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Modulation of calcium signalling by the endoplasmic reticulum in *Carassius* neurons

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ABSTRACT

It is known that endoplasmic reticulum (ER), being a calcium store participates in the regulation of intracellular calcium concentration. Ca-ATPase of the ER is one of the crucial agents providing the calcium-accumulating function of this intracellular structure. We studied the role of the ER in modulation of calcium signalling in *Carassius* neurons using a Ca^{2+}-imaging technique. We tested the role of the ER in the maintenance of a steady state calcium level in the cytoplasm and in modulation of Ca^{2+} transients evoked by cell depolarizations. The ER calcium stores were depleted using inhibitors of ER Ca-ATPase, which provided blocking of Ca^{2+} uptake by the ER. Our experiments firstly showed that the ER can significantly modulate the characteristics of intracellular calcium signals in *Carassius* neurons during their activity. These findings also indicate that the ER modulates the shape of Ca^{2+} signals rather than the basal level of intracellular Ca^{2+} in these neurons.

1. Introduction

At rest, the free calcium concentration in the cytoplasm of the cells is only 50–100 nM, while the respective concentration in the extracellular environment surrounding the cell is about 2 mM. The endoplasmic reticulum (ER) is one of the most important cellular structures participating in the maintenance of a low intracellular calcium level. The dysfunction of the ER can promote cell death in the case of ER stress [6], influences upon this intracellular structure, which is observed in neurodegenerative diseases [5,19]. The work of the relevant energy-dependent sarco/endoplasmic transport ATPase (pump), SERCA, provides absorption of calcium from the cytoplasm by the ER and the maintenance of a very high Ca^{2+} concentration gradient between the ER lumen ($[Ca^{2+}]_{ER}$) and the cytoplasm. An important feature of the SERCA-pump is a high dependence of the rate of calcium transport on the concentration of these ions in the ER; decreases in the $[Ca^{2+}]_{ER}$ dramatically increase the rate of accumulation of calcium in the ER. The ER function is also significantly regulated by the pH level [12,22].

Endoplasmic reticulum significantly affects time and amplitude characteristics of calcium signals in mammalian neurons [8,24]. An important role of this structure in coordination of intercellular and intracellular signals via long-term adaptive responses was established. The ER has also been identified as a source of rapid physiological signaling; it works as a dynamic calcium store [2,11,21] is activated by electrical or chemical stimulation of the cells. It was found that the ER also plays an important role in the distribution of cytosolic Ca^{2+} waves [1]; its ability to function as an intracellular calcium tunnel was demonstrated. Calcium ions are molecular mediators that integrate diverse signals in the ER. Within the structure of the calcium cascade, the ER serves as a "quick exchanger" of the reserve Ca^{2+}; it is able to release Ca^{2+} ions at the appropriate physiological stimulation [9,10,18]. Functioning of the ER as a dynamic calcium store requires the maintainance of a high concentration of Ca^{2+} within its lumen ($[Ca^{2+}]_{ER}$), within a 0.2/2 mM range. The ER preserves a significant amount of Ca^{2+} at the rest, and this amount can be immediately released from the ER in the case of neuron activation. However, the ER fails to maintain a large amount of calcium ions consistently in most central neurons; therefore, previous activation of the cells and refilling of the store are required for the release of Ca^{2+} from this store [20].

An alkaloid, thapsigargin is a specific inhibitor of calcium transport Ca^{2+}-ATPase in the ER. This agent can pass through the cell membrane and act in nanomolar concentrations. By binding with a molecule of Ca^{2+} ATPase, a promoter of tumor growth, thapsigargin irreversible blocks this enzyme. Among other specific blockers of Ca^{2+}-ATPase are cyclopiazonic acid (acting in micromolar concentrations) and BHQ (2,5-di-(tert-butil)-1,4-benzohidroquinalone). Cyclopiazonic acid and BHQ posses the same mechanism of blocking, and are also very specific for Ca^{2+}-ATPase of the ER and do not affect other ATPases. Ca^{2+}-ATPase of the ER can also be nonspecifically blocked by orthovanadate and fluoride ions.

