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Original Research Article

Different effects of lysophosphatidic acid on L-type calcium current in neonatal rat ventricular myocytes with and without H_2O_2 treatment

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ABSTRACT

L-type calcium current ($I_{Ca\text{-}L}$) alterations are implicated in various cardiac diseases, and the lysophosphatidic acid (LPA) level increases in several ischemic heart diseases. We investigated the effects of LPA on $I_{Ca\text{-}L}$ in normal and H_2O_2-treated neonatal rat ventricular myocytes. LPA treatment (24 h) increased the action potential duration (APD) and $I_{Ca\text{-}L}$ in normal ventricular myocytes, but it decreased these parameters in H_2O_2-treated myocytes. LPA increased the single-channel open probability of L-type calcium channels in both normal and H_2O_2-treated myocytes. LPA activated calcineurin (CaN) and induced the cytoplasm-to-nucleus translocation of nuclear factor of activated T-cells (NFAT) in H_2O_2-treated cardiomyocytes. In H_2O_2-treated cardiomyocytes, LPA decreased $Ca_v1.2$ mRNA and protein expression levels at 4 and 8 h, respectively. A CaN inhibitor (FK-506) prevented LPA-induced APD, $I_{Ca\text{-}L}$, and $Ca_v1.2$ mRNA and protein down-regulation. The LPA-induced $I_{Ca\text{-}L}$ increase in normal cardiomyocytes was CaN–NFAT signaling-independent, and LPA did not affect $Ca_v1.2$ mRNA or protein expression. In conclusion, LPA increases the $I_{Ca\text{-}L}$ in normal ventricular myocytes by increasing the single-channel open probability of L-type calcium channels, and LPA decreases $I_{Ca\text{-}L}$ in H_2O_2-treated cardiomyocytes via the CaN–NFAT pathway.

1. Introduction

Myocardial infarction is one of the most severe cardiovascular diseases [1,2]. The standard treatment for myocardial infarction is rapid reperfusion, which can lead to myocardial ischemia/reperfusion (I/R) injury [3,4]. I/R injury involves the release of reactive oxygen species (ROS) which are generated in large amounts during reperfusion and contribute to intracellular Ca^{2+} overload [5,6]. H_2O_2 is a major ROS molecule and contributes significantly to cellular injury, and it has been widely used in experiments to mimic ROS injury [7,8].

Lysophosphatidic acid (LPA) is a serum-derived phospholipid capable of activating specific G protein-coupled receptors (GPCRs) to evoke multiple cellular responses, including cell proliferation, cell survival, cell migration, cytoskeletal reorganization and ion transport [9,10]. The effects of LPA have been implicated in numerous pathophysiologic processes, including cancer progression, wound healing and vascular diseases [11,12]. LPA elicits multiple cellular responses by interacting with six known GPCRs termed LPA 1–6 [13,14]. Previous reports have suggested that LPA plays potential roles in the cardiovascular system, such as the promotion of platelet aggregation, stimulation of cardiac fibroblast proliferation, induction of vascular smooth muscle contraction, modulation of myocardial contractility and promotion of cardiac hypertrophy [15–17]. Platelets contribute to the production of LPA [18,19], which in turn stimulates platelet aggregation. LPA accumulates in patients with atrial fibrillation, acute coronary syndrome (ACS) and atherosclerosis [19–22], which are all related to platelet aggregation. Chen et al. reported that the LPA level increases significantly in patients with myocardial infarction (MI) [23]. All of these studies suggest that the concentration of LPA increases following ischemic heart disease. Moreover, LPA participates in the proliferation and apoptosis of cardiac fibroblasts [24], is involved in cardiac remodeling after myocardial infarction [25], and can induce arrhythmia in isolated working rat hearts [26].

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Wei et al. demonstrated that LPA can increase the electrophysiological instability of the adult rabbit ventricular myocardium by augmenting the L-type calcium current ($I_{Ca\text{-}L}$) [27]. In addition, we found previously that LPA increased the $I_{Ca\text{-}L}$ in isolated guinea pig ventricular myocytes (data not shown). However, these experiments were conducted in normal cardiac myocytes, and the potential effects of LPA on the electrophysiological properties of cardiomyocytes after ROS injury have not been investigated. Cardiomyocytes in infarcted and non-infarcted regions exhibit different characteristics [28–31]. Therefore, LPA may play different roles in damaged and undamaged cardiac myocytes after ROS injury. In this work, H_2O_2 was used to experimentally mimic ROS injury, and neonatal ventricular myocytes were used to examine (1) whether LPA exhibits different effects on the $I_{Ca\text{-}L}$ in myocytes with or without H_2O_2 treatment and (2) the possible signaling mechanisms involved.

2. Materials and methods

2.1. Animals and reagents

Neonatal Wistar rats were obtained from the Experimental Animal Center of Jilin University, China. All animal care and experimental procedures complied with the regulations of the ethics committee of Jilin University and the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. All reagents were purchased from Sigma (Saint Louis, MO, USA).

2.2. Cell culture and experimental procedures

Hearts were removed from 1- to 3-days-old rats, and the ventricles were minced and subjected to consecutive digestion with 0.6 mg/ml trypsin. Cell suspensions were centrifuged at 1000 × *g* for 5 min. The pellets were resuspended in DMEM (Gibco, Carlsbad, CA, USA) containing 20% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA), 0.1 mM bromodeoxyuridine, and 1% penicillin–streptomycin. The cell suspensions were pre-plated for 2 h at 37 °C to remove fibroblasts. The ventricular myocytes remaining in suspension were plated on 35 mm plates (Thermo Fisher Scientific, Rochester, NY, USA) and incubated in the above medium at 37 °C in humidified air with 5% CO_2 for 48 h.

