset), as described previously by others (26). Meanwhile, the slope of the relaxation in this hysteresis relationship in LPlasC displayed two phases, consistent with the biexponential process of the decline phase in the Ca<sup>2+</sup> transient described previously.

The role of SR Ca<sup>2+</sup> load in the LPlasC-induced increase in systolic and diastolic Ca<sub>2+</sub> was also examined in fura-loaded myocytes using caffeine (10–15 mM for ~5 s) pulses that have been used to estimate SR Ca<sup>2+</sup> content (4, 12). Figure 4A shows that, in the presence of LPlasC, caffeine induced a greater Ca<sup>2+</sup> transient. Figure 4B shows that LPlasC slowed the decay of the
Transient in the presence of caffeine, an indirect index of sarcolemmal Na⁺/Ca²⁺ exchange activity (2), compared with control. Combined data in Fig. 4 show that LPlasC increased the time constant of the decay of the caffeine-induced Ca²⁺ transient ~65%. Figure 4, D and E, summarizes data showing that LPlasC caused a 40–60% increase in the magnitude of the caffeine-induced Ca²⁺ transient when estimated using either the resting level (termination of electrical stimulation) or diastolic level as baseline. Results also show that the fractional Ca²⁺ release from SR in the steady-state twitch (i.e., a ratio of the magnitude of steady-state systolic Ca²⁺ transients to that of the caffeine-induced Ca²⁺ transient) was greater in the presence of LPlasC than in control. Note that the ~40% fractional Ca²⁺ release observed in control was consistent with the 43% fractional SR Ca²⁺ release reported previously by others using rabbit ventricular myocytes (4).
The time course of recovery of the systolic Ca\$^{2+}\$ transients after removal of caffeine (postcaffeine) was shown in Fig. 4C. Results show that 1) the magnitude of the first postcaffeine Ca\$^{2+}\$ transient in response to electrical stimulation during exposure to LPlasC was greater than that in control, suggesting an increased Ca\$^{2+}\$ influx, and 2) the rate of the recovery to a new steady state was best fit with a single exponential with a time constant of 8.6 and 9.5 s in the presence of LPlasC and in control, respectively. In C, an aftercontraction (*) was developing in the presence of LPlasC. D, inset, the first derivative of the Ca\$^{2+}\$ transient before and during LPlasC exposure. E: combined data of LPlasC-induced relative changes in parameters of Ca\$^{2+}\$ transients from 29 cells. LPlasC elicited significant effects on all parameters (P < 0.005, except for the decay time: P < 0.05).

Arrhythmiogenic effect of LPlasC. In many cells, LPlasC induced arrhythmias with different patterns and severity that varied from cell to cell. For example, 1 μM LPlasC elicited mild arrhythmias in one cell (Fig. 5A), whereas it induced more severe arrhythmias in another cell (Fig. 5B). In Fig. 5A, inset, potentiated contractions were preceded by early aftercontractions and oscillations. In contrast, primarily delayed aftercontractions were preceded by early aftercontractions and oscillations. In contrast, primarily delayed aftercontractions were observed in the cell shown in Fig. 5B. Figure 5C shows Ca\$^{2+}\$ transients during LPlasC-induced arrhythmias; an apparent increase in systolic Ca\$^{2+}\$ was followed by Ca\$^{2+}\$ transients in a pattern consistent with early and delayed aftercontractions 2 min after exposure to LPlasC. Figure 5C also shows that SR
Ca\(^{2+}\) load was reduced after the arrhythmia but gradually returned to the control level upon removal of LPlasC. When the concentration of LPlasC was increased to 10 \(\mu\)M, severe arrhythmias and contracture occurred in <1 min in all four cells tested (data not shown).

