Measurements of mitochondrial pH in cultured cortical neurons clarify contribution of mitochondrial pore to the mechanism of glutamate-induced delayed Ca\textsuperscript{2+} deregulation

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Abstract

To clarify the role of the mitochondrial permeability transition pore (MPT) in the mechanism of the glutamate-induced delayed calcium deregulation (DCD) and mitochondrial depolarization (MD), we studied changes in cytosolic (pH\textsubscript{c}) and mitochondrial pH (pH\textsubscript{m}) induced by glutamate in cultured cortical neurons expressing pH-sensitive fluorescent proteins. We found that DCD and MD were associated with a prominent pH\textsubscript{m} decrease which presumably resulted from MPT opening. This pH\textsubscript{m} decrease occurred with some delay after the onset of DCD and MD. This argued against the hypothesis that MPT opening plays a dominant role in triggering of DCD. This conclusion was also supported by experiments in which Ca\textsuperscript{2+} was replaced with antagonist of MPT opening Sr\textsuperscript{2+}. We found that in Sr\textsuperscript{2+}-containing medium glutamate-induced delayed strontium deregulation (DSD), similar to DCD, which was accompanied by a profound MD. Analysis of the changes in pH\textsubscript{c} and pH\textsubscript{m} associated with DSD led us to conclude that MD in Sr\textsuperscript{2+}-containing medium occurred without involvement of the pore. In contrast, in Ca\textsuperscript{2+}-containing medium such “non-pore mechanism” was responsible only for MD initiation while in the final stages of MD development the MPT played a major role.

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1. Introduction

Hypoxic–ischemic injury of brain tissue is determined by overstimulation of NMDA subtype of glutamate receptors and subsequent neuronal Ca\textsuperscript{2+} overload [1]. The glutamate-induced deterioration of [Ca\textsuperscript{2+}]\textsubscript{c} homeostasis (also referred to as calcium deregulation) is accompanied by mitochondrial dysfunction, increase in cytoplasmic Na\textsuperscript{+} concentration, and reduction of intracellular ATP content (for reviews, see [2–4]).

Previous investigations have shown that mitochondrial depolarization (MD) plays a crucial role in the mechanism of glutamate-induced deregulation of neuronal Ca\textsuperscript{2+} homeostasis [2]. However, the exact mechanism of MD is not fully understood yet. Initially, it was found that cyclosporin A (CsA) and some of its derivatives retard and diminish glutamate-induced secondary increase in MD and [Ca\textsuperscript{2+}]\textsubscript{c} in cultured central neurons [5,6]. Taking into account the ability of these compounds to antagonize the opening of the permeability transition pore (MPT) in isolated mitochondria [7], it was concluded that MPT underlies the glutamate-induced collapse of the mitochondrial potential (∆ψ\textsubscript{m}). However, this conclusion was questioned by the finding...
that in many cases the ability of CsA to prevent glutamate-induced MD and Ca\(^{2+}\) deregulation could not be reproduced [8–11]. These contradictions and some difficulties in interpreting the effects of CsA even upon MPT in isolated mitochondria [12] prompted us to apply a novel experimental approach to the solution of this problem, namely to study glutamate-induced changes in mitochondrial pH (pH\(_{\text{m}}\)) and their relationship to cytosolic Ca\(^{2+}\) and pH (pH\(_{\text{c}}\)) dynamics.

It is well known that glutamate application to cultured neurons induces a pronounced decrease in pH\(_{\text{c}}\) and that cytosolic acidification is Ca\(^{2+}\)-dependent [13–15]. However, the effect of glutamate exposure on neuronal pH\(_{\text{m}}\) has not been investigated so far. Meanwhile, pH\(_{\text{m}}\) regulates mitochondrial Ca\(^{2+}\) buffering capacity [16,17] and, hence, strongly affects ability of neuronal mitochondria to accumulate Ca\(^{2+}\) and prevent cytosolic Ca\(^{2+}\) overloading. Moreover, it is logical to expect that mitochondrial pore opening would induce fast pH\(_{\text{m}}\) decrease (since pH\(_{\text{c}}\) is smaller than pH\(_{\text{m}}\)). Hence, MPT blockade would prevent mitochondrial acidification. However, we studied here dynamic changes in pH\(_{\text{c}}\) and pH\(_{\text{m}}\) during prolonged glutamate challenge using pH-sensitive proteins selectively expressed in cytosol and mitochondria (cytYFP and mtYFP [18]). These measurements were combined with simultaneous monitoring of changes in [Ca\(^{2+}\)]\(_{\text{c}}\). In parallel experiments on sister cells we carried out simultaneous monitoring of glutamate-induced changes in [Ca\(^{2+}\)]\(_{\text{m}}\) and Δψ\(_{\text{m}}\). All measurements were performed both under control conditions and after treatments known to antagonize the MPT opening in isolated mitochondria, i.e., Ca\(^{2+}\)/Sr\(^{2+}\) replacement and treatment with cyclosporin A (CsA) [19].

