Induction of Cyclosporin A-Sensitive Pore in Mitochondria of Intact Neurons during Uncoupling of Oxidative Phosphorylation

T. P. Storozhevykh, A. I. Yuryavichyus, E. G. Sorokina, and V. G. Pinelis

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 131, No. 5, pp. 520-523, May, 2001
Original article submitted December 4, 2000

Using primary cultures of cerebellar granule cells we showed that Ca^{2+} transported into neurons under the effect of glutamate is accumulated and stored in mitochondria for a long time. Protonophore FCCP, an uncoupler of oxidative phosphorylation, stimulated the release of Ca^{2+} from mitochondria in a calcium-free medium in 81% glutamate-treated cells. Cyclosporin A and ATP-synthase blocker oligomycin decreased the number of cells with FCCP-induced Ca^{2+} release to 53 and 12%, respectively. Oligomycin partly prevented glutamate- and FCCP-induced decrease of intracellular ATP level.

Key Words: *neurons; mitochondria; mitochondrial pore; glutamate; cyclosporin A; oligomycin*

Recent studies demonstrated that Ca^{2+} entering neurons during their activation is accumulated in mitochondria [9,12]. Ca^{2+} uptake by mitochondria plays an important role in restoring intraneuronal Ca^{2+} homeostasis after stimulation of glutamate (Glu) receptors [8,13]. Ca^{2+} enters mitochondria via Ca^{2+} uniporter in the inner mitochondrial membrane due to transmembrane potential difference ($\Delta\Psi$~180 mV). Uncoupling of oxidative phosphorylation with protonophores (for example, FCCP phenylhydrazone) after Glu stimulation increases untracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in a calcium-free medium [9]. It is assumed that uncoupler-induced $\Delta\Psi$ collapse reverts the electrochemical potential for Ca^{2+} ions and they are transported back to the cytosol via the uniporter [6]. However, the experiments on isolated mitochondria showed that protonophore-induced Ca^{2+} release from mitochondria is insensitive to the uniporter blocker ruthenium red, whereas at low external Ca^{2+} concentration comparable to that in resting cell (50-100 nM) uniporter is blocked. Moreover, Ca^{2+} transport via the uniporter can be activated only by high $\Delta\Psi$ [3]. On the other hand, protonophores can induce the formation of a nonselective pore in the inner membrane of Ca^{2+}-loaded mitochondria [5]. Mitochondrial pore (MP) can be induced also in intact cells, for example in rat thymocytes [1]. Here we studied Ca^{2+} transport from deenergized mitochondria in cultured CNS neurons.

MATERIALS AND METHODS

The study was carried out on 7-9-day primary cultures of cerebellar granule cells obtained from 1-week-old Wistar rat pups [2]. $[Ca^{2+}]_i$ and $\Delta\Psi$ were measured spectrofluorimetrically on a Diamorph image analyser combined with a SPEX fluorimeter. Image analyser allowed recording of several neurons simultaneously. For $[Ca^{2+}]_i$ measurements the cells were loaded with Fura-2/AM (5 μM, Fluka) for 40 min, while $\Delta\Psi$ was measured after 10-min incubation with rhodamine 123 (5 μM, Molecular Probes). Thereafter, the cells were washed for 20 min with control saline containing (in mM): 130 NaCl, 5 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 20 HEPES, and 5 glucose pH 7.4. The cells on a cover-

Laboratory of Membranology, Center of Children Health, Russian Academy of Medical Sciences, Moscow. ***Address for correspondence:*** tstor@mail.ru. Storozhevykh T. P.

0007-4888/01/1315-0440$25.00 © 2001 Plenum Publishing Corporation

slip were transferred to a 0.2-ml perfusion chamber mounted on a stage of an inverted Nikon microscope. Fura-2 fluorescence was measured at 340 and 380 nm excitation and 505 nm emission wavelengths. Changes in $[Ca^{2+}]_I$ are presented as F_{340}/F_{380} ratio. Rhodamine 123 fluorescence was measured at 488 nm excitation and 535 nm emission wavelengths. The initial F_{488} of each cell was accepted as 100%. The increase in F_{488} fluorescence indicated $\Delta\Psi$ decrease. Intracellular ATP content was measured on a Lucy-1 luminometer using a luciferin-luciferase kit. Protein content was determined by the method of Lowry.

