CALCIUM SIGNALING IN CEREBELLAR NEURONS OF *CARASSIUS GIBELIO*

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Information about prof. E.A. Lukyanetz is described on p. 167.

Inroduction

Establisment of the mechanisms of survival of neurons of animals tolerant to hypoxia is one of the perspective trends in the search for effective medical treatment of pathological states induced by ischemia/hypoxia (Bickler, 2004; Bickler and Buck, 2007). Fresh-water fish *Carassius gibelio* pertains to such animal species. It has been demonstrated that free calcium ion concentration in the cytosol of neurons significantly grows at many pathological states, including hypoxia/ischemia, and this can result in apoptosis of these cells and their irreversible dying (Lukyanetz et al.; Stanika et al., 2002). Removal of excessive Ca^{2+} accumulated in the cytosol during excitation of neurons results from the functioning of several membrane systems. Among them, Na^{+}/Ca^{2+} exchanger (NCX), Ca^{2+}-ATPase of the plasma membrane (PMCA) and mitochondria are the most important in this respect. It is believed that NCX is one of the most powerful cellular mechanisms responsible for removal of Ca^{2+} from the cytosol (DiPolo and Beauge, 2006). The PMCA is more widely distributed; it possesses a higher affinity for Ca^{2+} but a smaller capacity. The high affinity of the PMCA for Ca^{2+} ions allows it to bind them even under conditions where their concentration is low. Therefore, it is be-

lieved that PMCA modulates calcium intracellular signals at low concentrations of such ions during normal functioning of the cells (Siegel et al., 2005), and the activities of NCX and PMCA are complementary factors.

Mitochondria are capable of significantly influencing spatial formation of calcium signals in neurons. Calcium transients induced by one and the same stimulation (depolarization of the plasma membrane via the action of a hyperpotassium solution) in different types of neurons differ significantly from each other in their characteristics (in particular, in the kinetics of decay and other parameters) (Shishkin et al., 2002). Thus, for example, in primary afferent neurons of the dorsal root ganglia (DRG neurones), the level of Ca^{2+} concentration after its transient increase showed the slowest return to the initial level in small (nociceptive) DRG neurons; the fastest recovery was obseved in central neurons of the spinal dorsal horn. It was hypothesized that such specific parameters of calcium signals depend, to a significant extent, on the peculiarities of spatial distribution of mitochondria in the mentioned cells. Taking into account the above data, the peculiarities of functioning of mitochondria in animal neurons tolerant to hypoxia are of special interest. It cannot be ruled out that these peculiarities are factors that are responsible for the high resistivity of central neurons of these organisms to hypoxia.

In presented studies, we examined the peculiarities of the functioning of membrane NCX, PMCA and mitochondria in neurons of the cerebellum of fish highly tolerant to hypoxia (*Carassius gibelio*) and also the peculiarities of intracellular calcium exchange related to the above phenomena.

Methods

Experiments were fulfilled on isolated cerebellar neurons of *Carassius gibelio* removed from the brain of 3-year-old fish; the technique for isolation was described in detail in (Lukyanets et al., 2009a; Lukyanets et al., 2009b; Lukyanets et al., 2009c). Briefly, for anesthetization, we used a technique of cooling anesthesia proposed by Karamyan (Karamyan, 1949) and partly modified in our laboratory. The fish was placed in a special pool put in a freezing chamber. The removed cerebellum was immediately immersed for 7 min in a cool DMEM solution (Sigma Aldrich, USA). The cerebellum was divided into parts and kept in the DMEM solution on ice; then, cerebellar tissue blocks were enzymatically treated for 30 min with a solution containing 0.1% protease (Sigma Aldrich, USA) and 0.1% trypsin (Sigma Aldrich, USA). After the treatment, they were washed out with an enzyme-free DMEM solution and further dispersed by passing through pipettes. The obtained single neurons were transferred on coverslips. To load the neurons with a calcium-sensitive indicator, the cells were kept in 5 μM fluorescent dye, Fura-2AM (Molecular Probes, USA) and 0.02% Pluronic F-127. After this procedure, they were incubated in the Tyrode solution for de-esterification of the dye.