2. Materials and methods

2.1. Primary cultures of cortical neurons

Cortical neurons were prepared from 1 to 3 days old newborn Wistar rats. Cortical tissue was minced in ice-cold Krebs salt solution (130 mM NaCl, 5.4 mM KCl, 20 mM HEPES, 0.4 mM KH\(_{2}\)PO\(_4\), 15 mM glucose, 0.5 mM MgSO\(_4\) and 3 mg/ml BSA (Sigma), pH 7.4), then the tissue was digested in Krebs solution with 0.8 mg/ml trypsin 1–300 (ICN) for 15 min at 36°C. Trypsin was inactivated by washing with Krebs solution containing 0.08 mg/ml trypsin inhibitor (Sigma) and 0.01 mg/ml DNase (Roche). Cells were dissociated by trituration and pelleted in Krebs solution containing 0.5 mg/ml trypsin inhibitor and 0.08 mg/ml DNase. They were then resuspended in Neurobasal Medium (Gibco) with supplement B-27 (Gibco), GlutaMax (Gibco) and penicillin/streptomycin (Gibco) and plated onto 25 mm glass coverslips, coated with poly-d-lysine (Sigma).

To monitor changes in mitochondrial or cytosolic pH, cells were transfected after 4–7 days in culture with the mitochondrially or cytosolic targeted yellow fluorescent proteins (mtYFP or cytYFP, respectively) [18,20] using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), following the protocol recommended by the manufacturer. Fluorescence analyses were carried out 2–4 days after transfection and usually recorded from one to four transfected neurons in a field.

2.2. Imaging of [Ca\(^{2+}\)]\(_{\text{c}}\) (or [Sr\(^{2+}\)]\(_{\text{c}}\)) and mitochondrial potential or pH

Cortical cultures were loaded for 40 min with 3 μM fura-2FF/AM (Teflabs, Austin, TX, USA) in incubator in the cell culture medium (K\(_{d}\) of fura-2FF for Ca\(^{2+}\) and Sr\(^{2+}\) is 5 μM and 55 μM, respectively [21]). For simultaneous measurements of [Ca\(^{2+}\)]\(_{\text{c}}\) (or [Sr\(^{2+}\)]\(_{\text{c}}\)) and mitochondrial potential rhodamine-123 (Rh123, 7.8 μM, equivalent to 3 μg/ml; Molecular Probes) was added to the non-transfected culture during the last 15 min of the fura-2FF loading period. Rh123 was selected as potentiometric probe because it is relatively nontoxic and insensitive to changes in the potential of plasmamembrane [22,23].

For simultaneous fluorescence measurements of mitochondrial or cytosolic pH and [Ca\(^{2+}\)]\(_{\text{c}}\) (or [Sr\(^{2+}\)]\(_{\text{c}}\)) cortical cultures transfected with pH-sensitive proteins mtYFP or cytYFP were loaded with fura-2FF as described above. Images were acquired on an epifluorescence inverted microscope Axiovert 200 (Zeiss, Germany) equipped with a 20× fluorite objective for simultaneous measurements of fura-2FF and Rh123 signals and 40× for recording of fura-2FF and signals of fluorescent proteins. [Ca\(^{2+}\)]\(_{\text{c}}\) (or [Sr\(^{2+}\)]\(_{\text{c}}\)) and mitochondrial potential or [Ca\(^{2+}\)]\(_{\text{c}}\) (or [Sr\(^{2+}\)]\(_{\text{c}}\)) and pH were monitored in single cells using excitation light provided by a Xenon arc lamp, the beam passing sequentially through 10 nm bandpass filters centered at 340 nm, 380 nm and 490 nm housed in a computer-controlled filter wheel (Sutter Instrument Co., CA, USA). Emitted fluorescence light was reflected by dichroic mirror through a 505–530 nm filter (fura-2FF) and 515–565 nm filter (Rh123, mtYFP, and cytYFP) placed in computer-controlled filter wheel. Images were acquired by CCD camera (Roper Scientific, USA). All imaging data were collected and analyzed using the Metafluor 6.1 software (Universal Imaging Corp., USA). The fura-2FF data are presented as the ratio of light excited × × × for recording of fura-2FF and signals of fluorescent proteins. [Ca\(^{2+}\)]\(_{\text{c}}\) (or [Sr\(^{2+}\)]\(_{\text{c}}\)) and mitochondrial potential or [Ca\(^{2+}\)]\(_{\text{c}}\) (or [Sr\(^{2+}\)]\(_{\text{c}}\)) and pH were monitored in single cells using excitation light provided by a Xenon arc lamp, the beam passing sequentially through 10 nm bandpass filters centered at 340 nm, 380 nm and 490 nm housed in a computer-controlled filter wheel (Sutter Instrument Co., CA, USA). Emitted fluorescence light was reflected by dichroic mirror through a 505–530 nm filter (fura-2FF) and 515–565 nm filter (Rh123, mtYFP, and cytYFP) placed in computer-controlled filter wheel. Images were acquired by CCD camera (Roper Scientific, USA). All imaging data were collected and analyzed using the Metafluor 6.1 software (Universal Imaging Corp., USA). The fura-2FF data are presented as the ratio of light excited at 340/380 nm. Rh123 is a single-wavelength dye and its initial fluorescence intensity may vary between cells; therefore, all Rh123 data have been normalized to the resting intensity.