If not otherwise stated, we employed un-stimulated cells as a control. Cardiomyocytes were cultured in serum-free media for 12 h prior to treatment. Different LPA concentrations (1, 5, 10 or 20 μmol/L) were then added to the serum-free medium for 2, 4, 8 or 24 h to test action potentials, currents, channel expression levels, CaN activity or NFAT translocation. After the myocytes were exposed to H_2O_2 (200 μmol/L) for 4 h in the H_2O_2-treated groups, the medium was replaced by LPA-containing DMEM (without any H_2O_2), and the myocytes were then cultured with 1, 5, 10 or 20 μmol/L LPA for 2, 4, 8 or 24 h in serum-free DMEM. In some experiments, FK-506 (5 μmol/L) was added to the culture medium and thoroughly washed out before recording APD, $I_{Ca\text{-}L}$, and $Ca_v1.2$ mRNA and protein levels.

2.3. Electrophysiological recordings

The whole-cell patch-clamp technique was used to record action potentials (APs) in current-clamp mode; both the whole-cell and cell-attached patch-clamp techniques were used to record calcium currents in voltage-clamp mode. APs and currents were obtained using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA) and pCLAMP 9.0 software (Axon Instruments, Foster City, CA, USA). Electrophysiological data were filtered at 1 kHz and sampled at 5 kHz. Patch pipettes with resistances of 1–3 MΩ were made from Pyrex borosilicate glass tubes using a horizontal microelectrode puller (Narishige PP-83, Tokyo, Japan) and were used to record calcium currents. The tip resistance was 3–5 MΩ during the AP recordings.

Myocyte APs were measured using a patch pipette filling solution contained in mM: 140.0 KCl, 2.0 EGTA, 1.8 $CaCl_2$, 2.0 $MgCl_2$, 5.0 HEPES and 10.0 glucose. The pH was titrated to 7.4 with KOH. The extracellular solution contained (in mM) 143 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 0.25 NaH_2PO_4 and 5.0 HEPES. The pH was titrated to 7.4 with NaOH. APs were elicited by 2 ms, twice-threshold pulses and were recorded at 1 Hz. The action potential duration (APD) was measured at 50% (APD_{50}) and 90% (APD_{90}) of repolarization. Cardiomyocytes that were not attached to neighboring cells were used for patch-clamp recordings.

The $I_{Ca\text{-}L}$ was measured using Na^+- and K^+-free external and internal solutions to avoid contamination by overlapping ionic currents. The cell membrane was clamped to a holding potential of −40 mV to inactivate the T-type calcium current. The $I_{Ca\text{-}L}$ was measured using 400 ms steps from −40 mV to +50 mV. Voltage pulses (in +10-mV increments) were applied at 10-s intervals to allow adequate time for calcium channel recovery from inactivation. Patch pipettes were filled with a solution containing (in mM) 130 CsCl, 5 EGTA, 5 HEPES and 2 ATP. The pH was titrated to 7.3 with CsOH. The extracellular bathing solution contained 135 mM tetraethylammonium-Cl (TEA-Cl), 5 mM 4-aminopyridine (4-AP), 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES and 10^{-6} mM TTX. The pH was titrated to 7.4 with CsOH. The currents are expressed as densities (pA/pF) to control for cell size variability. Leak currents were corrected by means of the leak subtraction procedure. The junction potential was always compensated for and was always smaller than 5 mV.

Single-channel currents were recorded from the holding potential of −50 mV, and patch membranes were depolarized at 1 Hz to 0 mV for 100 ms. Pipettes were pulled from Pyrex borosilicate glass tubes coated with insulating varnish and subsequently fire-polished. Single-channel recordings were filtered at 1 kHz using the Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA) and were sampled at 10 kHz. Data were acquired using pCLAMP 6.04 (Axon Instruments, Foster City, CA, USA) and a Digidata 1200 AD/DA data acquisition system. The mean open and closed times of the single channels were obtained by a double exponential fitting, and the single-channel open probability was calculated by the following equation: Po = total open time/total recording time. The pipette solution contained (in mM) 100 $BaCl_2$ and 10 HEPES. The pH was titrated to 7.4 with TEA-OH. The extracellular solution contained (in mM) 120 K-aspartic, 20 KCl, 10 glucose, 2 EGTA, and 10 HEPES. The pH was titrated to 7.4 with KOH [32].

2.4. RNA isolation and real-time RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and a sample of the total RNA (1000 ng) was reverse transcribed using the Takara PrimeScript RT Master Mix (TaKaRa, Tokyo, Japan). Reactions in SYBR Premix Ex Taq (TaKaRa, Tokyo, Japan) were performed using an ABI 7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA) with the following primers (Sangon, Shanghai, China): $Ca_v1.2$, sense (5′-AGCAACTTCCCTCAGACGTTTG-3′) and antisense (5′-GCTTCTCATGGGACGGTGAT-3′); and β-actin, sense (5′-CGTAAAGACCTCTATGCCAACA-3′) and antisense (5′-TAGGAGCCAGGGCAGTAAT-3′), as an internal control. Real-time PCR was performed in 20-μl reaction volumes using 10 pmol of primers. cDNA was amplified with an initial cycle of 95 °C for 2 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. A