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

The composition of the Tyrode solution used was the following (mM): NaCl, 125; CaCl*2*, 2; KCl, 2.5; MgCl, 1; HEPES, 20; and glucose, 10 (pH 7.4). All experiments were carried out at room temperature. Statistical processing was performed using OriginPro 8.0 software. All numerical data are presented as averaged ± s.e.m. The level of significance was estimated using one-way ANOVA; intergroup differences were considered significant at $P < 0.05$.

Role of membrane PMCA in calcium exchange

Calcium transients in the studied *Carassius* cerebellar neurons were evoked by depolarization of the plasma membrane by application of a high K^+ concentration (50 mM) solution. In response to the depolarization, the level of intracellular Ca^{2+} ($[Ca^{2+}]_i$) in cerebellar neurons rapidly increased; then we observed a rapid exponential decline in the $[Ca^{2+}]_i$, returning this value to the initial one within a relatively short time interval, Fig. 1.

The amplitudes and kinetic parameters of calcium transients at the beginning of the experiment and at the 25th min were close to each other, which was indicative of preservation of the normal viability of the studied cells in the course of the experiment. In *next* experiments, we tested the involvement of the membrane

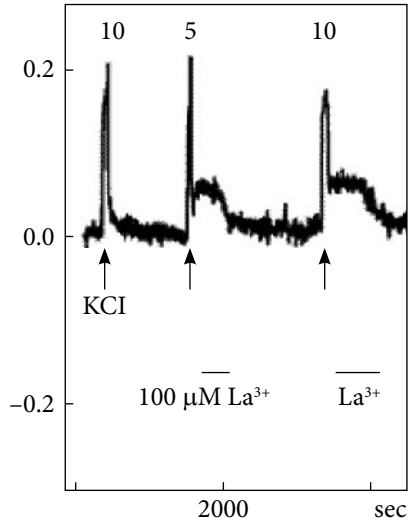

Fig. 1. Calcium transients in neurons of the cerebellum of the crucian carp (*Carassius gibelio*) induced by 5- and 10-sec-long depolarizations of the plasma membrane by applications of 50 mM KCl (indicated by arrows). Measurements were performed under control conditions and upon blocking of Ca^{2+}-ATPase of the plasma membrane in the presence of 100 μM La^{3+} in the extracellular solution (indicated by bars below the transient traces). Abscissa: Time, sec; Ordinate: Ratio of the magnitudes of fluorescent signals at two wavelengths (F/F_0), which reflects changes in the intracellular calcium concentration

PMCA in calcium exchange in these neurons. For that we used 100 μM La^{3+} for the extracellular solution (such a concentration of La^{3+} blocks the activity of PMCA and does not influence the activity of NCX (Shimizu et al., 1997)). Under control conditions, calcium transients were induced by depolarizations of the membrane using 5- or 10-sec-long applications of the hyperpotassium solution. With such durations of the above stimulation, the amplitudes of control calcium transients were mostly identical. The effect of La^{3+} was also tested using two durations of hyperpotassium solution application (5 and 10 sec). La^{3+}, similarly to many other inorganic cations, can block the activity of voltage-dependent calcium channels in the membrane. To esclude such a possibility, we applied the La^{3+}-containing solution immediately after the membrane depolarization. In this case, the amplitude of calcium transients after the blockade of PMCA showed no rise.

However, in all the cells studied, the level of Ca^{2+} did not return to the basal level but reached a somewhat higher value. Thus, we observed a certain "shelf" which existed as long as La^{3+} was applied (Fig. 1). This was indicative of blocking of the functioning of PMCA; as a result, Ca^{2+} ions were accumulated, and the basal level of $[Ca^{2+}]_i$ increased in the cytosol. After La^{3+} removing from the extracellular solution using washing out, the basal level of $[Ca^{2+}]_i$ returned to the initial value for nearly 1 min. Quantitative measurements of calcium transients induced by 3- or 5-sec-long KCl-depolarization showed that the basal level of $[Ca^{2+}]_i$ increased with respect to the control values in the presence of 100 μM La^{3+} by 31.4 ± 3.2% ($P < < 0.01$), and this effect was independent of the depolarization duration.