2.3. pH measurements

pH calibration was performed with the use of the following calibration solutions [13]: 5 μM nigericin, 1 μM FCCP, 134.2 mM K-gluconate, 1 mM MgCl\(_2\), and 20 mM HEPES. pH was adjusted by the addition of HCl or KOH. The pH-sensitivity of mtYFP and cytYFP is shown in Fig. 1. The data
were normalized and fitted to the three-parameter equation of a sigmoidal curve: 
\[
\frac{F_{490}}{F_0} = \frac{a}{1 + \exp(b(c - p\text{H}) \ln 10)}.
\]
The representative experiments \((n=4)\) yielded the following parameters: 
\(a = 1.029, b = 1.09, c = 6.45\) for mtYFP and 
\(a = 1.041, b = 1.385, c = 6.15\) for cytYFP. According to these measurements, pH in cytosol and mitochondrial matrix was 
\(7.2 \pm 0.1\) and \(8.0 \pm 0.1\), respectively.

2.4. Experimental procedures

The coverslips with cell culture were placed into the 300 µl experimental chamber at room temperature \((25 ^\circ \text{C})\) and washed with a standard physiological recording saline containing: 140 mM NaCl, 5.4 mM KCl, 1 mM MgCl\(_2\), 2 mM CaCl\(_2\), 5 mM glucose and 20 mM HEPES, pH adjusted to 7.4 with NaOH. Solution in the chamber was removed by peristaltic pump. Addition of solutions into the chamber was performed by pipette. Washout of solutions was made three times to completely remove old solution from the chamber. In experiments with cyclosporin A (CsA, 500 nM), the cells were preincubated with CsA for 1–1.5 h before experiments. In experiments with Sr\(^{2+}\) all solutions contained 2 mM Sr\(^{2+}\) instead of Ca\(^{2+}\), and before experiments cultures were placed for 1.5 h in Sr\(^{2+}\)-containing recording saline to exchange intracellular Ca\(^{2+}\) for Sr\(^{2+}\). Simultaneous measurements of [Ca\(^{2+}\)]\(_c\) (or [Sr\(^{2+}\)]\(_c\)) and \(\Delta \psi_m\) dynamics were performed in at least three independent experiments with neurons from the different cell preparations (15–20 cells were usually in a field).

All chemicals were from Sigma. CsA was a kind gift of Prof. P. Bernardi, University of Padua.

2.5. Isolation and purification of brain mitochondria

Non-synaptosomal mitochondria from the brains of male Sprague–Dawley rats, 200–250 g (Harlan, Indianapolis, IN) were isolated in mannitol-sucrose medium according to an IACUC approved protocol and purified on a discontinuous Percoll gradient [24]. Mitochondrial protein was measured by the Bradford method [25] using BSA as a standard.

2.6. Monitoring of respiration and \(\Delta \psi_m\) in isolated mitochondria

Mitochondrial respiration and membrane potential \((\Delta \psi_m)\) in isolated brain mitochondria was monitored simultaneously or separately in the incubation medium containing 125 mM KCl, 0.5 mM MgCl\(_2\), 3 mM glutamate, 3 mM succinate, 0.1% BSA, 10 mM HEPES, pH 7.4. The concentration of protein in the chamber was 0.25 mg/ml. \(\Delta \psi_m\) was monitored by following the concentration of tetraphenylphosphonium cation \((\text{TPP}^+)\) in the external medium with a TPP\(^+\)-sensitive electrode [26]. The monitoring of respiration and \(\Delta \psi_m\) was performed under continuous stirring at 37 °C in 0.3 ml chamber. All experimental traces shown are representative of at least three replicates.

2.7. Data analysis

To measure the time of the onset of delayed calcium deregulation \((t_{\text{DCD}})\), the ratio of fura-2FF was fitted by spline and differentiated. The first derivative of fura-2FF ratio has two peaks: the first peak corresponds to the [Ca\(^{2+}\)]\(_c\) increase in response to glutamate addition and the second peak corresponds to the DCD development. \(t_{\text{DCD}}\) is time interval between the onset of glutamate treatment and the time point on the left slope of the second peak where the derivative achieved 1/5 of its peak value. Similar procedure was used to evaluate time of the onset of strong mitochondrial depolarization \((t_{\text{MD}})\) which accompanied DCD.

The time of onset of secondary cytosolic or mitochondrial alkalization \((t_{\text{Cal}}\) or \(t_{\text{Mal}}\), respectively) was defined by the point at which the slope of the pH tracing became positive for at least three consecutive pH\(_c\) (or pH\(_m\)) measurements.

The time of onset of secondary mitochondrial acidosis \((t_{\text{Smac}})\) was defined by the point at which the slope of the pH\(_m\) tracing became negative for at least three consecutive pH\(_m\) measurements.