**Effects of LPlasC on \(I_{\text{Ca,L}}\) in patch-clamped ventricular myocytes.** We next examined whether LPlasC-elicted increases in systolic \(Ca\) and postcaffeine \(Ca^{2+}\) influx result from an enhancement of \(I_{\text{Ca,L}}\). In a perforated-patch configuration, \(I_{\text{Ca,L}}\) was monitored before, during, and after exposure to LPlasC in normal Tyrode solution. Figure 6A shows that the \(I-V\) curve of peak \(I_{\text{Ca,L}}\) was increased \(-10\%\) (measured at \(+10\) mV) in 20 s and reached a maximum \((-20\%,\) measured at 0 mV) after 80–130 s of exposure to 1 \(\mu\)M LPlasC. In addition, it seemed that LPlasC had a profound stimulatory effect at potentials between \(-20\) and 0 mV; e.g., it shifted the maximum peak \(I_{\text{Ca,L}}\) in response to depolarizing pulses from \(+10\) to 0 mV. Figure 6A also shows that LPlasC gradually increased peak outward current (measured at \(+60\) mV) and caused a leftward shift in the zero-current potential. The LPlasC-induced changes in \(I_{\text{Ca,L}}\) were reversible after washout (data not shown); however, this was preceded by a transient increase in current amplitude (rebound stimulation of \(I_{\text{Ca,L}}\); Fig. 6A). Superimposed selected current traces (measured at \(-10, 0,\) and \(+10\) mV) recorded before, during, and after exposure to LPlasC are shown in Fig. 6B. Summarized data show that LPlasC increased \(I_{\text{Ca,L}}\) by 22 \(\pm\) 3\% \((n = 5)\) within 1–2 min, whereas its rebound during LPlasC removal was 34 \(\pm\) 6% \((n = 3)\) above the control level. The LPlasC-induced increase in \(I_{\text{Ca,L}}\) and rebound stimulation are consistent with its inotropic effects, as shown in Fig. 1A.

Measurement of \(I_{\text{Ca,L}}\) and its rebound using the perforated whole cell patch-clamp configuration was often very difficult because of cell contracture and death. Thus some experiments were carried out in the conventional whole cell configuration. Figure 6C shows the \(I-V\) relationships of \(I_{\text{Ca,L}}\) (normalized to the peak amplitude of \(I_{\text{Ca,L}}\) at \(+10\) mV before LPlasC exposure) before, during, and after exposure to 1 \(\mu\)M LPlasC. Similar to findings obtained using the perforated-patch configuration, the results show that LPlasC increased \(I_{\text{Ca,L}}\) by 35 \(\pm\) 8% in 2 min \((n = 7, P < 0.002)\) and induced a rebound activation by 54 \(\pm\) 23% \((n = 4)\) upon its removal. In contrast to results in the perforated-patch recordings, no significant shift in the \(I-V\) curve of \(I_{\text{Ca,L}}\) (between \(-20\) and 0 mV) was observed in the whole cell configuration.

LPlasC-induced changes in \(I_{\text{Ca,L}}\) were also examined in \(Na^{+}\)- and \(K^{+}\)-free solutions to minimize the contamination by currents associated with \(Na^{+}\) and \(K^{+}\). Results showed that LPlasC increased \(I_{\text{Ca,L}}\) 1.07 \(\pm\) 0.03 \((n = 3)\), 1.12 \(\pm\) 0.02 \((n = 11)\), and 1.23 \(\pm\) 0.06 \((n = 5)\)-fold at 0.1, 1, and 2 \(\mu\)M, respectively. The increased \(I_{\text{Ca,L}}\) was then followed by a reduction to 68.6 \(\pm\) 4.6% \((n = 7)\) of the control level in the continued presence of 1 \(\mu\)M LPlasC. Subsequent removal of LPlasC also induced a transient rebound activation of \(I_{\text{Ca,L}}\) by 37.1 \(\pm\) 8.6% \((n = 6)\), similar to that observed in the presence of \(Na^{+}\) and \(K^{+}\), as shown in Fig. 6. The LPlasC-induced changes in the \(I-V\) relationship of \(I_{\text{Ca,L}}\) in \(Na^{+}\)- and \(K^{+}\)-free conditions were also comparable to that observed in normal Tyrode solutions. Meanwhile, outward currents were increased by 83.7 \(\pm\) 8.3% \((n = 8,\) measured at \(+60\) mV) during a long exposure (>2 min) to LPlasC. We also used \(Ba^{2+}\) as the charge carrier to replace \(Ca^{2+}\) in the perfusion solution to minimize \(Ca^{2+}\)-activated changes in current measurements and further confirm the LPlasC-induced changes in \(I_{\text{Ca,L}}\). Under these conditions, 1 \(\mu\)M LPlasC elicited a transient increase in \(I_{\text{Ba}}\), followed by a 30% decline after 3 min. Removal of LPlasC caused a 27% rebound activation of \(I_{\text{Ba}}\) above the control level.