RESULTS

Addition of 100 μM Glu (+10 μM glycine, 0 Mg^{2+}) caused a transient $[Ca^{2+}]_i$ increase in cerebellar granule cells (Fig. 1, *a*). Five min after termination of Glu treatment $[Ca^{2+}]_i$ returned to the initial level and the cells were treated with 1 μM protonophore FCCP (Sigma) in a calcium free medium. FCCP increased $[Ca^{2+}]_i$ in 81% examined neurons (n=275, Fig. 1, *a*). Since FCCP activates voltage-dependent Ca^{2+} channels in the plasma membrane and stimulates Ca^{2+} influx [7], the incubation medium in the post-glutamate period contained no calcium ions (+100 μM EGTA). Inhibition of Ca^{2+}-ATPase of endoplasmic reticulum with 5 μM thapsigargin (Fig. 1, *a*) did not affect secondary FCCP-induced $[Ca^{2+}]_i$ increase. Hence, this increase was due to the release of Ca^{2+} stored in deenergized mitochondria during Glu stimulation. FCCP had no effect on cells not treated with Glu. FCCP-induced $[Ca^{2+}]_i$ increase depended on the time interval between termination of Glu treatment and FCCP application: increasing this interval to 30 min almost completely eliminated the effect of FCCP (data not shown). It is important to note that FCCP-stimulated Ca^{2+} release from mitochondria was characterized by a 30 sec-5 min latency in different cells (Fig. 1, *a*). This lag-period from the addition of the protonophore to the post-glutamate $[Ca^{2+}]_i$ increase was also observed in cerebellar granule cells [9]. At the same time, our study showed that $\Delta\Psi$ collapse occurred simultaneously in all cells. The Glu-induced increase in $[Ca^{2+}]_i$ caused only a minor $\Delta\Psi$ decrease, whereas the subsequent 20-30-sec FCCP application caused $\Delta\Psi$ collapse in all cells (Fig. 1, *b*). We hypothesized the formation of a Ca^{2+} channel in the inner mitochondrial membrane, which occurred during this lag-period and was not determined by $\Delta\Psi$. This channel can be presented by nonselective MP (permeability transition pore). MP can be identified by its sensitivity to changes in Ca^{2+}, $\Delta\Psi$, and immunosuppressive drug cyclosporin A (CSA) [5]. Our experiments showed that FCCP-induced Ca^{2+} release from mitochondria depended on the way of neuron activation: FCCP caused no $[Ca^{2+}]_i$ increase in cells stimulated with 50 mM KCl instead of Glu (equimolar replacement of NaCl; Fig. 2, *a*). However, FCCP-induced $\Delta\Psi$ decrease was the same (data not shown). Ca^{2+}/H^+ ionophore ionomycin (1 μM) added for testing of mitochondrial Ca^{2+} content did not increase $[Ca^{2+}]_i$, which confirmed insignificant Ca^{2+} accumulation in mitochondria. These findings are at controversy to previously reported data [12]. Rapid inactivation of voltage-dependent Ca^{2+} channels resulted in $[Ca^{2+}]_i$ decrease despite the presence of K^+. It can be assumed that the decrease in $[Ca^{2+}]_i/[Ca^{2+}]_{matrix}$ gradient reverted Ca^{2+} flux from mitochondria to cytosol (by Na^+-dependent or -independent parthway [3]) and then from the cells to the medium, which prevented $[Ca^{2+}]_i$ increase in response to FCCP (Fig. 2, *a*). Direct measurements showed that the rate of Ca^{2+} sequestration in mitochondria was lower in the case of Ca^{2+} influx via voltage-dependent, compared to Glu-operated channels [12].

Preincubation with 1-2 μM CSA (Sigma, 1 h before Glu) decreased the number of neurons with post-glutamate FCCP-induced $[Ca^{2+}]_i$ increase to 53% examined cells (n=66) compared to 81% without CSA pretreatment (p<0.05). MP induction was also very sensitive to the blockade of mitochondrial ATP-synthase with oligomycin (2.5 μg/ml, Sigma; Fig. 2, *b*).

