Cytosolic increased labile Zn^{2+} contributes to arrhythmogenic action potentials in left ventricular cardiomyocytes through protein thiol oxidation and cellular ATP depletion

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ABSTRACT

Intracellular labile (free) Zn^{2+}-level ($[Zn^{2+}]_i$) is low and increases markedly under pathophysiological conditions in cardiomyocytes. High $[Zn^{2+}]_i$ is associated with alterations in excitability and ionic-conductances while exact mechanisms are not clarified yet. Therefore, we examined the elevated-$[Zn^{2+}]_i$ on some sarcolemmal ionic-mechanisms, which can mediate cardiomyocyte dysfunction. High-$[Zn^{2+}]_i$ induced significant changes in action potential (AP) parameters, including depolarization in resting membrane-potential and prolongations in AP-repolarizing phases. We detected also the time-dependent effects such as induction of spontaneous APs at the time of ≥ 3 min following $[Zn^{2+}]_i$ increases, a manner of cellular ATP dependent and reversible with disulfide-reducing agent dithiothreitol, DTT. High-$[Zn^{2+}]_i$ induced inhibitions in voltage-dependent K^+-channel currents, such as transient outward K^+-currents, I_{to}, steady-state currents, I_{ss} and inward-rectifier K^+-currents, I_{K1}, reversible with DTT seemed to be responsible from the prolongations in APs. We, for the first time, demonstrated that lowering cellular ATP level induced significant decreaeses in both I_{ss} and I_{K1}, while no effect on I_{to}. However, the increased-$[Zn^{2+}]_i$ could induce marked activation in ATP-sensitive K^+-channel currents, I_{KATP}, depending on low cellular ATP and thiol-oxidation levels of these channels. The mRNA levels of Kv4.3, Kv1.4 and Kv2.1 were depressed markedly with increased-$[Zn^{2+}]_i$ with no change in mRNA level of Kv4.2, while the mRNA level of I_{KATP} subunit, SUR2A was increased significantly with increased-$[Zn^{2+}]_i$, being reversible with DTT. Overall we demonstrated that high-$[Zn^{2+}]_i$, even if nanomolar levels, alters cardiac function _via_ prolonged APs of cardiomyocytes, at most, due to inhibitions in voltage-dependent K^+-currents, although activation of I_{KATP} is playing cardioprotective role, through some biochemical changes in cellular ATP- and thiol-oxidation levels. It seems, a well-controlled $[Zn^{2+}]_i$ can be novel therapeutic target for cardiac complications under pathological conditions including oxidative stress.

1. Introduction

Ionic zinc (Zn^{2+}) is a constituent of many proteins and enzymes in the human body, which plays important roles in cells and tissues [1]. It has been shown that even mild Zn^{2+}-deficiency has impact on human health, including heart function [2]. In general aspect, Zn^{2+} is not only involved in cellular redox cycle but also has been regarded as relatively nontoxic. However, the recent studies point out how high intracellular labile Zn^{2+} level ($[Zn^{2+}]_i$) can be a potent killer of numerous types of cells [1]. $[Zn^{2+}]_i$ is measured to be less than one-nanomolar under physiological conditions in mammalian cardiomyocytes [3,4], being much less than that of $[Ca^{2+}]_i$. It has been demonstrated that oxidants caused about 30-fold increase in $[Zn^{2+}]_i$ with only 2-fold increase in

$[Ca^{2+}]_i$ [3]. These data point out that high $[Zn^{2+}]_i$ can be considered as much more biologically toxic than that of generally realized. Supporting these above statements, early studies showed that any disruption in Zn^{2+}-homeostasis could be associated with severe disorders, including cardiac dysfunction [3,5].