Statistical analysis was performed with the aid of Excel and Prism 2.0 (Graphpad Software Inc., CA, USA) software.
3. Results

3.1. Glutamate-induced deregulation of $\text{Ca}^{2+}$ homeostasis and mitochondrial function is independent of mitochondrial permeability transition

Previously it was established that the biphasic $[\text{Ca}^{2+}]_c$ increase induced by overstimulation of glutamate receptors in cultured hippocampal neurons and cerebellar granule cells is associated with synchronous changes in mitochondrial potential ($\Delta \psi_m$) [5,23,26,27]. Indeed, in our first set of experiments on cultured cortical neurons co-loaded with fura-2FF and Rh123 and treated with 100 $\mu$M glutamate (in Mg$^{2+}$-free, 10 $\mu$M glycine-containing solution) we observed a similar $[\text{Ca}^{2+}]_c$–$\Delta \psi_m$ relationship (Fig. 2). The changes in $[\text{Ca}^{2+}]_c$ and $\Delta \psi_m$ during prolonged glutamate challenge could be divided into two phases. The first phase included the initial $[\text{Ca}^{2+}]_c$ peak and the latent period preceding the secondary vast $[\text{Ca}^{2+}]_c$ elevation. The duration of the latent period varied among the cells from seconds to minutes. This phase of $[\text{Ca}^{2+}]_c$ response was accompanied by a minor mitochondrial depolarization (MD). The second phase, so-called delayed $\text{Ca}^{2+}$ deregulation (DCD), included the fast $[\text{Ca}^{2+}]_c$ increase to a high $\text{Ca}^{2+}$ plateau level. The DCD development was always accompanied by a strong mitochondrial depolarization. To analyze synchronicity of development of MD and DCD we compared the time until the onset of MD and DCD in cells and found that mean of the $t_{\text{MD}}/t_{\text{DCD}}$ values calculated for each cell was 1.006 ± 0.009, and paired t-test showed no difference between the two values ($p = 0.74$, $n = 71$; Fig. 2C and D), which is in agreement with previous studies [26]. After long-term glutamate challenge high $[\text{Ca}^{2+}]_c$ plateau became resistant to external $\text{Ca}^{2+}$ removal indicating an irreversible impairment of $\text{Ca}^{2+}$ extrusion from the cell [2,28,29].

Our next goal was to examine dynamics of mitochondrial pH ($\text{pH}_m$) during glutamate treatment and compare it with the dynamics of changes in $\Delta \psi_m$. However, simultaneous measurements of $\text{pH}_m$ and $\Delta \psi_m$ were not achievable due to spectrum overlap of the YFP and Rh123 probes. Nevertheless, due to the strong correlation of the glutamate-induced changes in $[\text{Ca}^{2+}]_c$ and $\Delta \psi_m$ (see Fig. 2) we considered the association between $\Delta \psi_m$ and $\text{pH}_m$ equivalent to the $[\text{Ca}^{2+}]_c$–$\text{pH}_m$ relationship. Since $\text{pH}$ of the mitochondrial matrix was shown to be closely related to the cytosolic $\text{pH}$ ($\text{pH}_c$) [30–32], in the next set of experiments we measured both parameters during glutamate exposure.

It has been previously established with the use of conventional fluorescent probes that glutamate treatment of neurons...
leads to a pronounced decrease in pHc [13–15]. Our experiments with cytYFP-expressing cells confirmed and extended these observations. We found that the glutamate-induced changes in pHc have a biphasic time course. During the first phase of [Ca\(^{2+}\)]c elevation pHc was decreased from 7.20 ± 0.07 to 6.00 ± 0.05 (n = 8). Subsequently, after beginning of DCD the cytosolic acidification was partly reversed, and pHc reached 6.10 ± 0.03. This phenomenon was denoted as secondary cytoplasmic alkalization (SCal, Fig. 3A). Next, glutamate-induced changes in pHm were studied in mtYFP-expressing cells. The mitochondrial alkalization was observed during the first phase of glutamate treatment, and subsequently, the mitochondrial acidification was observed. This phenomenon was denoted as secondary mitochondrial acidification (SMac, Fig. 3D). The recovery of [Ca\(^{2+}\)]c and pHm was observed after prolonged glutamate washout (∼30 min).
expressing cells loaded with fura-2FF. Similarly to pHc, the changes in pHm could be subdivided into two main phases. The first phase comprised a transient pHm increase and a subsequent pHm fall from 8.0 ± 0.10 to 7.2 ± 0.04 (n = 17, Fig. 3B) followed by a substantial further decrease to 6.40 ± 0.04, which we denoted as secondary mitochondrial acidification (SMac). This process was observed invariably in all cells examined, but in some cells (5 of 17 cells) it was preceded by a transient alkalization (not shown). Importantly, the secondary pHm decrease strictly followed the changes of [Ca2+]c since glutamate removal at the early, reversible phase (Fig. 3E) that the secondary mitochondrial acidification (Fig. 3C).

Next, we analyzed the relationships between the timing of SCal, SMac and DCD (tSCal, tSMac, and tDCD, respectively). The analysis revealed exact synchronism between the onset of SCal and SMac (Fig. 3D), reflecting proton flux from the cytoplasm into the mitochondrial matrix, which may result from opening of mitochondrial permeability transition (MPT) or any other pore. However, tSMac and tSCal were significantly larger than tDCD, as revealed by paired t-test analysis, showing (Fig. 3E) that the secondary mitochondrial acidification developed after the onset of [Ca2+]c deregulation. Therefore, we concluded that DCD and synchronous MD were initiated without synchronous increase in H+ influx through the inner mitochondrial membrane, i.e. without MPT or any other pore opening. However, the final stages of DCD and high [Ca2+]c plateau were accompanied by pronounced mitochondrial acidification which may result from MPT opening.

In our further experiments we examined possible role of MPT in pHm and Δψm changes induced by glutamate.

