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Short communication

Nanosecond pulsed electric field induces calcium mobilization in osteoblasts

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ABSTRACT

Nanosecond pulsed electric field (nsPEF) has the ability to induce a host of intracellular biochemical processes in living cells as a function of parameter setting. In vitro experiments proved that nsPEF stimulation could remarkably promote biomineralization processes and new bone formation. However, the impact of nsPEF parameter settings on the calcium flux of osteoblasts, as well as the intracellular mechanisms underlying those observations have yet to be elucidated. In this study, live osteoblast-like MG63 cells were loaded with fluorescence indicator dye fluo-4 AM. nsPEF stimulation could induce intracellular calcium mobilization in MG63 cells with no refractory period. We confirmed that moderate output voltage (4–8 KV) and large pulse number (25) were required for efficient nsPEF irritation of MG63 cells. Additionally, nsPEF stimulation-induced calcium flux in MG63 cells was dramatically reduced with the treatment by ethylene glycol tetraacetic acid (EGTA), proving that the intake of extracellular calcium ions is crucial for increasing intracellular calcium concentration of nsPEF-treated MG63 cells. Our preliminary study investigated the mechanisms underlying nsPEF stimulation-induced calcium mobilization in osteoblast-like cells, and it has the potential to accelerate the application of nsPEF stimulation in new bone formation.

1. Introduction

Nanosecond pulsed electric field (nsPEF), characterized by high voltage, low energy and non-thermal effects, has attracted broad interest for its ability to generate a number of effects on biological systems. Dependent on electromechanical potentials that impair the barrier function of various membranous structures in living cells [1–3], nsPEF can initiate a variety of activities such as membrane-impermeable solutes uptake [4, 5], intracellular calcium release [6–10], cytoskeleton destruction [11], cell apoptosis [7, 12], platelet aggregation [13] and bacterial inactivation [14]. Many studies have proved that stimulation by electrical fields, especially nsPEF, could dramatically promote the formation of bone tissue, exhibiting great potential for applications in fields such as bone defect repair, dental implants and tissue engineering [15–28]. In addition, in vitro experiments demonstrated that multistep biomineralization processes in osteoblast-like cells were remarkably accelerated by electric fields stimulation [24–26, 29–31], and pulsed electric field stimulation could enhance the growth rates of embryonic chick tibiae [15, 16]. Unfortunately, although membrane defects induced by electric field stimulation have been intensively studied, the intracellular mechanisms underlying those observations have yet to be elucidated [1].

It is well known that cytosolic calcium plays a critical role in numerous biochemical processes such as ionic signaling, gene activation and metabolic pathway regulation [32]. Moreover, intracellular and extracellular calcium control the uptake and transport of taurine in osteoblasts respectively, which plays an important role in enhancing bone tissue formation and preventing bone loss [33]. Considering that nsPEF has the ability to induce calcium mobilization as mentioned previously [6–10], we concluded that intracellular calcium-dependent pathways may play a key role in nsPEF treatment induced new bone formation. However, to the best of our knowledge, not only the impact of parameter settings of nsPEF stimulation on calcium flux of osteoblasts but also the underlying mechanisms have never been investigated. In this present study, using cardiomyocytes as a positive control, we evaluated the impact of output voltage and pulse number of nsPEF stimulation on the intracellular calcium flux of osteoblast-like MG63 cells using the fluorescence indicator dye fluo-4 AM. Moreover, preliminary studies were performed to investigate the mechanisms underlying the nsPEF

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stimulation-induced intracellular calcium concentration increase in MG63 cells. We believe our study will promote the application of nsPEF stimulation for formation and calcification of new bone.

2. Materials and methods

2.1. Cell culture

Cardiomyocytes were prepared from adult Sprague-Dawley rats as previously described [34, 35], and stored in Tyrode's solution (pH = 7.35) consisting of 135 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 10 mM glucose and 5 mM HEPES. MG63 cells were obtained from the American Type Culture Collection (USA). MG63 cells were cultured in minimum essential medium (MEM; GIBCO, USA) containing 10% FBS (GIBCO, USA), 100 U ml^{-1} penicillin (Amresco, USA) and 0.1 mg ml^{-1} streptomycin (Amresco, USA) under standard cell culture conditions (37 °C, 100% humidity, 95% air and 5% CO_2). Cells were fed every two days. When cells grew to approximately 90% confluence, they were trypsinized with 0.25% trypsin/EDTA (HyClone, USA) and passaged into new cell culture plates (Corning, USA) at a splitting ratio of 1:3.

2.2. Application of nsPEFs

A pulse generator was assembled in our lab as previously described [36]. Briefly, it is composed of a direct current power source, coaxial cable, spark gap switches and transmission line pulse forming-networks. The waveforms were monitored using a DPO 4054 digital phosphor oscilloscope (Tektronix, USA). Time intervals between the pulses was approximately 1 s. In this paper, unless otherwise specified, nsPEFs of 60 ns duration and 10 kV output voltage were applied to stimulate cell samples.

