Original Paper

Electrophysiological Effects of Lysophosphatidylcholine on HL-1 Cardiomyocytes Assessed with a Microelectrode Array System

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Key Words

HL-1 cardiomyocytes • Lysophosphatidylcholine • Microelectrode array system • Electrophysiology • Field potential

Abstract

Background: Sudden death due to malignant ventricular arrhythmias is the most important cause of death in acute myocardial infarction. Improved knowledge about the pathophysiology underlying these arrhythmias is essential in the search for new anti-arrhythmic strategies. Lysophosphatidylcholine (LPC), a hydrolysis product of (membrane) phospholipid degradation, is one of the most potent pro-arrhythmic substances that accumulate in the human heart during myocardial ischemia. The aim of this study was to set up and validate an *in vitro* experimental system for studies on the effects of LPC on electrophysiological parameters in beating cardiomyocytes. **Methods and Results:** Spontaneously beating HL-1 cardiomyocytes were cultured on multielectrode array microchips for three days for the recording of electrical activities in the form of field potentials (FP). FPs were recorded at baseline and after addition of 2, 4, 8, 12, 16, 20, and 24 μM of LPC to the cell medium ($n=9$). We found that LPC could induce rapid effects on electrical parameters in the HL-1 cells. The overall half-maximal effective concentration (EC_{50}) of LPC was around $12\mu\text{M}$. The beating rate and peak-peak amplitude of FP thus decreased at concentrations $\geq 12\mu\text{M}$ and were inversely proportional to increased LPC concentration. The duration of FP was significantly prolonged with LPC above $12\mu\text{M}$ and was concentration-dependent. LPC delayed signal propagation, an effect which was mimicked by blocking gap junctions with heptanol and attenuated by pre-treatment with isoprenaline and atropine. Finally, asynchronous activity was induced by LPC at $>12\mu\text{M}$. **Conclusions:** LPC induced prompt and pronounced electrophysiological alterations that may underlie its observed pro-arrhythmic properties. Our *in vitro* model with HL-1 cells and microelectrode array system may be a useful tool for preclinical studies of electrophysiological effects of various pathophysiological concepts.

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Introduction

Malignant ventricular arrhythmias are the major cause of sudden death in acute coronary syndromes and heart failure [1]. In ischemia, these arrhythmias occur early after the onset of myocardial ischemia and are therefore difficult to treat timely [2]. Understanding and prevention of lethal arrhythmias is an important research and clinical objective. In order to achieve this goal we need relevant experimental models.

Prior studies suggest that early-phase arrhythmic events are caused by accumulating metabolites in the ischemic myocardium [3]. Lysophosphatidylcholine (LPC) is an endogenous amphiphilic product of membrane degradation produced through the action of phospholipase-A₂ (PLA₂). Under normal physiological conditions, accumulation of LPC is precluded by reacylation and hydrolysis [4]. In the setting of myocardial ischemia, activity of PLA₂ increases while reacylation and washout of LPC decreases. Within the first few minutes after the onset of ischemia, LPC increases by 50-60% with a protracted normalization [5, 6]. LPC exerts various pro-arrhythmic effects. It inhibits fast Na⁺ and inwardly rectifying K⁺ currents [7, 8]. LPC causes intracellular Ca²⁺ accumulation and decreases cell-to-cell coupling [9-12]. The consequences of these alterations are slowed conduction and abnormal rhythmic activity (i.e. triggered activity and delayed after-depolarization, facilitating intramyocardial re-entry).

There are several *in vitro* and *in vivo* experimental models for studies of electrophysiological mechanisms. Patch-clamp techniques are suitable to study specific ion channels and the technique can be applied to whole cells. A significant limitation is the invasiveness of the method on the cellular level and the fact that it is limited to a single cell or a membrane patch. Optical mapping methods are less invasive and can be performed in living cellular syncytia including Langendorf preparations, but involve fluorescent bleaching and are sensitive to methodological artefacts. Microelectrode array recording (MEA) is an *in vitro* system for extra-cellular measurements of currents in the form of field potentials (FP) [13]. FP have been extensively studied and it has been shown that the information gained from analysis of FP closely correlates to the information gained by more invasive methodology i.e. intracellular recordings [14]. Recent technological developments in the field of microelectronic fabrication and amplifier design have improved the signal to noise ratio and the quality of recordings by MEA are now comparable to patch-clamp recordings. Furthermore, MEA technology offers the possibility to analyze impulse-generation, impulse-propagation, wave front velocity, and arrhythmic activity simultaneously [15].