Although more and more researches were carried out on fishes in recent years, most experiments were performed on the zebrafish. The transparency of the body of the larvae of this species, a

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simple arrangement of the nervous system, and its small size allow experimenters to monitor the activity of the whole brain. However, these studies were focused mainly on the development of genetic and molecular tools for studying of the gene functions, in particular, genetic approaches for imaging of calcium were developed [7]. Some experiments on fishes were devoted to the investigations of calcium channels [13,25], synaptic transmission [3,4,16,17,23], and the visual system [23]. However, rather little is known about calcium signalling and properties of the ER in the *Carassius* neurons and possible protecting role of these phenomena in this hypoxia-tolerant species. Therefore, the aim of our experiments was to examine modulation of Ca^{2+} signaling by the ER in *Carassius* cerebellar neurons.

2. Materials and methods

2.1. Animals

Experiments were carried out on isolated cerebellar neurons of *Carassius gibelio* removed from the brain of fishes (mean body mass 70 g); the technique for isolation was described in detail earlier [14,15].

2.2. Isolation of cerebellar neurons

Briefly, we used a technique of cooling for anesthetization. The fish was placed in a special pool put in a freezing chamber at −19 °C for 5–10 min. The removed cerebellum was immediately immersed for 7 min in a DMEM solution (Sigma Aldrich, USA) cooled to +5 °C. After enzymatic treatment, blocks of the cerebellar tissue were washed out with the enzyme-free DMEM solution and divided into smaller fragments for further dispersion, by passing through pipettes. The obtained single neurons were transferred on the coverslips and placed to a recording chamber mounted on an inverted Olympus IX70 microscope (Olympus, Japan).

2.3. Fura-2 Ca^{2+} imaging analysis

To load the neurons with a calcium-sensitive indicator, the cells were kept in the Tyrode solution with the addition of 5 μM of a fluorescent dye, Fura-2AM, and 0.02% of a detergent, Pluronic F-127. The cells were loaded with the above dye for 30 min at room temperature (+22 P). After this procedure, the cells were incubated in pure Tyrode solution for 30–40 min for de-estherification of the dye.

The level of intracellular free calcium was measured using microfluorescent analysis. The dye Fura-2 was alternately excited by UV-light at two wavelengths (360 and 390 nm); a monochromator and an Imago-QE CCD camera (TILL Photonics, Germany) were used. Signals from the CCD camera were entered into a computer for further measurements of the ratio of intensities of fluorescent signals at two wavelengths ($R = F1/F2$). This ratio adequately reflects changes in the intracellular calcium concentration. Recording and processing of the data obtained were performed using TILvisION software (TILL Photonics, Germany).

2.4. Solutions and drugs

The composition of the Tyrode solution used was the following (mM): NaCl, 125; $CaCl_2$, 2; KCl, 2.5; MgCl, 1; HEPES, 20; glucose, 10 (pH 7.4). All experiments were carried out at room temperature. Most chemicals were obtained from Sigma–Aldrich (USA), while Fura-2AM and Pluronic F-127 were from Molecular Probes (USA).

2.5. Statistical analysis

Statistical processing was performed using OriginPro 8.0 software. All numerical data are presented below as means ± s.e.m. The level of significance was estimated using one-way ANOVA; intergroup differences were considered significant at $P < 0.05$.

3. Results

3.1. KCl-induced calcium transients in Carassius neurons

To study the role of the ER we first tested calcium transients evoked by membrane depolarization of *Carassius* cerebellar neurons induced by high KCl concentrations. We used the cells with clearly delineated membrane without visible damage and intracellular inclusions. We measured the intracellular level of Ca^{2+} as $R = F1/F2$ (see Methods). Applications of a hyperpotassium solution (50 mM KCl) evoked intense depolarizations of the plasma membrane. In response to the membrane depolarizations of neurons by KCl, a rapid increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) followed by a rapid exponential decline to the initial level of this parameter was observed. To test the cell viability and reproducibility of the responses, we usually used 5 s long KCl applications, (Fig. 1A).

The amplitude of calcium transients in response to membrane depolarization was stable and sustained throughout the period of the experiment (25–30 min). Fig. 1B shows values of the amplitude of Ca^{2+} transients evoked by KCl applications lasting 1, 5 and 10 s, that were normalized with respect to maximal in the set value. The amplitude and time parameters of calcium transients at the beginning of the experiment and in 25 min demonstrated similar values, indicative of the normal functioning of cells during the study, Fig. 1C.

