

# Mitochondrial deenergization underlies neuronal calcium overload following a prolonged glutamate challenge

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**Abstract** The purpose of our work was to study the relationship between glutamate (GLU)-induced mitochondrial depolarization and deterioration of neuronal  $\text{Ca}^{2+}$  homeostasis following a prolonged GLU challenge. The experiments were performed on cultured rat cerebellar granule cells using the fluorescent probes, rhodamine 123 and fura-2. All the cells, in which 100  $\mu\text{M}$  GLU (10  $\mu\text{M}$  glycine, 0  $\text{Mg}^{2+}$ ) induced only relatively slight mitochondrial depolarization (1.1–1.3-fold increase in rhodamine 123 fluorescence), retained their ability to recover  $[\text{Ca}^{2+}]_i$  following a prolonged GLU challenge. In contrast, the cells in which GLU treatment induced pronounced mitochondrial depolarization (2–4-fold increase in rhodamine 123 fluorescence), exhibited a high  $\text{Ca}^{2+}$  plateau in the post-glutamate period. Application of 3–5 mM NaCN or 0.25–1  $\mu\text{M}$  FCCP during this  $\text{Ca}^{2+}$  plateau phase usually failed to produce a further noticeable increase in  $[\text{Ca}^{2+}]_i$ . Regression analysis revealed a good correlation ( $r^2 = 0.88 \pm 0.03$ ,  $n = 19$ ) between the increase in the percentage of rhodamine 123 fluorescence and the post-glutamate  $[\text{Ca}^{2+}]_i$ . Collectively, the results obtained led us to conclude that the GLU-induced neuronal  $\text{Ca}^{2+}$  overload was due to the collapse of the mitochondrial potential and subsequent ATP depletion.

**Key words:** Mitochondrial depolarization; Neuronal  $\text{Ca}^{2+}$  overload; Glutamate neurotoxicity

## 1. Introduction

It is generally accepted that glutamate (GLU)-induced neuronal  $\text{Ca}^{2+}$  overload is an early key step in delayed nerve cell injury caused by hypoxic/ischemic insult [1]. However, the mechanism of this deterioration of  $\text{Ca}^{2+}$  homeostasis has not yet been clarified. Recently [2,3], it has been found that prolonged GLU treatment of cultured central mammalian neurons produced mitochondrial depolarization (MD) along with an increase in  $[\text{Ca}^{2+}]_i$  [4–6],  $[\text{Na}^+]_i$  [7,8],  $[\text{H}^+]_i$  [9,10] and a reduction in the neuronal ATP content [3,11]. In the present work, carried out on cultured rat cerebellar granule cells, we reveal for the first time: (i) the existence in these neurons of different patterns of GLU-induced changes in mitochondrial potential ( $V_{\text{mit}}$ ) and (ii) a close correlation between the MD and  $[\text{Ca}^{2+}]_i$  measured following a toxic GLU challenge. In

these experiments, the fluorescent probes rhodamine 123 (Rh 123) and fura-2/AM were used to monitor changes in  $V_{\text{mit}}$  and  $[\text{Ca}^{2+}]_i$ , respectively. The results obtained provide strong evidence in favour of the hypothesis [2,12] that it is the collapse of the  $V_{\text{mit}}$  that underlies the GLU-induced neuronal  $\text{Ca}^{2+}$  overload.