The effects of LPlasC on the kinetics and steadystate activation and inactivation of \(I_{\text{Ca,L}}\) were then examined using the conventional whole cell configuration. \(I_{\text{Ca,L}}\) was more stable in normal Tyrode solution during exposure to LPlasC; thus its inactivation was analyzed in normal Tyrode solution. The inactivation of \(I_{\text{Ca,L}}\) was best characterized as a double-exponential function, consistent with findings reported previously in \(Na^{+}\)- and \(K^{+}\)-free solutions containing 2 mM \((\text{Ca}^{2+})\) or \(\text{Ba}^{2+}\) (17). Results show that LPlasC accelerated the fast time constant \((\tau_{f})\) from 4.2 \(\pm\) 0.3 to 3.7 \(\pm\) 0.4 ms, \(n = 6, P < 0.05,\) paired \(t\)-test) but slowed the slow time constant \((\tau_{s})\) from 19.6 \(\pm\) 0.8 to 24.3 \(\pm\) 0.8 ms, \(n = 6,\) \(P < 0.005\) of \(I_{\text{Ca,L}}\) inactivation in response to a voltage pulse to \(+10\) mV. These results are consistent with
those observed in perforated-patch recordings (see Fig. 6B). In contrast, LPlasC increased both $\tau_\text{Ba}$ and $\tau_\text{Ca,L}$ by 15 ± 2 and 5 ± 2%, respectively ($n = 4$). Such discrepancy in effects on the $\tau_\text{Ca,L}$ and $\tau_\text{Ba}$ inactivation probably results from the initial increase in $I_{\text{Ca,L}}$ induced by LPlasC, which enhances $\text{Ca}^{2+}$-dependent inactivation.

Effects of LPlasC on the voltage dependency of steady-state inactivation ($f_\text{ss}$) and activation ($d_\text{ss}$) of $I_{\text{Ca,L}}$ were determined in Na$^+$- and K$^+$-free solutions before and during exposure for >3 min to 1 $\mu$M LPlasC when $I_{\text{Ca,L}}$ reached a quasi-steady state. Figure 7 shows that LPlasC caused a 5-mV depolarizing shift in holding potential ($V_h$) of $f_\text{ss}$ (from $-17.1 \pm 0.8$ mV, $n = 6$, in control, to $-12.3 \pm 0.8$ mV, $n = 5$, $P < 0.005$, paired t-test) without a significant change in the slope factor ($k$; control: $5.1 \pm 0.2$ mV; LPlasC: $5.8 \pm 0.4$ mV). Interestingly, LPlasC decreased the state of inactivation from 0.25 to 0.46 at $50$ mV (i.e., after a prepulse) and $I_{\text{Ca,L}}$ was observed in LPlasC. Results also show that LPlasC elicited a 6-mV depolarizing shift in $V_h$ of $d_\text{ss}$ (from $-1.7 \pm 1.5$ mV, $n = 6$, in control, to $-0.7 \pm 0.8$ mV, $n = 5$, $P < 0.01$; paired t-test) without a significant change in the slope factor ($k$; control: $5.1 \pm 0.2$ mV; LPlasC: $5.8 \pm 0.4$ mV).
5, in control, to +4.5 ± 1.8 mV, n = 5, P < 0.001) with a significant increase in k values (control, 6.4 ± 0.1 mV; LPasC, 7.2 ± 0.3 mV, P < 0.05). The LPasC-induced depolarizing shift in d resulted in an increase in I_{C_{a,L}} window current.