Later discoveries have revealed that the amount of $[Zn^{2+}]_i$ is tightly controlled at the level of uptake, intracellular sequestration, redistribution, storage, and elimination, and, thereby creating a narrow window of optimal $[Zn^{2+}]_i$ in cells [6]. Being itself redox inert, Zn^{2+} creates a redox active environment, when it binds to sulfur ligand, inducing conformational changes on the proteins [7] and initiating an activation process of many signaling molecules [8]. Labile Zn^{2+} can permeate membranes either directly through ligand-gated channels

[9–11] or voltage-activated Ca^{2+} channels, whereas it can modulate these channels, significantly [12–15]. Entry of Zn^{2+} can alter function and structure of sub-cellular organells and proteins, as well as gene expression [16–18], while it can be highly cytotoxic for cells [19]. Jang et al. reported that nitric oxide, NO, mobilizes intracellular Zn^{2+} in cardiac tissue *via* cyclic guanosine monophosphate/protein kinase G signaling pathway [20], while Prost et al. demonstrated that Zn^{2+} is both an intracellular and extracellular regulator of ATP-dependent K^+-channels (KATP-channels) [21]. Currently, $[Zn^{2+}]_i$ is likely recognized as an important intracellular second messenger due to a strong possiblity of its role in native pathways, while the exact mechanisms are still unknown. In this regard, it has been recently shown that M-type K^+-channels, controlled the excitability of neurons and muscles, are activated with increasing $[Zn^{2+}]_i$ *via* reducing the channel-dependency on phosphatidylinositol 4,5-bisphosphate in HEK 293 cells [22].

The increased $[Zn^{2+}]_i$ also modulates several types of ionic currents. It has been shown that increased $[Zn^{2+}]_i$ facilitates the downward regulation of the background Cl^--conductance [23] and inhibits cardiac L-type Ca^{2+} current [24], while both L-type and T-type of Ca^{2+}-channel currents are inhibited by external Zn^{2+} [24,25]. Alvarez-Colazo et al. have demonstrated the modulation of transmembrane Ca^{2+} movements *via* regulation of β-adrenergic stimulation with both intracellular and extracellular Zn^{2+} in rat ventricular cardiomyocytes [15]. It has been also demonstrated that there is close relationship between high $[Zn^{2+}]_i$ and increase production of reactive oxygen/nitrogen species (ROS/RNS) in living cells [26,27]. Additionally, we previously have shown that an increase in $[Zn^{2+}]_i$ could induce thiol oxidation of total cellular proteins and hyperphosphoryation in many proteins and kinases in contractile machinery of cardiomyocytes [28,29]. Since most of voltage-dependent ionic channels can be modulated by phosphorylation, nitrosylation, and specific protein thiol oxidation [30,31], it can be hypothesized a deleterious effect of high $[Zn^{2+}]_i$ on the electrical activity (*i.e.* action potential) of a single cardiomyocyte, which may further stimulate an induction of arrhytmia at cellular level. Although it has been already shown the blockage of L-type Ca^{2+} current by Zn^{2+} [24], depressed mechanical activity of papillary muscle strips under high Zn^{2+} exposure [27] and permeation of Zn^{2+} blocks through L-type Ca^{2+} channels [12] in cardiomyocytes.

Increased $[Zn^{2+}]_i$-associated alterations in excitability and ionic conductances of different cells are demonstrated by early studies [32,33], while later studies have implicated the regulation of intracellular homeostasis of divalent cations such as Ca^{2+} and Zn^{2+} *via* thiol groups in structural proteins and enzymes [7,34]. Importantly, although reversible and reproducible increases in $[Ca^{2+}]_i$ *via* Zn^{2+} and ATP has been demonstrated in human endothelial cells [35], the exact mechanisms underlying cardiac dysfunction *via* cellular high $[Zn^{2+}]_i$ are not clarified yet. From here, to examine the underlying mechanisms of high $[Zn^{2+}]_i$ associated alterations in the heart, at single cell level, we aimed to examine the effects of high $[Zn^{2+}]_i$ in freshly isolated cardiomoycytes from adult male rats by determination of action potential parameters, voltage-dependent K^+-channel currents (I_K) and ATP sensitive K^+-channel currents (I_{KATP}) using electrophysiological techniques. Second, we wanted to test whether high $[Zn^{2+}]_i$ exposures can affect the mRNA levels of these channels, the levels of protein thiol oxidations and cellular level of ADP to ATP ratio in cardiomyocytes.