3.2. CsA treatment or Ca2+/Sr2+ replacement unveils mitochondrial alkalization related to MD but preceding DCD

In order to further characterize the relationship between MPT and MD, we applied two approaches to modify the development of MPT which was monitored by following pHc and pHm changes. First, neurons were pretreated with cyclosporin A (CsA, 0.5 µM) for 1–1.5 h. As shown in Fig. 4A and B, CsA had no effect on the final extent of the secondary pHm and pHc changes accompanying the DCD. However, the dynamics of pHc changes was radically altered during prolonged glutamate exposure in the presence of CsA. MPT, characterized by the almost complete equilibration of mitochondrial and cytosolic pH, developed later in CsA treated cells, as compared to non-treated controls, as measured by the time difference between the development of DCD and SCal (Fig. 4C). Still, CsA had no impact on development of strong MD and DCD, since the occurrence and timing of these events remained unaltered (n = 71; Fig. 5). These findings, in agreement with previous reports [10,11], confirmed that MD and DCD were not initiated by MPT.

Interestingly, CsA, apart from delaying equilibration of the cytosolic/mitochondrial pH gradient, had an additional effect on the pHm response after DCD development. As shown in Fig. 6A and B, in 8 out of 15 cells we observed a large, transient pHm rise during the secondary [Ca2+]c increase. This alkalization (i) was specific to mitochondria, since no parallel changes were observed in pHc, (ii) showed heterogeneous timing with respect to the secondary increase in [Ca2+]c. Due to the immense heterogeneity of the effect, no conclusion could be drawn on the mechanism of the observed pHm changes; therefore, in our further experiments we used another approach to antagonize MPT opening, i.e. replacement of Ca2+ with Sr2+ in the incubating medium.

It is well established that virtually all cellular Ca2+ transport systems, are also able to transport Sr2+ [21,33–35]. However, in contrast to Ca2+, Sr2+ ions do not induce the MPT opening and antagonize Ca2+-induced activation of MPT due to the competitive interaction with Ca2+ at the common binding site [19].

Similarly to CsA treatment, and in agreement with our previous data obtained in hippocampal and cerebellar neurons [2,27], we found that Ca2+/Sr2+ replacement neither prevented nor postponed significantly the development of the secondary increases in fura-2FF (delayed Sr2+ deregulation, DSD) and Rh123 fluorescence (MD, Fig. 5C, D and F). Conversely, analysis of [Sr2+]c–Δψm relationship revealed that, in contrast to the synchronous development of MD and DCD in Ca2+-containing medium, in Sr2+-containing medium MD appeared before the beginning of secondary [Sr2+]c increase in 90% cells (n = 62, Fig. 5E). These results, on the one hand, further confirmed that MPT is not a prerequisite for the glutamate-induced MD, and, on the other hand, gave an excellent experimental model for further analyzing the relationship between MD, mitochondrial Ca2+/Sr2+ transport and H+ handling. Thus, in the next set of experiment we analyzed the relationship between DSD and pH dynamics in the cytosol and mitochondria.

Yet again, similarly to CsA treatment, Ca2+/Sr2+ replacement did not influence the extent of pHc changes, i.e. the magnitude of cytosolic acidification and the following alkalization remained the same (Fig. 4A), but significantly slowed down the development of SCal (see Fig. 4C). In contrast, both the dynamics and extent of pHm changes during the second phase of [Sr2+]c increase was dramatically altered, Ca2+/Sr2+ replacement prevented the equilibration of cytosolic and mitochondrial pH gradient (see Fig. 4A and B). Moreover, in all experiments (n = 11) the secondary [Sr2+]c increase was accompanied by pronounced pHm elevation, which we denoted secondary mitochondrial alkalization (SMal: Fig. 6C). A comparison of times of the onset of DSD and secondary mitochondrial alkalization showed that tSMal/fDSD was 0.87 ± 0.02. Moreover, parallel comparison of the relationship between tSMal/fDSD and tMD/fDSD by paired t-test showed that both SMal and MD developed significantly earlier than DCD, indicating a close association between these events (Fig. 6D).
3.3. Sr\(^{2+}\) causes MPT-independent but \(P_1\)-dependent loss of \(\Delta \psi_m\) in isolated brain mitochondria

Based on Ca\(^{2+}\) transport measurements with isolated mitochondria it is generally accepted that Ca\(^{2+}\) influx into the matrix lowers \(\Delta \psi_m\) and raises pH gradient since H\(^+\) extrusion compensates for the Ca\(^{2+}\) uptake [36,37]. We assumed that similar mechanism might account for the parallel alkalization and MD in Sr\(^{2+}\)-containing medium. Since Ca\(^{2+}\)-induced matrix alkalization and MD in isolated mitochondria could be reversed by inorganic phosphate (\(P_1\)) in the next set of experiments we tested the effect of \(P_1\) and ruthenium red (RR), which blocks Ca\(^{2+}\) uniporter, on the Sr\(^{2+}\)-induced MD in isolated brain mitochondria.