Effects of LPasC on steady-state membrane currents. Figure 8A demonstrates that nearly identical quasi-steady-state I-V relationships were generated using voltage-pulse or voltage-ramp protocols in a ventricular myocyte. With the use of the voltage-ramp protocol, Fig. 8B shows I-V relationships of the steady-state membrane current before, during, and after exposure to 1 μM LPasC. Results show that, after 5 min of exposure, LPasC caused a 60–80% increase in the steady-state outward current in the voltage range between +40 and +80 mV without altering I_{K1} (measured between −70 and −110 mV). Similar to its effect on the peak outward current, the effect of LPasC on the steady-state outward current was irreversible. Comparable results were obtained using the perforated-patch configuration in which 1 μM LPasC increased the steady-state outward current (measured at +60 mV) 2.1 ± 0.2-fold without significant change in I_{C_{a,L}} window current.

Effects of LPasC on AP and cell contraction in patch-clamped ventricular myocytes. Figure 9 shows selected traces obtained from continuous, simultaneous recordings of AP (A) and contraction (B) in a myocyte before, during, and after exposure to LPasC. Exposure for 3 min to 0.1 μM LPasC caused 35 and 16% prolongations of AP duration at 25 and 75% of repolarization, respectively (Fig. 9A), concomitant with a small increase in systolic shortening (Fig. 9B). A subsequent increase in the concentration of LPasC to 1 μM resulted in a substantially prolonged AP duration accompanied by a ∼10-mV depolarization of diastolic membrane potential and a 20% increase in the magnitude of contraction at 40 s. When the diastolic membrane potential depolarized dramatically toward −20 mV in the presence of LPasC (e.g., an afterdepolarization was observed at 45 s), contraction became smaller. The basal (diastolic) level of contraction was increased...
gradually with time during exposure to LPlasC, consistent with CS measured in intact cells (Fig. 1A) and the increased diastolic Ca\textsubscript{i} (Fig. 2B). Upon rapid removal of LPlasC, AP partially recovered, whereas cell contraction was transiently increased by 134% before returning to the control level, similar to that shown in Fig. 1A. Similar results were observed in three other experiments.

Although the time course and exact pattern of LPlasC-induced changes in AP configuration varied from cell to cell, LPlasC-induced changes in AP duration were consistent with our previous findings (20). The occurrence of diastolic membrane potential depolarization (toward approximately −20 mV) and afterdepolarization was 68 and 53%, respectively, in 1 μM LPlasC (n = 19) and increased to 100 and 82%, respectively, during exposure to 2–10 μM LPlasC (n = 11). The LPlasC-induced membrane depolarization was also concentration and time dependent, e.g., depolarization to −29.5 ± 5.2 mV (n = 9), −22 ± 4.0 mV (n = 5), and −13.3 ± 1.4 mV (n = 3) was observed at 1, 3, and 10 μM, respectively. The onset of membrane depolarization induced by 10 and 1 μM LPlasC occurred within 15 s and 3 min, respectively. Afterdepolarizations (without sustained depolarization) were observed at 0.1 μM LPlasC in only one out of nine experiments. When afterdepolarization and membrane depolarization occurred, cells tended to round up and die unless LPlasC was removed immediately.