2.3. Fluo-4 AM loading analysis

Following the previously reported method [37], the intracellular calcium mobilization within MG63 cells was visualized in real-time using the fluorescence indicator dye of fluo-4 AM (ThermoFisher, USA), and calcium mobilization within cardiomyocytes was conducted as a positive control. Briefly, cells were rinsed twice with low sodium HEPES buffer (LSHB) comprising of 135 mM NaCl, 1 mM $CaCl_2$, 4 mM KCl, 1.2 mmol $MgCl_2$, 1.2 mM NaH_2PO_4, 10 mM glucose, and 5 mM HEPES. Then, the cells were incubated in 1 ml of loading solution consisting of LSHB, 0.3 mM ouabain (Sigma, USA), 2.5 μM fluo-4 AM, 0.02% pluronic acid (ThermoFisher, USA), and 1.25 mM probenecid (ThermoFisher, USA) at 37 °C for 5 min in the dark. After rinsing twice with LSHB buffer, the cells were incubated for an additional 15 min in the LSHB buffer to allow for complete esterase cleavage of the fluo-4 AM.

2.4. Influence of nsPEFs parameter settings on intracellular calcium flux of MG63 cells

As shown in Supplementary Fig. 1, for nsPEF experiments, cardiomyocytes and MG63 cells were, respectively, seeded onto the surfaces of sterile glass coverslips, which were placed on the center of glass-bottom culture dishes (Mat Tek, USA). After 24 h of adhesion, the cells were loaded with fluo-4 AM and maintained in LSHB buffer as previously described. Then, the culture dishes containing fluo-4 AM-loaded cells were, respectively, placed on the microscope stage. A pair of pure Ti electrodes was inserted into the LSHB buffer and brought into contact with the margins of coverslip oriented in the horizontal direction, aiming to conduct nsPEF stimulations. Cells cultured on the coverslip surfaces served the purpose of maintaining the cells within nsPEFs. To clarify these processes, we present a schematic diagram shown in Supplementary Fig. 1. Moreover, according to the equation "electric field intensity (KV cm^{-1}) = output voltage (KV)/width of the coverslip (cm)", the electric field intensity can be easily manipulated by changing the output voltage or coverslip width. In this study, electrode separations of 6 mm and 3 mm were used for cardiomyocytes and MG63 cells respectively.

The Ca^{2+} flux in the fluo-4 AM-loaded cells was detected in real-time by a Zeiss LSM 5 (Germany) live confocal laser scanning microscope. The fluorescence was excited at 494 nm and detected at 516 nm with a fluorescence microscope. Series of experiments were performed to investigate the impact of parameter settings on the intracellular calcium flux of osteoblast-like MG63 cells. First, to conduct a positive control check, the fluorescence of fluo-4 AM-loaded cardiomyocytes was measured in real-time by a confocal laser scanning microscope for 100 s, and nsPEF stimulation was applied to cardiomyocytes between 45 s and 75 s since detection. Second, for the attached fluo-4 AM-loaded MG63 cells located on the coverslip surfaces, the fluorescence intensities of the cells were measured at every 5 s for 30 s. During this period, MG63 cells were stimulated by nsPEF between 5 s and 15 s since detection. At the same time, the cells were visualized by a confocal laser scanning microscope. Third, an experiment was conducted to determine whether there existed a refractory period in nsPEF-treated MG63 cells. The fluorescence of the fluo-4 AM-loaded MG63 cells was detected in real-time by a fluorescence microscope, and MG63 cells that were stimulated by nsPEF with a pulse number of 25 were immediately treated by nsPEF again once the fluorescence intensity of calcium fell to the baseline level. Fourth, to clarify the correlation between output voltage of nsPEF and calcium mobilization in MG63 cells, the fluorescence changes of the fluo-4 AM-loaded MG63 cells after stimulation by 1 pulse of nsPEF were measured with various output voltages (2 KV, 4 KV, 5 KV, 6 KV, 8 KV and 10 KV) respectively. Finally, we measured the fluorescence changes of the fluo-4 AM-loaded MG63 cells after exposure to nsPEF with pulse numbers of 1 and 25, investigating the effect of pulse number on calcium mobilization in MG63 cells.

2.5. Investigating the mechanism of calcium release

To eliminate the effect of extracellular calcium ions, 1 mM $CaCl_2$ in LSHB assay buffer was substituted by 1 mM ethylene glycol tetraacetic acid (EGTA; named EGTA-containing buffer). MG63 cells were incubated in 1 ml of loading solution consisting of EGTA-containing buffer, 0.3 mM ouabain, 2.5 μM fluo-4 AM, 0.02% pluronic acid, and 1.25 mM probenecid at 37 °C for 5 min in the dark as mentioned before, and then rinsed twice with EGTA-containing buffer. Using pristine cells as controls, MG63 cells in fresh EGTA-containing buffer were immediately stimulated by 1 pulse of nsPEF, and the fluorescence intensity changes were measured by a confocal microscope.

2.6. Statistical analysis

Using a photometric method, F0 was derived by subtracting the background from the baseline, and fluorescence intensity (F) was calculated by subtracting the mean of the baseline from the peaks. Four biological replicates were conducted for each experiment. Three fields each containing one cell sample were analyzed for all biological replicates (12 cell samples in total), and each measurement was repeated 4 times. The fluorescence intensity change ($\Delta F = F - F0$) of each group was presented as the mean ± standard deviation. Student's *t*-test was used to determine the significant differences among the groups, and p values <0.05 were considered statistically significant.

3. Results and discussion

3.1. Pulse generator

Pulse generator that consisted of a direct current power source, coaxial cable, spark gap switches and transmission line pulse forming