The aim of this study was to set up and validate an experimental *in vitro* model for studies of electrophysiological alterations during LPC challenge of cardiomyocytes (HL-1 cells) using MEA technology.

Materials and Methods

HL-1 cell culture

HL-1 cell lines, which are derived from immortalized murine atrial cells, were a gift from Dr. William Claycomb (Louisiana State University Medical Center, New Orleans, LA). They express various ion channels, including Na⁺, K⁺ and Ca²⁺ channels and have a phenotype similar to adult atrial cardiomyocytes [16]. The cells were grown in Claycomb medium (JRH Biosciences, KS) supplemented with 10% fetal bovine serum (JRH Biosciences, KS), 2 mM L-glutamine, 100 μM norepinephrine, 100 U/ml penicillin and 100 μg/ml streptomycin on flasks pre-coated with gelatin-fibronectin (BD Biosciences, PA). The medium was changed every 24 h. In the experiments the cells were kept at 37°C in room air. Under these circumstances the cells are stable for long periods of time, far exceeding the time frame of the pharmacological challenge.

Solution preparations for LPC experiment

1-Palmitoyl-*sn*-glycero-3-phosphocholine, i.e. LPC-16 (in this article referred to as LPC), was purchased as powder (>99% purity) (Sigma-Aldrich, St. Louis, MO, USA). In order to solubilize LPC in phosphate-

buffered saline (PBS) as micelles, it was first dissolved in chloroform/methanol and then dried under a stream of nitrogen, re-suspended in PBS, and sonicated [17]. LPC solutions were prepared in different concentrations by diluting the LPC solution in cell culture medium. LPC was added to the cell medium and stirred gently with a pipette before recording.

Atropine and isoprenaline (ISO) were obtained in clinically available ampules. Heptonal was purchased from Sigma-Aldrich (H3003, Sweden). These agents were added directly into cell culture medium at each desired testing concentration.

Protocol of pharmacological treatment in LPC experiment

HL-1 cells were exposed to LPC at 2 μ M, 4 μ M, 8 μ M, 12 μ M, 16 μ M, 20 μ M and 24 μ M, respectively. All concentrations were tested in one MEA plate and washout was performed 3 times with cell media between each two neighbouring concentrations. Electrical signals were recorded before and 3 min after LPC additions of each concentration (Fig. 1A).

In subsequent experiments, HL-1 cardiomyocytes were pre-treated with muscarinic receptor antagonist (Atropine, 50 μ M) or β -adrenoceptor agonist (isoprenaline, ISO, 10 μ M) 3 min before LPC challenge. The atropine/isoprenaline cell medium was removed before adding LPC. Finally, we tested heptanol (a selective gap-junction blocker) at 200, 250, 500, 600 and 700 μ M in the same manner as LPC. Heptanol at 800 μ M, put out signals completely and no analysis at this dose was possible.

MEA recordings

HL-1 cardiomyocytes (100 000-250 000) were seeded on sterile gelatine-coated MEA plates. This coating was chosen because it was superior both to no coating and to gelatine/fibronectin coating as judged by better cell adherence and better signal quality in the MEA recordings. The cells were allowed to grow for three days in culture until homogenous growth and synchronous beating was observed in light microscopy. Cell cultures not showing this pattern, i.e. those showing insufficient growth, overgrowth, or no beating, were discarded. After the attachment and confluence of beating HL-1 cardiomyocytes, extracellular recordings were performed using a MEA data acquisition system (Multi Channel Systems, Reutlingen, Germany). The standard substrate-integrated MEA culture dish contains 60 Titanium Nitride coated gold electrodes (30 μ m diameter) arranged in an 8x8 electrode grid with an inter-electrode distance of 200 μ m. The system allows simultaneous recording of extracellular potentials from all electrodes at a sampling rate of 10 kHz. The integral heating device maintains the temperature stable at 37°C.