DISCUSSION

LPlasC accumulates after activation of a membrane-associated, Ca\textsuperscript{2+}-independent PLA\textsubscript{2} in ventricular myocytes during short intervals of hypoxia and is a potent arrhythmogenic lipid metabolite (20). With the use of multiple technical approaches, the present study demonstrated that 1) LPlasC elicits positive inotropic, positive lusitropic, and arrhythmogenic effects in adult ventricular myocytes, 2) LPlasC-induced changes in

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**Fig. 6.** Effects of LPlasC on the current-voltage (I-V) relationship of peak I\textsubscript{Ca,L}. A: I-V relationships of I\textsubscript{Ca,L} obtained before, during, and after exposure to LPlasC by applying 25-ms voltage pulses to potentials between −50 and +60 mV from a holding potential of −40 mV using a perforated-patch configuration. The use of 0.2 mM Cd\textsuperscript{2+} to block I\textsubscript{Ca,L} was for clarification of I\textsubscript{Ca,L} in normal Tyrode solution. B: superimposed current traces elicited by voltage pulses to potentials of −10, 0, and +10 mV before (C), during (L), and after (W) exposure to 1 μM LPlasC. Cell membrane capacitance: 94 pF. C: I-V curves of I\textsubscript{Ca,L} obtained before (C), during (L), and after (W) removal of 1 μM LPlasC using a conventional whole cell configuration. I-V curves were constructed by scaling maximum I\textsubscript{Ca,L} to 1 in each cell before exposure to LPlasC.

**Fig. 7.** LPlasC-induced alterations in the voltage dependency of steady-state inactivation and activation of I\textsubscript{Ca,L}. Steady-state inactivation (I) and activation (\(I_{\text{h}}\)) of I\textsubscript{Ca,L} were determined using a gapped double-pulse protocol (see MATERIALS AND METHODS) and curve-fit by Boltzmann equations as follows: \(H_{\text{max}} = \frac{1}{1 + \exp(V - V_{0.5})},\) where \(V_{0.5}\) is the half-maximum inactivation potential and \(k\) is the slope factor for \(I_{\text{h}}\), and \(G_{\text{max}} = \frac{1}{1 + \exp(-(V - V_{0.5})/k)}\), where \(V_{0.5}\) is the half-maximum activation potential for \(I_{\text{h}}\). G is conductance, and \(G_{\text{max}}\) is maximal conductance. Data represent means ± SE from 5–6 experiments.
contractile function are paralleled by its effects on intracellular free Ca\(^{2+}\) concentration and SR function, 3) LPPlasC increases Ca\(^{2+}\) influx and \(I_{\text{Ca,L}}\), and 4) LPPlasC-induced changes in contractile function are accompanied by anticipated effects on AP. The increased Ca\(^{2+}\) influx, SR Ca\(^{2+}\) content, and SR Ca\(^{2+}\) release lead to AP prolongation and augmented Ca\(^{2+}\) transients, thereby increasing contractility and/or inducing arrhythmias.

LPPlasC increases the systolic and diastolic state of cell contraction with maximum effects in 5–6 min, followed by a small decrease. Simultaneous recordings in fura-PE3-loaded cells showed that LPPlasC-induced increases in systolic and diastolic free Ca\(^{2+}\) levels precede its effect on contraction. LPPlasC increases \(I_{\text{Ca,L}}\), and the first electrically elicited Ca\(^{2+}\) transient after SR Ca\(^{2+}\) has been emptied by caffeine, supporting our hypothesis that LPPlasC-induced inotropic effects are initiated by an increase in Ca\(^{2+}\) influx via \(I_{\text{Ca,L}}\). In addition, the time course for CS to reach a quasi-steady state was longer than that for the Ca\(^{2+}\) transient during exposure to LPPlasC (Fig. 2A), suggesting an incremental increase in the Ca\(^{2+}\) sensitivity of contractile proteins. The LPPlasC-induced left shift of the CS-Ca\(_{\text{Ca}}\) trajectory during early relaxation supports this suggestion and is consistent with an increase in myofilament Ca\(^{2+}\) sensitivity demonstrated previously by others using adult rat ventricular myocytes (26).

