Dynamic changes of $[Ca^{2+}]_i$ in cerebellar granule cells exposed to pulsed electric fields

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Abstract Intracellular free Ca²⁺ concentration ([Ca²⁺]_i) in embryonic chick cerebellar granule cells loaded with fluo-3/AM and exposed to a single pulsed electric field was investigated using a confocal laser scanning microscope and fluorescent microscope equipped with CCD video imaging system. The results showed that [Ca²⁺]_i increased immediately and rose to the peak rapidly as the cells exposed to a single pulsed electric field. The amplitude and rate of the increases of [Ca²⁺]_i depend on the intensity of external electric field. In the presence of Ca²⁺ chelant EGTA or Ca²⁺ channels blocker La³⁺ in the pulsation solutions, the increase of [Ca²⁺]_i was still observable. It was also observed that [Ca²⁺]_i of different intracellular areas in the cell elevated simultaneously while the peak of the increase of [Ca²⁺]_i in the poles of the cell preceded to the peak in its somata and recovered to a plateau within a short time.

Keywords: pulsed electric field, Ca²⁺, granule cells, fluo-3/AM, confocal laser scanning microscopy.

Calcium ion as an important second messenger is sensitive to various environmental stimuli. Whether the transcription factors are activated or not can be specifically determined according to the amplitude and frequency of intracellular Ca^{2+} concentration^[1]. Meanwhile, elevated $[Ca^{2+}]_{i}$ controls a diversity of cell functions. It has hence become important for scientists to research the relation between the external electromagnetic fields and the change of [Ca²⁺]_i. Some experimental results have shown that extremely low frequency field is effective on modifying Ca^{2+} fluxes^[2]. [Ca²⁺]; change not only nonlinearly depends on field intensities but also on field frequency^[3]. Pulsed electric stimulation evokes rapid rise of $[Ca^{2+}]_i$ in the rat granular cells and the elevated [Ca²⁺]_i nonlinearly depends on the frequency and intensity of external electric field, but the increase of $[Ca^{2+}]_i$ is blocked in absence of Ca^{2+} in the external medium^[4]. A further experiment has revealed that L-type and N-type Ca²⁺ channels in smooth muscle cells of gall bladder of guinea pig could be opened by the pulsed electric field when the electric intensity was 100 V/cm, pulsed width was 0.5 ms, frequency was $1-16 \text{ Hz}^{[5]}$. A theoretical model was presented by Eichwald, of which the cytosolic calcium oscillations were controlled by receptors and external electric field had influence on cell signal transduction^[6]. By using Fura-2/AM ratio-fluorescent method, the effects of single pulsed electric field on [Ca²⁺]; and cytosolic cAMP (cyclic Adenosine

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Monophosphate) were previously studied in suspended embryonic chick brain cells, we observed that the electric field apparently induced $[Ca^{2+}]_i$ to rise and the elevation was associated with electric field intensity and embryonic chick age when the intensity was from 0.5 kV/cm to 2.0 kV/cm and the pulse width was 100 us.after the external medium Ca²⁺ was chelated by EGTA, $[Ca^{2+}]_i$ still increased obviously. The results suggested that pulsed electric field not only induced transport of extracellular Ca²⁺ across the plasma membrane into the cell but also induced release of Ca²⁺ from subcellular Ca²⁺ stores in embryonic brain cells. The induced increase of $[Ca^{2+}]_i$ was related to the pulse intensity^[7].

Confocal laser scanning microscope has offered a valuable method to study $[Ca^{2+}]_i$ change in the spatial localization and rapid kinetic process. The aim of this study was to research the temporal and spatial $[Ca^{2+}]_i$ changes induced by exposure to pulsed electric fields using confocal laser scanning microscope and a fluorescent microscope equipped with CCD video imaging system. The experimental results revealed that $[Ca^{2+}]_i$ increased significantly in the granular cells exposed to the external electric field even there is no Ca^{2+} influx, suggesting that electric field activated intracellular calcium stores to release as well as Ca^{2+} channels in membrane opening.

1 Materials and methods

1.1 Cell culture

Primary-cultured cerebellar granular cells were used in this study. The granule cells of embryonic chick were isolated from fertilized white Leghorn eggs, obtained from Beijing Agriculture University, and incubated at 37 °C. According to the studies on biophoton emission of intact brains and suspended brain cells^[8,9], the cultured granule cells were prepared from 8-day-age to 14-day-age embryonic chick (E8, E10 and E14). Whole cerebella were isolated and transferred into Mg^{2+/} Ca²⁺-free buffer, trimmed and cut into small pieces. After being blown with a pipette and filtered with 200 mesh net, the cerebellar cells were cultured in medium DMEM with 10% fetal calf serum. The cells were plated in a 35-mm culture dish containing glass coverslips (1.5×3 mm) which had been precoated with rat tail collagen. The isolated cells (6×10⁶ cell/mL) were cultured for 24 h at 37 °C with 5% CO₂.