Analysis of MEA recordings

All data were analyzed off-line using dedicated software MC_Rack version 3.9 (Multi Channel Systems, Reutlingen, Germany) or a customized toolbox programmed with MATLAB (The Mathworks, Natick, MA, USA). The following parameters were analyzed as previously described [18]: beating rate (BR), FP duration (FP_{dur}), rise time of FP upstroke (FP_{rise}) and peak-peak FP amplitude (FP_{AMP}). The nomenclature is demonstrated in Fig. 1B. FP_{rise} is the time span required for the decline of the voltage from baseline to FP_{Min} (Fig 1B). It has been demonstrated that FP_{rise} correlates well with the upstroke of the action potential [19].

Within a single MEA chip, FP morphologies varied between electrodes and FP_{Max} (the last positive peak in FP) was not always easily detected in the offline analysis. One specific electrode with a clearly detectable FP_{Min} and FP_{Max} was chosen for analysis; 20 successive FPs from this electrode were averaged to derive mean values for the predefined parameters. The pharmacological effects of tested compounds on electrophysiological properties of HL-1 cells can be revealed by the repeated, comparative analysis of these electrical parameters on the same electrode before and after the compound addition within the same preparation. In addition, a concentration-effect curve for each parameter was developed after normalization for the parameters at baseline. The half maximum effect concentration (EC_{50}) was then derived by fitting the concentration-effect curve with Boltzmann function.

Propagation velocity was evaluated as previously described [20]. We included the MEA plates that had initial signals (pacemakers) lying at one of the four edges of the 8x8 electrode matrix. The total propagation distance was a constant of around 1400 μ m. Propagation velocity was then evaluated in the form of propagation time, i.e. the time required for signals propagating from the "pacemaker" site to the other edge of the electrode matrix (Fig. 6D).

Statistical analysis

Data are presented as the mean $\pm$ S.E.M. Repeated measures ANOVA was used for the analysis of FP parameters after the exposure to the different LPC and heptanol concentrations. Concentration-effect was

Fig. 1. A) Screen-dump from MC Rack (recording software for MEA system) with typical field potential (FP) signals from the HL-1 cell syncytium. Each square in the data window represents the corresponding unipolar FP recording from a single electrode. Observe various forms, amplitude and noise in the signals. B) Magnification of a clearly recorded FP with standardized nomenclature. Beating rate is calculated as signal spikes per second.

fitted with Boltzmann function for calculation of half maximum effect concentration (EC_{50}). Computer software IBM SPSS Statistics 19.0 (IBM Corporation, Somers, NY) and Origin 8.0 (Northampton, MA) were used for analysis of the data. Statistical significance was defined as $P < 0.05$.

Fig. 2. Example of the effects of lysophosphatidylcholine (LPC) on beating rate (BR). A) Single electrode (FP) recording at different concentrations of LPC. Note that with increased concentration of LPC there is significant decrease in BR. B) Concentration-effect curve fitted with a Boltzmann function. * $P < 0.05$ compared with baseline.

Results

Properties of spontaneous electrical activity in the HL-1 syncytium under basal conditions

After ~3 days of growth in culture, spontaneously and synchronously beating HL-1 cells were present on the MEA plates. The cells produced characteristic FP signals (Fig. 1A). The signals were sustained for hours and did not change over time under experimental conditions. However, within a MEA plate, the morphologies of FP from the 60 electrodes varied (Fig. 1A). This variability in FP morphologies within one MEA chip can be attributed to several factors including variable relative position between electrode and cell, confluence state of the cells, inconsistent signal propagation direction among electrodes, damages to some electrodes, etc. However, we found that there was only one dominant pacemaker detected within a single MEA plate during the experiments, and no significant difference was documented in BR among individual electrodes. In one MEA plate, many cells may have pacemaker properties, but only the one with the highest intrinsic frequency (automaticity) acts as pacemaker. Signals recorded from the other electrodes in the plate therefore share the same BR as the effective