Role of membrane NCX in calcium exchange

To estimate the involvement of the membrane NCX, we replaced Na^+ ions in the extracellular solution by Li^+. As was mentioned above, the NCX-pm cannot operate under conditions where Na^+ ions are replaced by Li^+, while the NCX-mit, in this case, preserves a normal functioning mode (Castaldo et al., 2009). Such situation allows one to use Li^+ ions in the studies of the role of the NCX-pm with no interference with the normal functioning of the NCX-mit. To study the action of Li^+ ions, we used theprotocol of experiments similar to that used in the experiments above; two durations of membrane depolarization (5 and 10 sec) under the KCl solution were used. As can be seen in Fig. 2, the effect of Li^+ was similar to that of La^{3+}.

In the presence of Li^+ ions, the amplitude of Ca^{2+} transients remained unchanged, while the level of cytoplasmic $[Ca^{2+}]_i$ after the decline of these transients did not fully return to the initial value. This level increased with respect to the control by 36.6 ± 2.1% ($P < 0.01$); the level of such shifts was independent of the cell depolarization duration. After washing out, the basal level of $[Ca^{2+}]_i$ returned to the initial value for 1-2 min. In contrast to the action of La^{3+}, we observed a significant decrease in the amplitude of KCl-induced Ca^{2+} transients in the experiments with Li^+ followed by its washout (Fig. 2). After 6-7 min, however, the

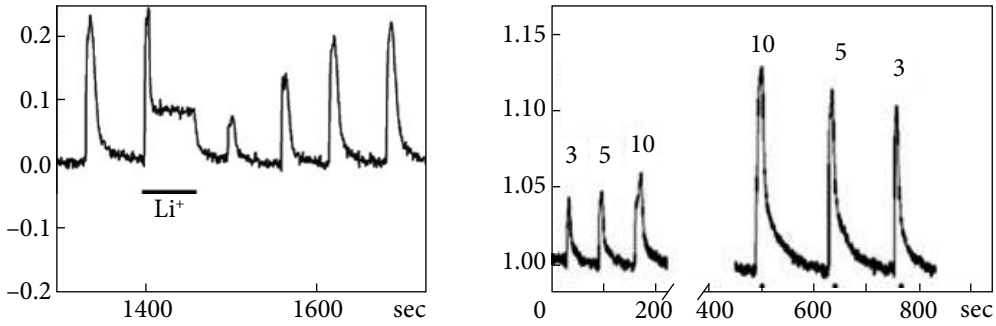

Fig. 2. Calcium transients in neurons of the cerebellum of the crucian (*Carassius gibelio*) induced by depolarization of the plasma membrane using 3-sec-long applications of 50 mM KCl. Measurements were performed under control conditions and upon blocking of the membrane Na^+/Ca^{2+} exchanger after replacement of Na^+ in the extracellular solution by Li^+ ions. The other designations are the same as in Fig. 1

Fig. 3. Effect of a mitochondrial blocker, CCCP, on calcium transients in cerebellar neurons of the crucian. Changes in the amplitude and shape of calcium transients under conditions of application of a hyperpotassium solution, which had different durations, from 1.0 to 20 sec (indicated above transients) are illustrated. The first three transients were recorded under control conditions (50 mM KCl), and the other three transients (indicated by points) were recorded under conditions of application of a solution containing 10 μM CCCP together with KCl. The other designations are the same as in Fig. 1

amplitude of these transients gradually returned to the control level. This phenomenon can be explained by the inability of Li^+ ions to replace Na^+ in the course of depolarization of the neuronal membrane mediated by the functioning of Na^+ channels. It should be noted that the described effects were clearly pronounced and reproduced in all tested neurons. Therefore, the obtained data indicate that the activities of PMCA and NCX of the plasma membrane in the *Carassius* cerebellar neurons are rather significant. Such activities contribute to a considerable extent to the functioning of the calcium signaling system in neurons of animals studied.