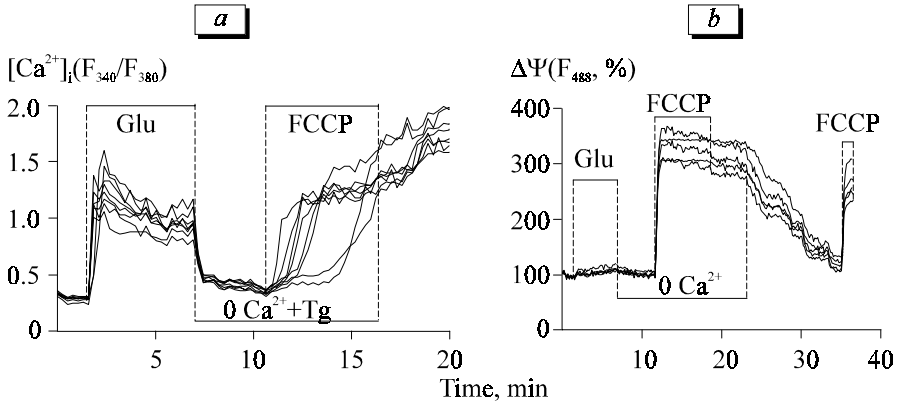

Fig. 1. Changes in $[Ca^{2+}]_i$ (*a*) and mitochondrial potential $\Delta\Psi$ (*b*) in rat cerebellar neurons (n=8) during FCCP (1 μM) action in the post-glutamate period. Increase in F_{488} fluorescence corresponds to $\Delta\Psi$ decrease. Here and in Fig. 2 and 3: Glu: glutamate, 100 μM (+10 μM glycine, 0 Mg^{2+}); 0 Ca^{2+}: calcium-free medium with 100 μM EGTA; Tg: thapsigargin, 5 μM.

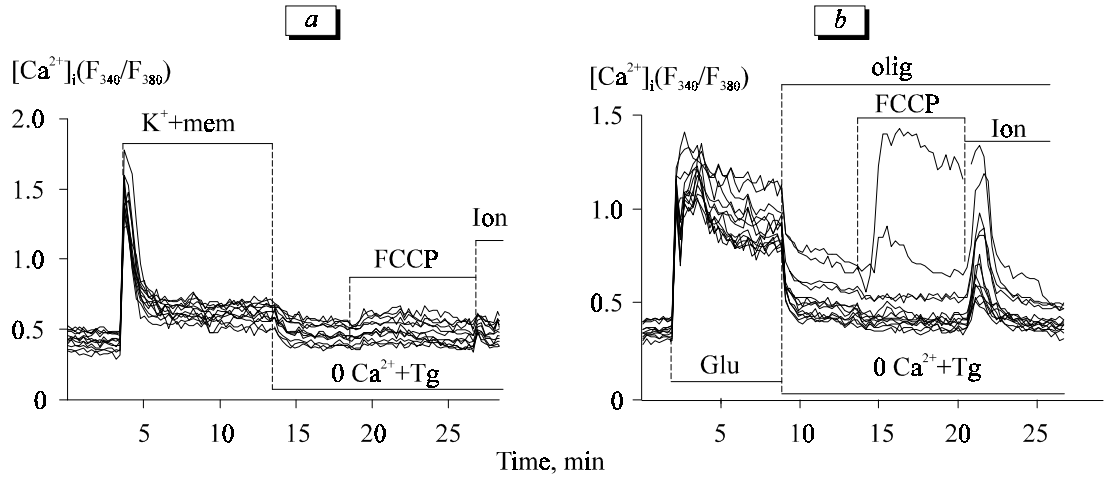

Fig. 2. Effect of uncoupler FCCP on $[Ca^{2+}]_i$ in rat cerebellar neurons (n=12) after KCl depolarization (*a*) and in the post-glutamate period against the background of mitochondrial ATP-synthase inhibition with oligomycin (*b*). *a*) absence of $[Ca^{2+}]_i$ increase; *b*) prevention of FCCP-induced $[Ca^{2+}]_i$ increase during post-glutamate period. K^+: 50 mM KCl; Ion: ionomycin, 1 μM; mem: memantine, NMDA channel blocker, 50 μM; olig: oligomycin, 2.5 μg/ml.