Fig. 3. Example of the effects of lysophosphatidylcholine (LPC) on field potential amplitude (FP_{AMP}). A) A representative FP at different LPC concentrations. The low dose (8 μM) increased the signal amplitude (non-significantly) but the amplitude then decreased significantly with higher doses. B) Concentration-effect curve fitted with a Boltzmann function. * $P < 0.05$ compared with baseline.

pacemaker. FP_{AMP} showed a large variability in individual electrodes, within and between MEA plates (Fig. 1A). Among those plates tested, the largest range of FP_{AMP} within a chip was between 9 and 890 μV . In contrast, there was no significant difference of FP_{dur} between electrodes within a MEA plate. Due to the homogeneity of HL-1 cardiomyocytes [16], HL-1 syncytium within a plate shares the same pattern of depolarization and repolarization and thus show the same duration of FP once they are electrically activated. However, FP_{dur} may be incorrectly measured using automatic calculation with MC_Rack software or Matlab programmed tools if FP's with undetectable FP_{Max} are included in the analysis.

Electrophysiological effects of LPC on HL-1 cells

Addition of LPC had a concentration-dependent slowing effect on the BR of HL-1 cells (Fig. 2A). BR decreased steadily with increasing concentrations of LPC. EC_{50} for BR was $11.47 \pm 1.05 \mu M$ (Fig. 2B). Similarly, LPC caused a concentration-dependent decrease in FP_{AMP} (Fig. 3A, B). EC_{50} for FP_{AMP} was $14.20 \pm 1.60 \mu M$. FP_{dur} increased significantly at higher

Fig. 4. Effects of lysophosphatidylcholine (LPC) on field potential duration (FP_{dur}). A) A representative FP at different LPC concentrations. Note prolongation of FP_{dur} with increasing LPC concentration. Here only individual FP's are depicted, but the FP_{max} is more accurately detected by averaging FP's. B) Concentration-effect curve fitted with a Boltzmann function. * P<0.05 compared with baseline.

LPC concentrations with EC₅₀ of 12.06±1.78 μM (Fig. 4). For all of the above parameters we noticed, at the lower concentrations of LPC, an initial trend in the opposite direction to the main finding (i.e. increase in BR and FP_{AMP} and shortening in FP_{dur}) Figs. 2B, 3B and 4B.

At LPC concentrations above 12 μM we saw an increase in the incidence of premature beats (from outside the pacemaker region) and asynchronous beating patterns in the HL-1 syncytium (Fig. 5). The asynchronous beats were polymorphic (of different morphologies) and had irregular coupling intervals. FP_{rise} was prolonged in a concentration-dependent way. The prolongation of FP_{rise} was significant at doses of >8 μM (Fig. 6A). EC₅₀ for FP_{rise} was 12.74 ±1.29 μM.

Delayed signal propagation in the HL-1 syncytium after LPC

We included a total of 5 plates in the analysis of signal propagation. During LPC administration there was no change in the direction of propagation, but a concentration-dependent prolongation in signal propagation time (Fig. 6B). The maximum propagation time at the highest concentration of LPC (24 μM) was delayed by 52.25 ±10.07 ms compared to baseline (P< 0.05).

The delayed signal propagation might be attributed to either decreased fast influx of Na⁺ or inhibition of gap junctions. We therefore conducted experiments by blocking gap junctions with heptanol. At baseline there was no significant difference in propagation time between heptanol and LPC experiments. The propagation time was significantly increased by heptanol in a concentration-dependent manner (Fig. 6C, D). The maximum delay induced by heptanol was, however, significantly less than that induced by LPC. This suggests that the LPC-induced signal propagation delay may not be exclusively due to blockage of gap junctions, but may partly be related to slower cellular depolarization (i.e. prolonged FP_{rise}). At low doses of LPC (2-8 μM), when there was no prolongation of FP_{rise}, the propagation time showed a trend towards delay, possibly due to impaired function of gap junction. Heptanol did not significantly modify FP_{AMP} and FP_{dur} at the tested concentrations (Fig. 6E), in contrast to LPC administration, but decreased BR.