As shown in Fig. 7, in the absence of \(P_1\) addition of Sr\(^{2+}\) to isolated brain mitochondria loaded with the potential sensitive probe tetraphenylphosphonium (TPP\(^+\)) led to strong MD. Importantly, this depolarization could be completely reversed by the successive addition of RR and \(P_1\). This suggests that development of MD is determined by two factors: (i) electrophoretic Sr\(^{2+}\) influx via uniporter and (ii) increase in pH gradient across the inner mitochondrial membrane. The later might occur due to the compensatory enhancement of H\(^+\) extrusion by the respiratory chain following Sr\(^{2+}\) entry into mitochondria. Activation of mitochondrial Pi/OH\(^-\)-antiporter caused by addition of \(P_1\) decreases pH gradient which restores the mitochondrial potential [38].

RR + \(P_1\) produced complete recovery of \(\Delta \psi_m\) in mitochondria depolarized by Sr\(^{2+}\) indicating that neither MPT nor any other pore opening was involved in development of the MD (Fig. 7A). The resistance of the Sr\(^{2+}\)-induced MD to the MPT blockers (ADP, ATP, and CsA) was revealed in the experiments with both suspension [39] and single rat brain mitochondria [40]. In contrast, when isolated brain mitochondria were incubated with Ca\(^{2+}\), RR + \(P_1\) did not cause complete recovery of \(\Delta \psi_m\) (Fig. 7B), presumably, due to
Fig. 5. Relationship between the \([\text{Ca}^{2+}]_c\) and mitochondrial potential during glutamate treatment in the presence of antagonists of mitochondrial permeability transition, cyclosporine A (CsA, 500 nM) or Sr\(^{2+}\). The neurons loaded with fura-2FF and Rh123 and treated with CsA (1–1.5 h) were exposed to glutamate. The presence of CsA did not prevent either DCD (A) or strong mitochondrial depolarization (B). The presence of Sr\(^{2+}\) instead of Ca\(^{2+}\) also did not prevent development of the secondary increase in \([\text{Sr}^{2+}]_c\) and strong mitochondrial depolarization (C and D). The panel (E) shows that the treatments known to antagonize MPT opening (CsA or Sr\(^{2+}\)) did not delay the onset of DCD. Note that, in contrast to synchronous development of DCD and MD in Ca\(^{2+}\)-containing medium, in Sr\(^{2+}\)-containing medium MD appeared before the onset of delayed strontium deregulation (DSD). (E) Neither MPT antagonist decreased proportion of the cells exhibited DCD and strong mitochondrial depolarization in response to glutamate. The data presented are average over at least three experiments, mean ± S.E.M.

MPT opening in a fraction of mitochondria (see also [39]). Indeed, in the incubation medium without exogenous P\(_i\) the major difference between Sr\(^{2+}\) - and Ca\(^{2+}\)-induced effects consisted in inability of added P\(_i\) to recover \(\Delta \psi_m\) collapsed by Ca\(^{2+}\) while Sr\(^{2+}\)-induced depolarization could be readily reversed by P\(_i\) (Fig. 8). Additionally, P\(_i\) activated respiration that could be due to a decrease in ΔpH and/or facilitation of substrate transport into mitochondria. Interestingly, cyclosporin A (CsA) failed to prevent Ca\(^{2+}\)-induced depolarization produced in the absence of exogenous P\(_i\). However, CsA decisively helped to recover Δ\(\psi\) after addition of P\(_i\) to mitochondria previously depolarized by Ca\(^{2+}\) (Fig. 8B and
Fig. 6. Effect of MPT antagonists on pHm dynamics. The neurons were transfected with mtYFP, loaded with fura-2FF, and placed in Sr2+-containing medium or incubated with CsA (500 nM, 1–1.5 h). The secondary [Ca2+]c increase (A) or high [Ca2+]c plateau (B) in the presence of CsA were accompanied by strong mitochondrial alkalization. Panel (C) shows typical neuronal response to glutamate in Sr2+-containing medium. Note that the secondary increase in [Sr2+]c was accompanied by a large mitochondrial alkalization which onset is marked by arrow. Panel (D) demonstrates that both strong MD and SMal began to develop before DSD.

C). This strongly suggested that, in contrast to Sr2+, Ca2+ produced depolarization involving the MPT. Thus, on the basis of obtained with Ca2+/Sr2+ replacement in the experiments with primary neurons or isolated brain mitochondria, we concluded that Ca2+ accumulation into mitochondria during prolonged glutamate treatment per se is sufficient to provoke mitochondrial depolarization accompanied by alkalization of the mitochondrial matrix. Yet, Ca2+ accumulation inevitably leads to MPT, which augments or, at least, preserves the depolarized state of the mitochondrial membrane, but as well lowers mitochondrial pH close to its cytosolic value.

4. Discussion

4.1. Relationship between pHm and [Ca2+]c during glutamate treatment

This work continues and extends previous studies of mechanisms responsible for regulation and destabilization of [Ca2+]c homeostasis in the brain neurons subjected to glutamate treatment [2,4]. In this study we performed comparative analysis of the changes in mitochondrial and cytosolic pH (pHm and pHc) with concurrent changes in [Ca2+]c and Δψm during and following prolonged glutamate exposure.