In the next experiments, we conducted the measurements of Ca^{2+} transients under control conditions, where we induced membrane depolarizations using different durations of KCl applications (1, 3, 5 and 10 s, Fig. 2A, the left part of the trace). The amplitudes of calcium transients at all tested time values of stimulation were similar each other or slightly increased during prolonged depolarizations under control conditions. However, the time of returning of the Ca^{2+} concentration to the basal level and relative amount of Ca^{2+} that entered the cell during depolarizations increased with increasing of the duration of KCl applications (Fig. 2A).

3.2. The effect of cyclopiazonic acid (ER Ca^{2+}-ATPase blocker)

To study the role of the ER in Ca^{2+} signalling of fish neurons, we prevented ER Ca^{2+}-absorbing function by blocking ER Ca^{2+}-ATPase (SERCA). We used a specific blocker of Ca^{2+}-ATPase, cyclopiazonic acid (CPA), in a concentration of 20 μM, which reversibly blocks ER Ca^{2+}-ATPase activity. To test the CPA effect, we applied the protocol similar to that in the control, using different time periods of KCl applications (1, 3, 5 and 10 s, respectively), but in the presence of the blocker in the applied solution (Fig. 2A, the right part of the trace). As is shown, the amplitude of Ca^{2+} transients increased significantly after blocking of Ca^{2+}-ATPase at all time intervals used. After every application, washing out with the control solution was performed. This was necessary to prevent cell death because of Ca^{2+} overloading. Quantitative measurements showed that the amplitude (A) of Ca^{2+} transients at 3 s and 5 s long depolarizations in the presence of the blocker, increased by 96.1% ± 0.09% ($p < 0.01$) and 79.7% ± 0.12% ($p < 0.05$), respectively, as compared with the control values (Fig. 2B and D). The relative amount of Ca^{2+} entering the cell during depolarization (S) also increased in all cases. This value we calculated as the integral value of the area under a graph

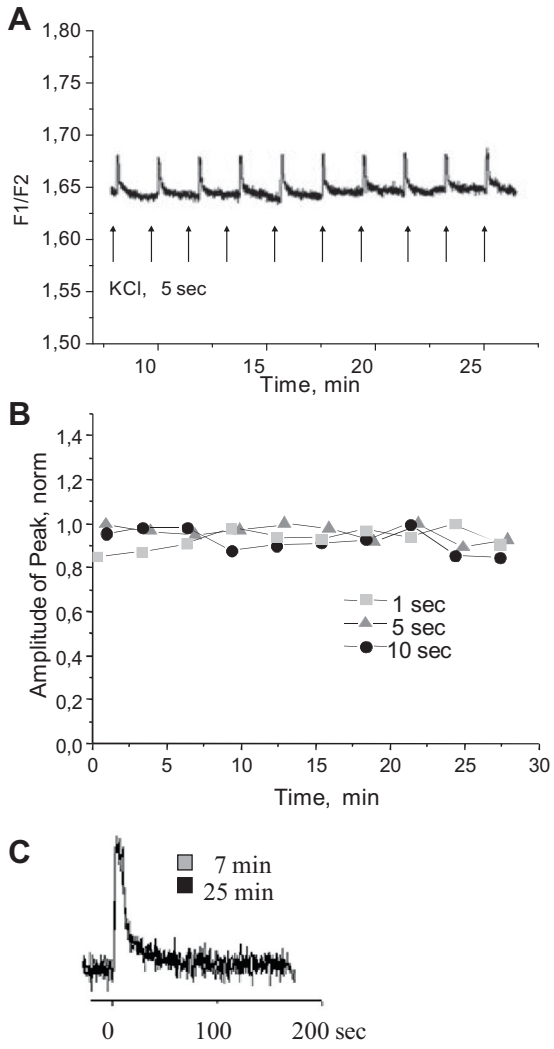

Fig. 1. Reproducibility of Ca^{2+} transients in *Carassius* cerebellar neurons. (A) Ca^{2+} transients evoked by 5 s long KCl (50 mM) applications (indicated near the peaks). (B) Amplitude values of Ca^{2+} transients evoked by 1, 5 and 10 s long KCl applications during a long-lasting experiment are shown. The values are normalized with respect to maximal ones in the set. (C) Temporal properties of Ca^{2+} transients at the beginning (7 min) and the end (25 min) of the experiment.