Fig. 5. Snap shots from a single electrode during lysophosphatidylcholine (LPC) challenge. At higher concentrations of LPC, extra beats are seen with varying coupling intervals and morphology.

Isoprenaline and atropine induced attenuation of LPC effects

In order to further explore the negative chronotropic effects (i.e. decreased BR) of LPC on the HL-1 syncytium and the contribution of β -adrenergic receptors and/or muscarinic receptors, we first pre-treated HL-1 cells with ISO at 10 μ M for 3 min before LPC application. 10 μ M of ISO has been verified to have a strong positive chronotropic effect on HL-1 cells in our previous experiments. ISO pre-treatment did not alter the effects of LPC on FP parameters (i.e. BR, FP_{AMP} and FP_{dur} ; data not shown). However, we noticed that the LPC-induced decrease in propagation velocity was significantly attenuated after ISO pre-treatment (Fig. 7). On the other hand, ISO could not reverse the already reduced signal propagation velocity induced by LPC when administered afterwards (data not shown). Similarly, we tested the effects of muscarinic receptor blocker pre-treatment with atropine. We first defined the effective concentration of atropine and found that 50 μ M had a strong positive chronotropic effect on HL-1 cardiomyocytes. As for ISO, we showed that pre-treatment with atropine significantly attenuated the LPC-induced delay in signal propagation while no other apparent effects were observed on LPC-induced changes of the FP parameters.

Discussion

According to previous research LPC, which is accumulated during ischemia, has profound electrophysiological effects on cardiomyocytes. The aim of this study was to set up and validate an experimental *in vitro* model for studies of electrophysiological alterations during LPC challenge of cardiomyocytes (HL-1 cells) using MEA technology. We demonstrated *in vitro* inhibition of LPC on several electrophysiological properties of HL-1 cardiomyocytes and an attenuation of LPC-induced signal propagation delay with the pre-treatment of ISO and atropine. This *in vitro* model with HL-1 cells and MEA system may thus be a useful tool for preclinical studies of electrophysiological effects of different pathophysiological concepts, including myocardial ischemia.

Fig. 6. Effects of lysophosphatidylcholine (LPC) and heptanol on field potential rise time (FP_{rise}) and signal propagation time. A) and B) Concentration-effect propagation time and rise time curves for LPC fitted with a Boltzmann function. C) Concentration-effect propagation time for heptanol fitted with a Boltzmann function. D) Representative colour maps showing slowed propagation with heptanol. E) Effect of heptanol on beating rate (BR), field potential amplitude and duration (FP_{AMP} and FP_{dur} respectively) * $P < 0.05$, significant dose-dependent effect.

LPC is an amphiphilic metabolite that is promptly released during myocardial ischemia in mammalian hearts, including the human heart. During ischemia, the production of LPC is increased by the action of phospholipase- A_2 while the catabolism of LPC is markedly reduced resulting in rapid LPC accumulation [6, 21, 22]. Free concentrations of LPC in the ischemic myocardium is typically in the range of 10-30 μM , similar to the concentrations used in

Fig. 7. Pre-treatment effect of isoprenaline (ISO) and atropine on lysophosphatidylcholine (LPC) induced depression of electrical activities of HL-1 cardiomyocytes. There is a significant effect of ISO and atropine in preventing the LPC-induced signal propagation delay. * $P < 0.05$, compared with LPC administration.

our model [23]. Our results are therefore likely to reflect the cellular effects of LPC during myocardial ischemia in the clinical setting.