Our data also show that LPPlasC increased \(I_{\text{Ca,L}}\) by ~12%, smaller than the ~100% increase in the Ca\(^{2+}\) transient of regular twitch and the 43% increase in the first postcaffeine electrically evoked Ca\(^{2+}\) transient.

![Fig. 8. Effect of LPPlasC on steady-state membrane currents in adult ventricular myocytes. A: in conventional whole cell patch-clamp recordings, currents were elicited in normal Tyrode solution using a voltage-step pulse (inset on right) and ramp (inset on left; see MATERIALS AND METHODS) protocol. The I-V curve was constructed by plotting the current amplitude at the end of test pulses (○) vs. corresponding test potentials or the current magnitude in response to a hyperpolarizing voltage ramp (indicated by arrow). B: steady-state whole cell membrane currents elicited using a voltage-ramp protocol (as shown in the inset of A) before (○), during (●), and after (△) exposure to 1 μM LPPlasC for 5–7 min. EtOH, ethanol. Data represent means ± SE.](http://ajpcell.physiology.org/)

![Fig. 9. LPPlasC-induced changes in action potential (AP) and simultaneously recorded contraction. Selected AP (A) and CS (B) traces, which were elicited and acquired at 1 Hz, were obtained at indicated time points before, during, and after exposure to LPPlasC in one experiment of simultaneous and continuous recordings of AP and CS. Continuous traces of the whole experiment were recorded on chart paper. Because the acquisition duration for each trace was set ~255 ms, some APs with a longer duration were only partially acquired.](http://ajpcell.physiology.org/)
Several possibilities could account for this difference in the LPlasC-induced changes in these parameters. First, \( I_{Ca,L} \) might be underestimated because the observed concomitant increase in outward currents masks the true magnitude of the increased \( I_{Ca,L} \). Second, LPlasC-induced prolongation of \( I_{Ca,L} \) inactivation, increases in channel availability and \( I_{Ca,L} \), window currents, and reduction in \( I_{Ca,L} \) steady-state inactivation during depolarization would enhance Ca\(^{2+}\) influx and thereby increase Ca\(^{2+}\) transients to values greater than the measured increase in \( I_{Ca,L} \). Third, LPlasC amplifies calcium-induced calcium release by increasing available SR Ca\(^{2+}\) release and/or the Ca\(^{2+}\) content of SR. This is supported by our data showing increases in the magnitude of caffeine-induced Ca\(^{2+}\) transient, fractional Ca\(^{2+}\) release, and \( +dR/dt_{max} \) elicited by LPlasC. Fourth, LPlasC reduces Ca\(^{2+}\) efflux via sarcolemmal Na\(^{+}\)/Ca\(^{2+}\) exchange, thereby increasing net Ca\(^{2+}\) gain in each cycle. This possibility is supported by our data showing that LPlasC increases the diastolic Ca\(^{2+}\) and shortening level. Fifth, LPlasC decreases Ca\(^{2+}\) buffering power, i.e., the same Ca\(^{2+}\) influx and SR Ca\(^{2+}\) release results in a greater increase in Ca\(^{2+}\). Studies in rat ventricular myocytes have suggested that a decrease in Ca\(^{2+}\) buffering power at elevated Ca\(^{2+}\), such as that induced by caffeine, increases the decay rate of free Ca\(^{2+}\) (10). This seems unlikely because our data showed that LPlasC slows the decay rate of the caffeine-induced Ca\(^{2+}\) transient. Taken together, LPlasC increases Ca\(^{2+}\) influx via L-type Ca\(^{2+}\) channels, SR function, and myofilament Ca\(^{2+}\) responses.