Role of mitochondria in calcium exchange

To elucidate the role of the mitochondria of cerebellar neurons of the crucian in Ca^{2+} signaling, we added the protonophore CCCP (carbonyl cyanide m-chlorophenylhydrazone) to the extracellular solution. This agent applied at a concentration of 10 μM blocks accumulation of Ca^{2+} ions by the mitochondria and does not influence other cellular structures. We used protocol with varied periods of action of the hyperpotassium solution, from 1.0 to 20 sec. Along with control application of the solution containing 50 mM KCl, we also used the same hyperpotassium solution but with the addition of 10 μM CCCP, which suppressed the uptake of Ca^{2+} ions by the mitochondria yet prior to the stimulation. As such

measurements showed, the amplitude of calcium transients after the above blocking of the functioning of the mitochondria increased; in this case, it increased proportionally to the increase in the duration of KCl application. The return of the Ca^{2+}level to the basal level after the development of transients was observed in all ofthe cells studied (Fig. 3). This indicates that functioning of the mitochondria noticeably influences the shape of the calcium signals.

The results of measurements of the parameters of calcium transients induced by 3 sec-long and 5 sec-long depolarization showed that the amplitude of Ca^{2+} transients in the presence of 10 μM CCCP increased with respect to the control values by 49.4 ± 0.1% ($P < 0.05$) upon 3 sec-long episodes of KCl application and by 41.9 ± ± 0.1% ($P < 0.05$) upon 5 sec-long KCl applications. Obtained data are indicative of the existence of significant calcium-storing activity of the mitochondria in neurons of the crucian cerebellum and also of a significant contribution of the above organelles to the functioning of the Ca^{2+} signal system in neurons of these animals.

Discussion

The presented data have shown that all the tested neurons of the *Carassius gibelio* cerebellum responded by a significant increase in the basal level of $[Ca^{2+}]_i$ upon the action of La^{3+} used in low concentrations, while the time parameters of calcium transients *per se* only slightly changed. This fact confirms that PMCA exists in the cytoplasmic membrane and demonstrates its significant activity. We also found that the above transport system effectively provides cleaning of the cytoplasm from Ca^{2+} excess, since the basal level of calcium transients demonstrated a one-third increase with respect to their control values after switching off the functioning of PMCA pump using its blocker (La^{3+}). The functioning of this pump practically did not depend on the duration of test depolarization.

We established that another system of cleaning the cytoplasm from Ca^{2+} excess, namely NCX, also exists in the plasma membrane of *Carassius* cerebellar neurons. After switching off the functioning of NCX using Li^{+} ions, the basal level of $[Ca^{2+}]_i$ from which Ca^{2+} transients start forming, increased by 36% or more with respect to the control values. The functioning of this transport system did not depend on the duration of depolarization either. These findings allow us to believe that the functioning of PMCA and NCX in neurons of the *Carassius gibelio* cerebellum significantly influences intracellular Ca^{2+} exchange providing the maintenance of an adequate basal $[Ca^{2+}]_i$ level in these neurons.

In our experiments, we revealed that the decay phase of KCl depolarization-induced intracellular Ca^{2+} transients slows down significantly with inhibition of accumulation of Ca^{2+} ions by the mitochondria. In addition, the amplitude of the transients increases noticeably. Therefore, our experiments indicate that the mitochondria are to a significant extent involved in the clearance of the cytoplasm of neurons from excessive Ca^{2+} accumulated during depolarization. Basing on our data, we can hy-

pothesize that mitochondria are characterized by a high sensitivity to local changes in the intracellular calcium concentration, in particular of those related to functioning of the endoplasmic reticulum (Pochynyuk et al., 2002). In addition, there are data showing that the peculiarities of functioning of mitochondria also depend on the specificity of spatial distribution of these organelles in the cell (Kostyk et al., 2005). It is obvious that further investigation of the above aspects is needed.

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