One of our main findings is that the primary [Ca2+]c increase and small MD during glutamate challenge was associated with a “primary mitochondrial acidification” preceded by a small transient pHm elevation. A transient small pHm increase at the onset of glutamate challenge seems to result from the enhanced proton extrusion in response to mitochondrial Ca2+ uptake. Such a phenomenon of Ca2+-induced pHm elevation was observed by many authors in experiments with isolated mitochondria (for references, see [30,36,39]) and recently was reported in neurons stimulated by glutamate [41]. The subsequent pHm decrease after transient pHm increase during the first phase of [Ca2+]c response seems to be a reflection of glutamate-induced cytosolic acidosis caused by plasmalemmal Ca2+/H+-pump suggesting that mitochondria can passively modulate pHc dynamics. Moreover, development of this mitochondrial acidification should also reduce mitochondrial ability to retain Ca2+ and affect composition of calcium-phosphate precipitates [16,17] formed during glutamate treatment [11].

The second major finding is that the secondary [Ca2+]c increase (DCD) and, hence, MD in all neurons was accompanied by delayed enhancement of mitochondrial acidification.
Fig. 7. Depolarization of isolated brain mitochondria induced by Ca\(^{2+}\) (A) or Sr\(^{2+}\) (B) and subsequent repolarization by ruthenium red and inorganic phosphate (Pi). Mitochondrial membrane potential was followed in the incubation medium without Pi. Additions: Ca\(^{2+}\) or Sr\(^{2+}\), 300 \(\mu\)M; Ruthenium Red (RR), 1 \(\mu\)M; Pi, 3 mM; 2,4-dinitrophenol (2,4-DNP), 80 \(\mu\)M.

(see Fig. 3). Existence of noticeable (to 3 min) delay between the onset of MD and the secondary pH\(_m\) fall suggests that MD was initiated without help of any pores. Third, strong Ca\(^{2+}\) load and development of MD promoted MPT opening which was seen in our experiments as profound fall in pH\(_m\) and small pH\(_c\) increase. Note that dynamics of [Ca\(^{2+}\)]\(_c\) and pH\(_m\) during development of DCD was “parallel”: [Ca\(^{2+}\)]\(_c\) recovery resulted in pH\(_m\) recovery (see Fig. 3C) whereas high [Ca\(^{2+}\)]\(_c\) and MD plateau was accompanied by a low pH\(_m\) plateau (see Fig. 3B). These findings suggest that dynamics of [Ca\(^{2+}\)]\(_c\) and pH\(_m\) during and after DCD development are tightly linked and it is likely that MPT serves as linking factor between these parameters.

4.2. Mechanisms of the secondary mitochondrial acidification

Theoretically, MPT opening at the onset of DCD should greatly enhance H\(^+\) influx from cytosol into the matrix resulting in decrease in pH gradient across the inner mitochondrial membrane, i.e. a considerable pH\(_m\) decrease and, possibly, pH\(_c\) increase. Our results are in agreement with these predictions: DCD was accompanied by delayed pH\(_m\) decrease and pH\(_c\) increase. Moreover, pH gradient between the cytosol and mitochondrial matrix was significantly decreased from 0.80 ± 0.17 (in resting cells) to 0.3 ± 0.1 (after development of the secondary pH\(_m\) decrease). “Incomplete” equilibration of pH difference between the cytosol and mitochondria during the secondary pH\(_m\) decrease is, presumably, determined by MPT opening only in a subpopulation of neuronal mitochondria [11].

The results obtained with the use of MPT antagonist, Sr\(^{2+}\), also supported our hypothesis that the secondary
mitochondrial acidification in Ca\textsuperscript{2+}-containing medium is a result of MPT opening. It is known that Sr\textsuperscript{2+} ions accumulated by mitochondria do not induce MPT opening and inhibit Ca\textsuperscript{2+}-induced MPT opening [19]. Hence, the secondary mitochondrial acidification in the presence of Sr\textsuperscript{2+} cannot result from MPT opening by Ca\textsuperscript{2+} which may be released during glutamate treatment from the intracellular stores such as endoplasmic reticulum. Presumably, the secondary Sr\textsuperscript{2+}-induced pH\textsubscript{m} decrease may result from an increase in the proton conductance of inner mitochondrial membrane and lead to collapse of pH difference between the cytosol and mitochondrial matrix. Another possibility is that this secondary pH\textsubscript{m} fall ensued from Sr\textsuperscript{2+} removal from the matrix by mitochondrial Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger and subsequent Na\textsuperscript{+} extrusion by mitochondrial Na\textsuperscript{+}/H\textsuperscript{+} exchanger. We found that after development of secondary pH\textsubscript{m} decrease in Sr\textsuperscript{2+}-containing medium pH gradient between the neuronal cytosol and mitochondrial matrix remained the same as in resting cells (0.82 ± 0.18 in rest vs. 0.67 ± 0.07 during secondary pH\textsubscript{m} fall). This means that, despite strong mitochondrial depolarization and profound pH\textsubscript{m} decrease, H\textsuperscript{+} permeability of the inner mitochondrial membrane in the presence of Sr\textsuperscript{2+} was not increased. Therefore, the secondary Sr\textsuperscript{2+}-induced pH\textsubscript{m} decrease results not from increase in permeability of inner mitochondrial membrane (including MPT opening) but from Sr\textsuperscript{2+}/H\textsuperscript{+} exchange.