## 2. Materials and methods

Primary cerebellar granule cell cultures were prepared from the cerebella of 7–8-day-old Wistar rats using a procedure described earlier [13]. The cells were grown in minimal Eagle's medium containing 25 mM KCl. The experiments were carried out on 7–8-day-old cultures. Fluorescence measurements in individual neurons were performed using the Spex spectrofluometric system (NJ, USA) described earlier [14]. The HEPES-buffered salt solution (HBSS) contained (in mM): 145 NaCl, 5 KCl, 1.8  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 20 HEPES, and 5 glucose. Sucrose was added to bring the osmolarity up to 320 mosmol. Rh 123 was dissolved in aqueous solution and the cells were exposed to medium containing 10  $\mu\text{g}$  of the dye/ml for 10 min at room temperature. The dye was well retained after washing of the cells with HBSS. Rh 123 fluorescence was excited at 488 nm and measured at 530 nm. To measure the changes in  $[\text{Ca}^{2+}]_i$ , the rest of the neurons were first incubated for 1 h at room temperature with 5  $\mu\text{M}$  fura-2/AM in HBSS and then washed with HBSS and left for 30 min to allow fura-2/AM deesterification and equilibration between the  $\text{Ca}^{2+}$ -bound and  $\text{Ca}^{2+}$ -free forms. Some neurons were co-loaded with both fluorescent probes in order to carry out measurements of  $V_{\text{mit}}$  and  $[\text{Ca}^{2+}]_i$  in the same neuron. These cells were first incubated for 50 min with fura-2 and then during the last 10 min with a fura-2 plus Rh 123 mixture. Since our system (Spex spectrofluorimeter) did not allow us to perform simultaneous monitoring of both the Rh 123 and fura-2 signals, we restricted ourselves to infrequent switching of the system from  $V_{\text{mit}}$  monitoring to measurements of  $[\text{Ca}^{2+}]_i$  (see later). The  $[\text{Ca}^{2+}]_i$  results are not presented in calibrated form because of the uncertainty arising from the use of different calibration techniques.

Fura-2/AM and Rh 123 were purchased from Molecular Probes; all other chemicals were from Sigma.

## 3. Results

Application of 100  $\mu\text{M}$  GLU (in  $\text{Mg}^{2+}$ -free, 10  $\mu\text{M}$  glycine-containing solution) to resting cells induced an increase in Rh 123 fluorescence, indicating depolarization of the inner mitochondrial membrane [15]. However, both the time course and the extent of MD varied among the cells over a wide range. In contrast, the GLU-induced changes in  $[\text{Ca}^{2+}]_i$  measured in parallel experiments with fura-2-loaded sister neurons exhibited only relatively small variations. Considerable differences in  $[\text{Ca}^{2+}]_i$  dynamics in these cells were revealed only in the post-glutamate period (see later). Fig. 1 demonstrates three major patterns of changes in Rh 123 fluorescence, henceforth referred to as patterns A–C of MD. The proportions of cells exhibiting these MD patterns varied between different culture series despite the apparently identical conditions for nerve cell cultivation. Thus, in some cultures most of cells

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**Abbreviations:**  $[\text{Ca}^{2+}]_i$  and  $[\text{Na}^+]_i$ , cytosolic  $\text{Ca}^{2+}$  and  $\text{Na}^+$  concentrations, respectively; pH<sub>i</sub>, cytosolic pH; fura-2/AM, acetoxymethyl ester of fura-2; NMDA, *N*-methyl-D-aspartate; GLU, glutamate; NMDG, *N*-methyl-D-glucamine; HBSS, HEPES-buffered salt solution; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone;  $V_{\text{mit}}$ , mitochondrial potential; MD, mitochondrial depolarization