The LPlasC-induced elevation in diastolic Ca\(^{2+}\) could account for the increased diastolic state of contraction. The return of Ca\(^{2+}\) to baseline during diastole depends primarily on SR Ca\(^{2+}\) uptake (contributing 70% and being a fast component) and the normal mode of Na\(^{+}\)/Ca\(^{2+}\) exchange (28% and a slow component) in rabbit ventricular myocytes (2, 3). Our data show that LPlasC increases \( -dCa^{2+}/dt_{max} \), attributable to an enhanced SR Ca\(^{2+}\) uptake, but prolongs the slow phase of the Ca\(^{2+}\) transient, attributable to a reduced Na\(^{+}\)/Ca\(^{2+}\) exchange activity. These changes were paralleled by a LPlasC-induced shift in the decay phase of the Ca\(^{2+}\) transient from a single to a double exponential function. In addition, LPlasC caused a 60% increase in the time constant of the decay phase of the caffeine-induced Ca\(^{2+}\) transient, an indirect measure of sarcolemmal Na\(^{+}\)/Ca\(^{2+}\) exchange resulting from the absence of SR uptake function (2). Moreover, in the presence of 10–15 mM caffeine, the steady state of free Ca\(^{2+}\) during LPlasC exposure was 70 nM higher than that in control (Fig. 4B). Thus, these results support the notion that LPlasC decreases Ca\(^{2+}\) efflux via sarcolemmal Na\(^{+}\)/Ca\(^{2+}\) exchange.

An intriguing finding was rebound stimulation of contraction (Figs. 1 and 9) and \( I_{Ca,L} \) (Fig. 6) observed upon removal of LPlasC in some myocytes. The phenomenon of rebound \( I_{Ca,L} \) stimulation has been shown during withdrawal of ACh (28, 29) and carbachol (CCh), a muscarinic agonist (5). This rebound activation of \( I_{Ca,L} \) was suggested to be responsible for the initiation of delayed afterdepolarizations in cat atrial myocytes (29) and to result from an increase in cAMP that is mediated by nitric oxide-induced cGMP-mediated inhibition of phosphodiesterase (29). In contrast, a study in ferret right ventricular myocytes showed that a cGMP-dependent pathway is not involved in the rebound \( I_{Ca,L} \) stimulation observed upon removal of CCh (5). In the present study, the rebound activation of contraction and \( I_{Ca,L} \) upon LPlasC removal was paralleled by a rapid recovery of AP duration from the preceding shortened AP duration. Interestingly, we have not observed any rebound stimulation of Ca\(^{2+}\) transients under the same experimental conditions. Comparable changes in \( I_{Ca,L} \) were obtained using perforated- and conventional patch-clamp recordings; however, the role of intracellular second messengers in LPlasC removal-induced rebound could not be completely excluded and requires further investigation.

LPlasC at 1 \( \mu \)M also elicits arrhythmias in intact myocytes, fura-loaded cells, and patch-clamped cells. The pattern of arrhythmia is consistent with a combination of early and delayed afterdepolarizations. LPlasC-induced arrhythmias apparently result from an increase in free Ca\(^{2+}\) and membrane potential depolarization. Both early and delayed afterdepolarizations can be elicited by Ca\(^{2+}\) overload (for review, see Ref. 7). Our data show that LPlasC increases SR Ca\(^{2+}\) release and could thereby cause depolarization of membrane potential and delayed afterdepolarization, as demonstrated by others (24). Thus LPlasC-induced Ca\(^{2+}\) overload could account for its arrhythmogenic effect.