2. Materials and methods

2.1. Animals and isolation of cardiomyocytes

We used 3-month old Wistar male rats (200–250 g). All rats had free access to standard rat chow and water. Rats were anaesthetized by pentobarbital sodium (30 mg/kg, i.p.). Cardiomyocytes were isolated from left ventricle of heart using a standard enzymatic digestion method, as mentioned previously [3]. Briefly, the hearts were rapidly removed and mounted onto the Langendorff-perfusion system leaving

perfused the hearts retrogradely through the coronary arteries with a nominal Ca^{2+}-free solution for 5–6 min (in mmol/L; 117 NaCl, 5 KCl, 4.4 NaHCO₃, 1.5 KH₂PO₄, 3.6 MgCl₂, 20 HEPES, 20 taurine and 10 glucose at pH 7.4, bubbled with 100% O_2 at 37°C), and then followed by a further 30–35 min perfusion with the same solution containing collagenase (1-1.2 mg/mL Collagenase Type 2, Worthington, USA). Following full-perfusion, the hearts were placed into Ca^{2+}-free solution and the left ventricles were removed and minced into small pieces. Following gentle massage of the small pieces through a nylon mesh, left ventricular cardiomyocytes were dissociated and then washed with the collagenase-free solution. Subsequently, Ca^{2+} was increased in a graded manner to a final concentration of 1 mmol/L. All cells were kept at 37 °C (in 1 mmol/L Ca^{2+} containing solution) and cells were used within 8-h of isolation. We only used Ca^{2+} tolerant rod-shaped cells for all types of electrophysiological examinations.

This study was specifically reviewed and approved by the Ankara University, with reference number 2016-13-137 and was carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised at 1996).

2.2. Electrophysiological measurements

Average cell capacitance, *Cm* of freshly isolated cardiomyocytes were 192.5 ± 8.7 pF (n = 50 cells). Liquid junction potential was compensated before establishing the gigaseal. No leak or capacitance subtractions were performed during the current and voltage recordings.

Freshly isolated left ventricular cardiomyocytes were used to determine a single cell action potential under electrical stimulation with a frequency of 0.5 Hz *via* injecting small depolarizing pulses using an Axoclamp patch-clamp amplifier with current-clamp mode (Axopatch 200B amplifier, filtered at 3 kHz, sampled and digitized at 5 kHz using a Digidata 1200 A analog-to-digital converter and a software of pCLAMP 10.0; Axon Instruments). The bathing solution contained (in mmol/L) NaCl 137, KCl 4, MgCl₂ 1.8, CaCl₂ 1.8, Na-HEPES 10, glucose 10 and pH at 7.4. The pipette solution for whole-cell patch-clamp contained (in mmol/L) KCl 130, HEPES 25, MgATP 3, NaGTP 0.4 at pH = 7.2. Action potential duration from repolarization phase at 25, 50, 75, 90% (APD25, 50, 75, 90), the resting membrane potential, and the maximum amplitude of action potentials were calculated from original records (at least 10 records/cell).

Whole-cell voltage-dependent K^+-channel currents (I_K) in freshly isolated cardiomyocytes were recorded, as described previously [15,36]. All recordings were performed at room temperature. The patch pipettes had a resistance of 1.5–3 MΩ with an internal solution (as mmol/L: KCl 130, HEPES 25, MgATP 3, NaGTP 0.4; pH = 7.2).

For determination of transient outward K^+-current (I_{to}), the difference between the peak and last part of the recorded K^+-currents, while inward rectifier K^+-currents (I_{K1}) and the steady-state K^+-currents (I_{ss}) were determined from the last 200 ms part of every K^+-current and from holding current to the 'quasi'-steady-sate level, respectively, as described previously [15,36]. The measured current values were divided by cell membrane capacitance to present them as current density (in pA/pF). Application of Zn^{2+} was performed either internally with ZnCl₂ (in patch-pipette) or externally with a zinc-ionophore pyrithione (ZnPT; 1-Hydroxypyridine-2-thione zinc-salt).

The ATP sensitive K^+-channel currents (I_{KATP}) were measured in freshly isolated ventricular cardiomyocytes using whole-cell voltage-clamp technique as described previously [37]. Briefly, we used a voltage-protocol including descending ramps from + 50 to −120 mV at 0.034 mV/ms from a holding of −70 mV. The holding potential between voltage-clamp protocols was −70 mV with interval time of 20 s. K_{ATP} currents were calculated as the difference current between baseline current (in the presence of 40 μM glibenclamide) and the current recorded after application of 2 μM FCCP (Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone). Application of Zn^{2+} was performed