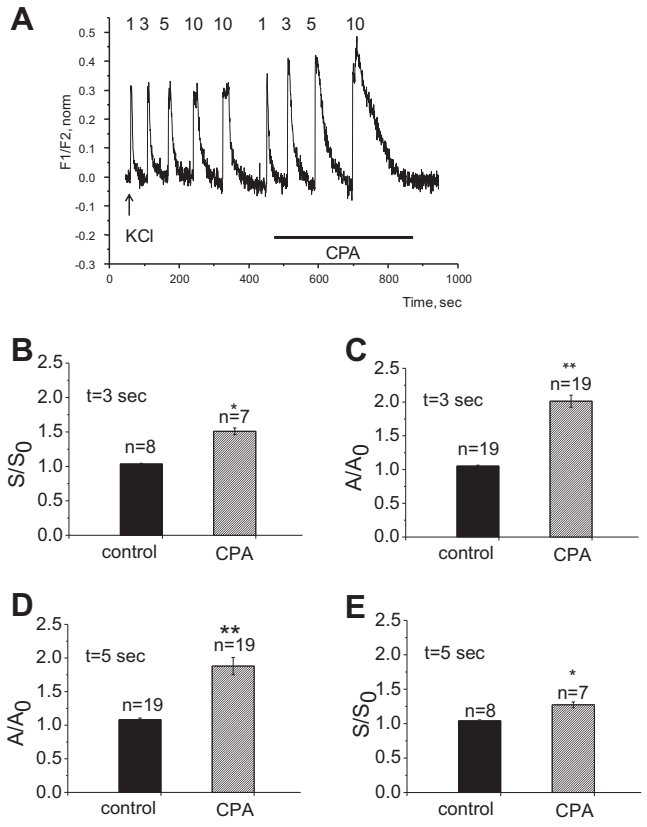

Fig. 2. Action of CPA on Ca^{2+} transients in *Carassius* cerebellar neurons. (A) Changes in the amplitude and shape of Ca^{2+} transients after 50 mM KCl applications of different durations (1, 3, 5 and 10 s, indicated near the peaks). Measurements were performed in the control and in the presence of a Ca^{2+}-ATPase blocker (20 μM CPA). (B–E) Quantitative estimation of the CPA effects on Ca^{2+} transients caused by membrane depolarizations. Changes in the amplitude of Ca^{2+} transients (A/A_0) caused by KCl application lasting 3 s (B) and 5 s (D); the values are normalized with respect to control ones (A_0). (C) and (E) The changes in the amount of Ca^{2+} entering the cell during depolarizations, represented as areas under Ca^{2+} transients (S/S_0) during KCl application lasting 3 (C) and 5 s (E). Values are normalized to control ones (S_0). Bars correspond to the control values (black) and 20 μM CPA ones (gray). $^{*}P < 0.05$, $^{**}P < 0.01$ in comparisons with the control.

for each transient observed in the experiment. As can be seen from the above figure this index also significantly increased relatively to the control values, but to a lesser degree. The respective increments were 47.4% ± 0.05% ($p < 0.01$) for 3 s long stimulation and 23.1% ± 0.04% ($p < 0.05$) for 5 s long one, (Fig. 2C and E).

3.3. The effect of thapsigargin (another ER Ca^{2+}-ATPase blocker)

Switching off of Ca^{2+}- accumulating function of the ER could be also provided by another selective blocker of SERCA. In this set of the experiments, we used 20 nM thapsigargin (Tg), which is also a specific blocker of ER Ca^{2+}-ATPase. For this purpose, we applied the same protocol as in the previous case, with two time intervals of KCl-induced membrane depolarization, 3 and 5 s long (Fig. 3A). The amplitude of Ca^{2+} transients in the presence of the blocker increased by 71.4% ± 0.09% ($p < 0.01$) for 3 s long stimulation and 56.3% ± 0.1% ($p < 0.05$) for 5 s long one, as compared with the control values (Fig. 3B and D). Fig. 3C and E illustrates integral Ca^{2+} entry values (areas under each Ca^{2+} transient obtained in the experiment). The Figure shows that the area also grew, but to a significantly lesser extent than the amplitude. The average increments were 33.6% ± 0.11% ($p < 0.05$) for 3 s long stimulation and 28.3% ± 0.04% ($p < 0.05$) for 5 s long ones. Thus, the data obtained are clearly indicative of the presence of ER Ca^{2+}-ATPase activity in cerebellar neurons of *Carassius* and a significant contribution of the ER to Ca^{2+} signaling system of neurons in this fish.