Our finding that Ca\textsuperscript{2+}/Sr\textsuperscript{2+} replacement prevented Ca\textsuperscript{2+}-induced decrease in pH gradient between the cytosol and mitochondrial matrix suggests that this decrease resulted from opening of mitochondrial proton channel activated by Ca\textsuperscript{2+} and inhibited by Sr\textsuperscript{2+}, i.e. MPT. This conclusion is supported by a comparison of mitochondrial morphology in situ during glutamate treatment in Ca\textsuperscript{2+}- and Sr\textsuperscript{2+}-containing media which showed that Sr\textsuperscript{2+}, in contrast to Ca\textsuperscript{2+}, does not induce mitochondrial swelling [39]. Thus, the secondary pH\textsubscript{m} fall and a decrease in pH gradient between the mitochondrial matrix and cytosol in Ca\textsuperscript{2+}-containing medium is a result of MPT opening.

4.3. Mechanism of the secondary pH\textsubscript{m} increase during glutamate treatment

Next, we addressed question how glutamate treatment may cause mitochondrial alkalization during DCD (or DSD). As discussed above, addition of Ca\textsuperscript{2+} to respiring mitochondria in the absence of inorganic phosphate (P\textsubscript{i}) induces MD and pH\textsubscript{m} elevation which may be suppressed by P\textsubscript{i} addition [36,39,42,43]. The presence of P\textsubscript{i} triggers an electroneutral OH\textsuperscript{−} efflux via P\textsubscript{i}/OH\textsuperscript{−}-antiporter and, thus, decreases the proton gradient contributing to MD. Our data obtained in experiments with isolated brain mitochondria indicate that the Sr\textsuperscript{2+}-induced MD may include two components: the electrogenic uniporter-mediated Sr\textsuperscript{2+} influx and the pH gradient created by imbalance between the enhanced H\textsuperscript{+} extrusion and H\textsuperscript{+} influx into the matrix. The Ca\textsuperscript{2+}-induced MD also includes these two components but, in addition, it implicates a third component, MPT opening.

Based on these data, it is conceivable that the secondary increase in pH\textsubscript{m} during a prolonged glutamate challenge may result from imbalance between H\textsuperscript{+} extrusion mediated by the respiratory chain and a compensatory OH\textsuperscript{−} efflux via P\textsubscript{i}/OH\textsuperscript{−}-antiporter. It seems that H\textsuperscript{+} influx/efflux imbalance results from a suppression of compensatory phosphate influx via P\textsubscript{i}/OH\textsuperscript{−}-antiporter. Another possibility is that this imbalance between proton fluxes may ensue from some other exchange processes such as mitochondrial K\textsuperscript{+}/H\textsuperscript{+} - or Na\textsuperscript{+}/H\textsuperscript{+}-exchange. However, our data obtained in experiments with isolated brain mitochondria suggest that P\textsubscript{i}/OH\textsuperscript{−}-exchange plays dominant role in the regulation of Δψ\textsubscript{m} and pH\textsubscript{m}. Therefore, it is likely that long-term continuing Ca\textsuperscript{2+} (or Sr\textsuperscript{2+}) influx and accumulation in the mitochondrial matrix during glutamate treatment suppresses activity of mitochondrial P\textsubscript{i}/OH\textsuperscript{−}-antiporter. This suppression may be determined by saturation of the buffering capacity of mitochondria due to excessive accumulation of Ca\textsuperscript{2+}(or Sr\textsuperscript{2+})–P\textsubscript{i} complex in the matrix or depletion of intracellular phosphate.

4.4. Mechanisms of strong mitochondrial depolarization (MD) during glutamate treatment

We showed that in both Ca\textsuperscript{2+}- and Sr\textsuperscript{2+}-containing media MD occurred without mitochondrial pore opening since this MD at initial stages was not accompanied by a decrease in pH gradient between the cytosol and mitochondrial matrix. Moreover, in 30% cells in the presence of Ca\textsuperscript{2+} and in 100% neurons in the presence of Sr\textsuperscript{2+} MD was accompanied by mitochondrial alkalization and, hence, increase in pH gradient between cytosol and mitochondrial matrix. It is clear that in this case MD results from mitochondrial Ca\textsuperscript{2+} (or Sr\textsuperscript{2+}) uptake which is uncompensated by OH\textsuperscript{−} (see above). Therefore, MD accompanied by mitochondrial alkalization reflects conversion of mitochondrial potential into pH gradient between the mitochondrial matrix and cytosol.

The MD in the absence of pH\textsubscript{m} changes (seen in the majority of neurons under control conditions) seems to result from enhancement of mitochondrial Ca\textsuperscript{2+} uptake due to impairment of Ca\textsuperscript{2+} extrusion systems of plasma membrane [28,29]. However, in this case Ca\textsuperscript{2+} uptake is compensated by P\textsubscript{i}/OH\textsuperscript{−} antiporter which prevents development of mitochondrial alkalization.

Thus, in any case strong glutamate-induced MD in Ca\textsuperscript{2+}-containing medium is initiated by MPT-independent mechanism which leads to opening of the MPT maintaining strong MD at plateau level.

Conflict of interest

The authors do not have any conflicts of interest.
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References

[37] V. Petronilli, C. Cola, P. Bernardi, Modulation of the mitochondrial cyclosporin A-sensitive permeability transition pore. II. The minimal