It is also worth mentioning that the LPlasC-induced increase in \( I_{Ca,L} \) was detected in 1–2 min and followed by a decline, whereas contraction and Ca\(^{2+}\) transients continue to rise to a plateau in 5–6 min, followed by a reduction. The discrepancy in the time course of LPlasC-induced changes in these two parameters could have resulted from a disturbance of the cell membrane in patch-clamped myocytes, which becomes more severe during LPlasC exposure. For example, the arrhythmias induced by LPlasC are more severe and occur more often in perforated-patch-clamped cells than in those with the conventional whole cell configuration or in intact myocytes. Combined effects of lipid metabolites and ionophores (nystatin and amphotericin B) could account for the disruption of membrane stability. In some cells, increases in peak and steady-state outward currents were observed during prolonged exposure to LPlasC (Fig. 6, A and B). This could have been mediated by Cl\(^{-}\) currents and/or a nonselective current because the zero-current potential was shifted from −70 mV to potentials between −20 and 0 mV with a relatively linear I-V relationship, a current similar to that reported by others (6). Thereafter, myocytes rarely recovered. In addition, the glibenclamide-sensitive ATP-sensitive K\(^{+}\) current (\( I_{K,ATP} \)) was observed in some cells during and after exposure to LPlasC and could have been responsible for the observed ineffectuality of myocytes with an extremely short AP duration and a membrane potential of ap-
proximately −80 mV (unpublished data). LPlasC-associated changes in AP configuration are determined by the net balance of its effects on all membrane currents. Because of the high membrane resistance that exists at the negative slope between −60 and −20 mV of the I-V curve in rabbit ventricular myocytes, a small increase in inward currents (e.g., \(I_{\text{Ca,L}}\) or nonspecific current) can depolarize membrane potential. On the other hand, an increased outward \(K^+\) current (e.g., \(I_{\text{K,ATP}}\)) can drive the membrane potential toward \(K^+\) equilibrium potential. This could account for the observed unstable AP configurations and delayed afterdepolarizations induced by LPlasC. Lipid metabolites, including LPlasC, have been suggested to alter the lipid microenvironment of ion channels (15). The distinct physical and chemical properties of LPlasC and its distribution in membrane phospholipids could alter lipid-protein interactions, thereby leading to alterations in gating properties of ion channels and/or transporters (15). Although measurements of membrane currents and APs in patch-clamped cells provide important information, lipid metabolite-induced disturbances in cell membrane structure and function make the correlation with physiological measurements in intact cells more difficult.

In comparison with other amphiphilic metabolites, LPlasC exerts distinct effects on cardiac contractile and electrical properties at 1 \(\mu M\), a concentration comparable to those observed in the plasma of animal models of myocardial ischemia or in coronary sinus effluent from patients with ischemic myocardium (21). Similar to LPlasC, palmitoylcarnitine (but not LPC) has been reported to elicit a transient increase in \(I_{\text{Ca,L}}\) in guinea pig ventricular muscle measured in normal Tyrode solution, consistent with its positive inotropic effect (1). In contrast, a study in rabbit ventricular myocytes suggested that 5 \(\mu M\) palmitoylcarnitine decreases \(I_{\text{Ca,L}}\) 10 min after exposure (30). LPlasC (1 \(\mu M\)) appears to have more profound effects on \(I_{\text{Ca,L}}\) than palmitoylcarnitine (5 \(\mu M\)). In contrast to other studies, we found that most myocytes could not survive in LPlasC at concentrations of >3 \(\mu M\) for ≥3 min in patch-clamp experiments. Thus LPlasC appears to be more potent at causing arrhythmias and cell death than LPC (6) and palmitoylcarnitine (30).

In summary, the present data show that LPlasC causes increases in \(I_{\text{Ca,L}}\), intracellular free \(Ca^{2+}\), and myofilament \(Ca^{2+}\) sensitivity, prolongs AP duration, and augments contractility. Such changes are often followed by early and/or delayed afterdepolarizations and sustained membrane depolarization, resulting from an abnormal AP duration that is probably mediated by intracellular \(Ca^{2+}\) overload. Myocytes would eventually become inexcitable because of substantial depolarization or hyperpolarization, probably resulting from LPlasC-induced nonspecific currents or \(I_{\text{K,ATP}}\), respectively. Such rapid, dramatic electrophysiological and mechanical changes in the heart function could occur under many pathophysiological conditions, including ischemia/reperfusion, hypoxia, cytokine-related cardiac dysfunction, and sudden cardiac death.

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