4. Discussion

Our previous investigations showed that several participants of intracellular calcium signalling, such as mitochondria [15], membrane Ca^{2+}-ATPase (PMCA) and membrane Na^{+}/Ca^{2+} exchanger (NCX) [14], significantly influence intracellular calcium exchange providing the clearing of the cytoplasm from Ca^{2+} excess. We have shown that PMCA and NCX participate in the maintenance of an adequate basal Ca^{2+} level in *Carassius* neurons [14], whereas the mitochondria provide a relatively small impact on the basal Ca^{2+} level but significantly modulate temporal characteristics of Ca^{2+} signals [15]. In this our study, we found that cyclopiazonic acid and thapsigargin evoked in all tested neurons of the *Carassius* cerebellum near twofold increases in the peak amplitude of Ca^{2+} transients. This fact confirms the important role of the ER as a Ca^{2+} store in neurons of this fish species. We found that ER Ca^{2+}-ATPase

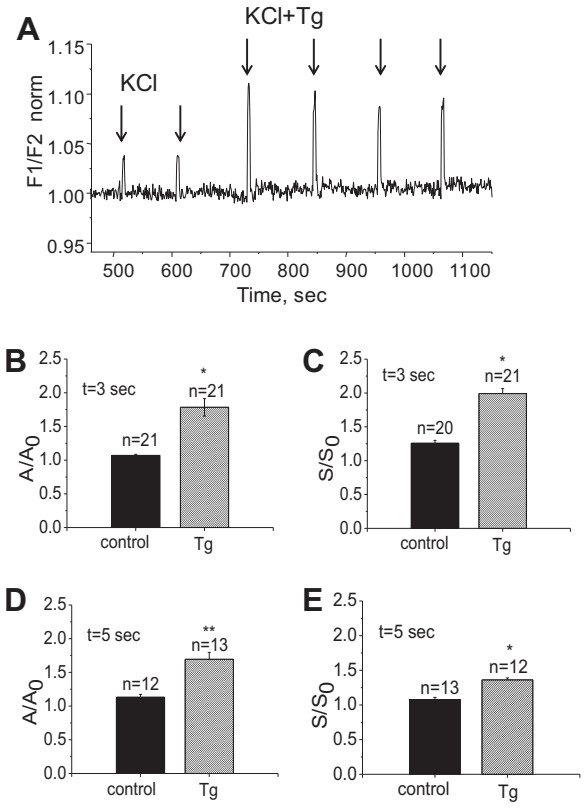

Fig. 3. Action of thapsigargin on Ca^{2+} transients in *Carassius* cerebellar neurons. Relative changes in the amplitude of Ca^{2+} transients (A/A_0) caused by KCl application lasting 3 (B) and 5 s (D), and changes in the amount of Ca^{2+} entering the cell during depolarization (represented as areas of Ca^{2+} transients, S/S_0) at KCl applications lasting 3 (C) and 5 s (E). The values obtained in the control and in the presence of 20 nM Tg are presented. Values are normalized to control one. $^{*}P < 0.05$, $^{**}P < 0.01$ in comparisons with the control.

provides the clearance of the cytoplasm of Ca^{2+} excess, which has entered the cell during KCl stimulation. The amount of Ca^{2+} depends on the duration of depolarization.

The fact that an increase in the Ca^{2+} transient amplitude is much greater than an increase in the area of such transient after the blocker action may indicate that SERCA pump is activated immediately after the beginning of Ca^{2+} entry the cell, while it exert lesser effects within more remote intervals after stimulation. A lower efficiency of the blockers at longer stimulations of cells also agrees with such explanation. These data suggest that the ER is significantly involved in the clearance of the cytoplasm from Ca^{2+} excess during stimulation of the cells and plays a significant role in the short-term modulation of calcium signals in fish neurons. By this property, the ER differs from the plasmatic membrane components (PMCA and NCX) [14], and is similar, in a certain senses in its function to mitochondrial one. Both types of the cell organelles provide considerable modulation of Ca^{2+} signals [15].

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