1.2 Electric field exposure

In this study, the following four different media were used as the pulsation solutions: the Hanks' solution containing Ca²⁺ (1.3 mmol/L) but Mg²⁺-free, the Hanks' solution containing EGTA (2.5 mmol/L, final), the Hanks' solution containing La³⁺ (2 mmol/L, final) and the Hanks' solution containing Fe³⁺ (2 mmol/L, final). The Hanks' solution contained (mmol/L): 137 NaCl, 5 KCl, 1.3 CaCl₂, 0.4 KH₂PO₄, 0.6 Na₂HPO₄, 3.0 NaHCO₃, 5.6 glucose, 20 HEPES, 0.2 μ mol/L glycine. Electric stimulation was exerted using a pair of parallel acupuncture pins with the diameter of 0.5 mm and the length of 33 mm. The silver acupuncture pins were separated by 2 mm and submerged in the pulsation solution contained in a dish specifically designed for the

experiment. In the center of the dish, a hole (diameter 20 mm) was made and sealed with a coverslip $(22 \times 22 \text{ mm})$. The glass coverslip with the cultured cells was placed between the pins. The stimulation was performed by using a single pulse generator (made by Tsinghua University, DR-1). The electrode pins were paralleled with the longitudinal axis of the exposed cell. After monitoring the fluorescent image of a measured cell under the microscope and recording the baseline of fluorescence, one single electric pulse was applied to the cell while dynamic changes of the fluorescent intensities were recorded continuously.

1.3 Fluorescent mark

The coverslips with cells were washed twice and then incubated in Hanks' solution containing fluo-3/AM (Sigma Chemical CO, 2 μ mol/L, final) for 1 h at 37°C. The coverslip was gently rinsed five times in Hanks' solution in order to remove excess dye.

1.4 Measurement of $[Ca^{2+}]_i$

Measurements of $[Ca^{2+}]_i$ were carried out at room temperature in a confocal laser scanning microscope (ACAS 570) with two methods: frame scanning method (FSM) and point scanning method (PSM). With the FSM, image delay-time (Scanning time) was selected less than 5 s. Using PSM, the monitored region located between one pole and the nucleus along its longitudinal axis of the cell, such as the secondary area showed in fig. 4, was scanned for 10 ms or 100 ms. The data of measured fluorescence were recorded by the software (ACAS 570 Master program) and analyzed by using statistic program (from Dr. Popp). A fluorescent microscope supplied with CCD video imaging system was also used for measuring $[Ca^{2+}]_i$. Using this system, 165 images were recorded within 16.5 s. The $[Ca^{2+}]_i$ was described in the fluorescent intensity ratio $F/F_0^{[10]}(F)$, the fluorescent intensity after electrical field stimulation; F_0 , the fluorescent intensity before electrical field stimulation). The increase rate of F/F_0 was shown as the ratio F/F_0 changed with time.

2 Results

2.1 Dynamic changes of $[Ca^{2+}]_i$

By using the PSM of the confocal laser scanning microscopy, the dynamic process of $[Ca^{2+}]_i$ changes can be caught by using scanning time of 0.01 s. Exposure of granular cells to the pulsed electric field evoked a biphasic Ca^{2+} response that consisted of an initial rapid rise of $[Ca^{2+}]_i$ from a baseline to a peak and a subsequent recovery over a short time to a low sustained plateau. The amplitude and rate of elevated $[Ca^{2+}]_i$ was associated with electric intensity (fig. 1). From fig. 1, the fluorescent intensity represented by F/F_0 was positively accompanied with the applied pulsed electric field intensity; the elevated rate of F/F_0 also became fast with the elevated electric intensity. However, when the external electric field intensity was increased to 1.0 kV/cm, the rate of F/F_0 obviously seemed to be related to the activation of ATPase (see another report). After specific chelant EGTA chelated external Ca^{2+} of the medium, $[Ca^{2+}]_i$ changes were observed using the PSM of the confocal laser scanning microscopy. As shown by fig. 2, the amplitude of $[Ca^{2+}]_i$ increase was obviously less than that of external medium containing Ca^{2+} . However, $[Ca^{2+}]_i$ still rose slowly after being stimulated by pulsed electric field.



Fig. 1. By using the PSM of the confocal laser scanning microscopy, $[Ca^{2+}]_i$ dynamic changes of cultured E8 cerebellar granule cells can be caught after electric field stimulation. The scanning time was 0.01 s. The curves from the top to the bottom are: 1, *E*=1.5 kV/cm; 2, *E*=1.0 kV/cm; 3, *E*=0.5 kV/cm. The arrow shows the start time point applying electric field stimulation.

Fig. 2. By using the PSM of the confocal laser scanning microscopy, $[Ca^{2+}]_i$ dynamic changes in cultured E_0 granule cells were measured after electric field stimulation when external Ca^{2+} in the medium was chelated by adding EGTA. The scanning time was 0.01 s. The curves from top to bottom were: 1, 1.3 mmol/L extracellula Ca^{2+} ; 2, free extracellular Ca^{2+} . The arrow shows the start time point pulsed electric field stimulation was applied.