LPC has been reported to affect several ion-channels. It reduces the upstroke of the action potential - an effect linked to inhibition of the peak sodium current [8]. This causes slowing of conduction, membrane depolarization and post-repolarization refractoriness [24]. Furthermore, LPC increases late inward Na^+ current via the formation of peroxynitrite [25] and inhibits Na^+/K^+ ATP-ase [26], resulting in intracellular Na^+ accumulation and consequently Ca^{2+} accumulation (through the $\text{Na}^+/\text{Ca}^{2+}$ exchanger [9]). These effects prolong action potential duration (APD) and increase APD dispersion - both of which can be pro-arrhythmic and consistent with the present observation. LPC has been demonstrated to have an inhibitory effect on the normal pacemaker activity [27]. Sodium currents along with pacemaker currents (I_f) play an important role in pacemaker activity of the HL-1 cells [28]. In our model, inhibitory effects of LPC on the inward Na^+ current and pacemaker current are reflected by the decrease in FP_{AMP} , BR, propagation velocity, and prolonged duration of FP rise time.

LPC also has complex effects on several potassium currents. The inwardly rectifying K^+ current (I_{K1}) can be inhibited by LPC [7], which would be expected to cause prolongation of the APD. APD has been shown before to correlate well with FP_{dur} [14]. In support, we found that FP_{dur} was prolonged with higher concentrations of LPC. However, at low concentrations of LPC, the delayed rapid rectifier potassium current (I_{Kr}) can be activated and induce shortening of the APD [29]. This may explain the non-significant trend for shortened FP_{dur} observed at lower concentrations of LPC in the present study.

LPC is a potent inhibitor of myocardial gap junction function [30]. We found that signal propagation was significantly delayed by LPC. Compared to heptanol (a selective gap-junction blocker), LPC seems to affect propagation time to a similar extent. The delayed signal propagation caused by gap-junction inhibition is, however, likely to be further aggravated by decreased inward Na^+ current and increased intracellular Ca^{2+} overload [31] induced by LPC.

In additional series of experiments, we unexpectedly found that the LPC-induced delay in the signal conduction of HL-1 cells could almost be completely prevented by pre-treatment with ISO or atropine. The mechanism for this is not clear, but it may be related to ISO-induced potentiation in gap junction function. It has been reported that β -adrenoceptors activation up-regulates gene expressions of Cx40 and Cx43, two important proteins of gap junction in cardiomyocytes, through a protein-kinase A (PKA) dependent pathway [32]. The short pre-treatment period (three minutes) suggests that other mechanisms than up-regulation of connexin genes may be contributing in our system.

In conclusion, this is the first study to examine effects of LPC on HL-1 cardiomyocytes using a multichannel MEA system. We have verified that LPC induces several electrophysiological alterations of the cardiomyocytes. An important advantage of the method is the possibility to analyze the net-effects of all cellular events elicited by experimental perturbation (e.g. caused by addition of an exogenous substance) on electrical properties, frequency, and rhythm, which provides the possibility to perform high-throughput screening of different pro-arrhythmic and anti-arrhythmic substances. The information gathered from this model can be complementary to that obtained from the existing *in vitro* cellular models and *in vivo* animal models.

Among the limitations of our cell model is that immortalized HL-1 cell-line preserves characteristics more similar to atrial than ventricular cardiomyocytes and may differ from normal adult cardiomyocytes. In this study we have chosen one substance (LPC), but several other substances are likely to participate in the electrophysiological changes seen during ischemia. We have neither monitored nor experimentally manipulated pH or electrolyte composition.

The incubation time of LPC on the HL-1 cells is relatively short in the present study. The observed electrophysiological effects of LPC in our model were almost completely reversible by washout. This indicates that LPC is incorporated into the sarcolemma and interact directly with membrane channels and pump, but does not cause irreversible damage to the cell membrane (i.e. electroporation), at least not during short term challenge with LPC.

To further understand the participation of individual ion channels in the observed electrophysiological alterations, various blockers and enhancers of specific ion channels could be utilized in the present model in the future.

Acknowledgements

The study was supported by grants from the Swedish Heart and Lung Foundation, the Swedish Research Council, Gothenburg Medical Society, Sahlgrenska University Hospital, and the Swedish Medical Society.

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