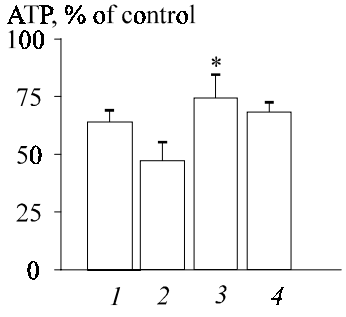

Fig. 3. Changes in ATP level in cerebellar neurons. *1*) 10 min of 100 μM Glu action; *2*) 10 min Glu+5 min 1 μM FCCP (experiment record see on Fig. 1, *a*); *3*) 10 min Glu+5 min FCCP+oligomycin (experiment record see on Fig. 2, *b*); *4*) 10 min oligomycin. Control ATP level: 5.6 nmol/mg protein. *p<0.01 compared to series 2.

No secondary FCCP-induced $[Ca^{2+}]_i$ increase in the post-glutamate period was observed in 88% examined neurons (n=102) after oligomycin pretreatment. This was associated with preserved mitochondrial Ca^{2+} level (positive ionomycin test, Fig. 2, *b*). Oligomycin did not affect FCCP-induced deenergization of mitochondria. It is known that FCCP-induced deenergization of mitochondria is accompanied by inhibition of aerobic ATP synthesis and reversion of ATP-synthase resulting in hydrolysis of ATP formed during glycolysis [10]. Measurements of ATP in cerebellar granule cells (Fig. 3) showed that FCCP added during the post-glutamate period induced a more pronounced ATP decrease compared to that caused by Glu (47% of the control level). Oligomycin added to FCCP-containing medium increased ATP level to 74% of the control (Fig. 3). We assumed that combined action of FCCP and oligomycin does not decrease the content of ATP to a critical level necessary for MP induction. It was shown that cytoplasmic ATP via hexokinase or direct complexatation with adenine nucleotide translocase on the inner mitochondrial membrane can inhibit MP formation [4]. Moreover, oligomycin can inhibit MP by increasing ADP in mitochondrial matrix [3] and/or inhibiting binding of proapoptotic protein Bax to mitochondrial membrane [11].

Thus, our findings indicate the possibility of MP induction in Glu-stimulated neurons during uncoupling of oxidative phosphorylation. Rapid Ca^{2+} release from mitochondria occurs through MP. Apart from mitochondrial Ca^{2+} level and mitochondrial membrane potential, cytoplasmic ATP concentration may also contribute to MP induction. The decrease in ATP content in brain cells below the critical level induced by ischemia/hypoxia can cause the formation of irreversible MP [5].

The authors are grateful to B. V. Cherhyak for the helpful discussion and to N. P. Vinskaya for preparation of cell cultures.

The study was supported by the Russian Foundation for Basic Research (grant No. 99-04-49181).

REFERENCES

1. B. V. Cherhyak, *Biokhimiya*, **64**, Iss. 8, 1097-1104 (1999).
2. N. Andreeva, B. Khodorov, E. Stelmashuk, *et al., Brain Res.*, **22**, 150-157 (1991).
3. P. Bernardi, *Physiol. Rev.*, **79**, 1127-1155 (1999).
4. G. Beulner, K. Ruc, B. Riede, and D. Broliczka, *Biochim. Biophys. Acta*, **1368**, 7-18 (1998).
5. M. Crompton, *Biochem. J.*, **341**, 233-249 (1999).
6. T. E. Gunter and D. R. Pfeiffer, *Am. J. Physiol.*, **258**, C755-C786 (1990).
7. S. K. Juthberg and T. Brismar, *Cell Mol. Neurobiol.*, **17**, No. 4, 367-377 (1997).
8. B. Khodorov, V. Pinelis, T. Storozhevykh, *et al., FEBS Lett.*, **393**, 135-138 (1996).
9. L. Kiedrowski and E. Costa., *Mol. Pharmacol.*, **47**, 140-147 (1995).

10. A. Leyssens, A. Nowicky, L. Petterson, *et al., J. Physiol.*, **496**, Pt. 1, 111-128 (1998).
11. S. Matsuyama, O. Xu, J. Velours, and J. Reed, *Moll. Cell,* **1**, No. 3, 327-336 (1998).
12. T.-I. Peng and J. T. Greenamyre, *Mol. Pharmacol.*, **53**, 974-980 (1998).
13. O. Vergun, J. Keelan, B. Khodorov, *et al., J. Physiol.*, **519**, No. 2, 451-466 (1999).