2.3 Dynamic changes of $[Ca^{2+}]_i$ without extracellular Ca^{2+} influx

In the presence of the lanthanum ion La^{3+} , a non-specific calcium channel blocker^[11], in the pulsation solution, cells exposed to the electric field with the intensity from 0.5 kV/cm to 1.5 kV/cm resulted in significant increase of $[Ca^{2+}]_i$ compared with the baseline calcium levels before stimulation (fig. 3). In order to keep the same concentration of ions in the extracellular solution, the Hanks' solution containing Fe³⁺ at the same concentration as La^{3+} was used as the controls.

2.4 FSM results

Fig. 4 shows $[Ca^{2+}]_i$ changes in the somata and poles of E10 chick cerebellar granule cells cultured for 24 h and exposed to 1.0 kV/cm electric field. The dynamic process in poles was apparently different from that in the somata. From fig. 4(b) which shows the first 70 s of fig. 4(a), it was clear that the $[Ca^{2+}]_i$ in the poles was earlier elevated to their peaks than that in the somata.

3 Discussion

Electric field-induced cytosolic elevated calcium may arise from transmembrane influx and release from intracellular stores. Under the electric field stimulation, the electric potential of membrane would change, inducing the calcium ion channels to open, the extracellular calcium ion enter cells and the intracellular $[Ca^{2+}]_i$ to rise.



Fig. 3. A fluorescent microscope with CCD video imaging system was used to catch the intracellular calcium change before and after electric stimulation. E10 cerebellar granule cells cultured 24 h were suspended in the Hanks' solution containing 2 mmol/L FeCl₃ (upper left) or 2 mmol/L LaCl₃ (bottom left). (a) $[Ca^{2+}]_i$ changes in different electric field intensities. 1, *E*=1.5 kV/cm; 2, *E*=1.0 kV/cm; 3, *E*=0.5 kV/cm. (b) Statistical results of $[Ca^{2+}]_i$ changes in two kinds of extracellular solution under the different electric stimulation (*n*=3,**p* <0.01).



Fig. 4. By using the FSM of the confocal laser scanning microscopy, $[Ca^{2+}]_i$ dynamic changes of E10 cerebellar granule cells cultured for 24 h can be caught after electric field stimulation. The top and the bottom left images were the same cell at different time before and after stimulation. The bottom right image was the dynamic change of $[Ca^{2+}]_i$ in the four located areas. The electric field intensity was 1 kV/cm. The curves from the top to the bottom are the mean florescent intensity in the different areas. 1, The third area; 2, the second area; 3, the first area; 4, the fourth area; 5, the fifth area (extracellular). (b) is the enlarged part of (a).

The result showed that $[Ca^{2+}]_i$ increased in cells exposed to pulsed electric field when there was no extracellular calcium ion, the increase of $[Ca^{2+}]_i$ maybe came from intracellular Ca^{2+} stores. Like in other excitable cells, there are two principal intracellular calcium stores in the granular cells, IP₃ receptors and ryanodine receptors (RYRs) stores, and the release of Ca^{2+} through the RYR receptor depends on the influx of external Ca^{2+} . What becomes clear is that pulsed electric field can induce $[Ca^{2+}]_i$ to rise after adding EGTA to chelate the extracellular Ca^{2+} or adding LaCl₃

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to block calcium channels in the pulsation solution, suggesting that the increase of $[Ca^{2+}]_i$ by the electric field may be related to the IP₃ receptor store^[12] opening. The above results conform with the previous studies of external electric field influences on suspended embryonic chick brain cells. Dibirdik and Kristupaitis^[13,14] reported that exposure of DT40 lymphoma B-cells to 60 Hz low energy electromagnetic fields led to rapid increase of IP₃, but Miller^[15] presented evidence that showed no significant effect of 60-Hz electromagnetic field on IP₃ production. Comparing our experimental results with those of Dibirdik, Kristupaitis and Miller, it could have a more complex mechanism to explain the effect of external electric field on the change of $[Ca^{2+}]_i$ in cells which should be viewed not only in terms of substances of influx and release of Ca^{2+} but also in terms of the interactions of fields formed by ions. Therefore further experimental and theoretical researches should be explored.

From thin sections of E10 chick granule cells, we observed under an electric microscope that endoplasmic reticulum (ER) was rich in the somata whereas microtubules (MT) in the poles respectively. As exposed to the pulsed electric field, the change of $[Ca^{2+}]_i$ in the granule cells depends on the intracellular areas. In the somata, as the second and third areas of fig. 4 show, $[Ca^{2+}]_i$ changes synchronously after stimulation. However, in the poles such as the first and the fourth areas of fig. 4, $[Ca^{2+}]_i$ rises up to their peaks earlier than in the somata. The phenomena were possibly related with cellular structures.

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