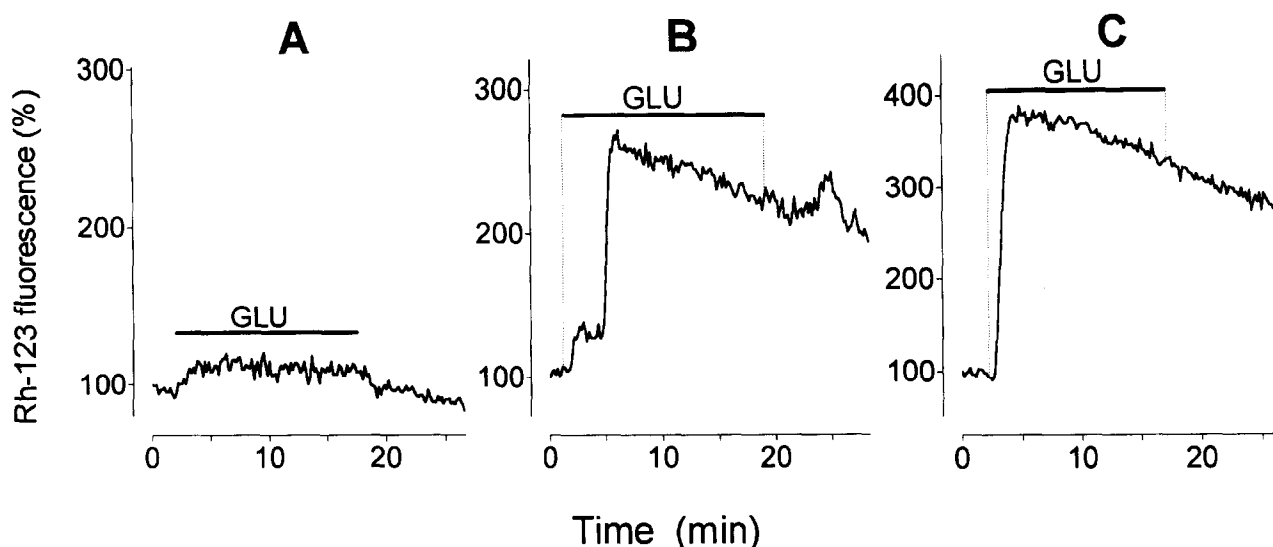


Fig. 1. Three major patterns of changes in Rh 123 fluorescence in individual cerebellar granule cells induced by 15 min application of 100  $\mu$ M glutamate (GLU) in  $Mg^{2+}$ -free, 10  $\mu$ M glycine-containing solution. Fluorescence recorded as a percentage change from the resting level and plotted against the time (min). An increase in fluorescence was interpreted as the result of mitochondrial depolarization on the basis of the method described in [15].

exhibited an A pattern of MD: the Rh 123 fluorescence increased by only 5–30% (mean  $\pm$  S.D.  $13.98 \pm 2.2$ ,  $n = 20$ ) and returned after the termination of GLU application. This slight MD could be transiently increased by short-term applications of the protonophore FCCP (Fig. 2A), or the blocker of mitochondrial electron transport,  $CN^-$  (Fig. 2B). Repeated FCCP pulses during GLU challenge led to a steady increase in Rh 123 fluorescence (see Fig. 2A,  $n = 9$ ). Another manipulation, which allowed us to produce an immediate increase in GLU-induced MD, was the replacement of  $Na^+$  in the GLU solution by NMDG [16]. The origin of such  $Na^+$  dependence of GLU effects on  $V_{mit}$  will be considered in more detail elsewhere.

In other cultures, most of the cells exhibited a B or C pattern of MD (see Fig. 1B,C). In cells with the B pattern of MD, the initial Rh 123 fluorescence rose by 25–39% (mean  $\pm$  S.D.  $39 \pm 7.3$ ,  $n = 5$ ) during a first step and then after a 2–9 min lag increased steeply to values which exceeded the initial fluorescence level by 80–250% (mean  $\pm$  S.D.  $174 \pm 33.7$ ,  $n = 5$ ). In cells exhibiting the C pattern of MD, the first step of MD was absent, and the initial Rh 123 fluorescence increased by 100–300% (mean  $\pm$  S.D.  $173 \pm 39.3$ ,  $n = 8$ ) just after the onset of the GLU challenge. The rate of this one-step MD varied between cells. After prolonged GLU application, Rh 123 fluorescence either remained at a high quasi-plateau level or partially recovered to a low plateau level (not illustrated). Application of  $CN^-$  or FCCP during a pronounced GLU-induced MD (B and C patterns) usually produced only a relatively small additional MD (not illustrated).

In parallel experiments on fura-2-loaded sister cells, we examined the effects of  $CN^-$  and FCCP pulses on  $[Ca^{2+}]_i$  before, during and after prolonged GLU challenge. Application of 3–5 mM NaCN to resting cell in most cases ( $n = 20/25$ ) failed to induce noticeable changes in the baseline  $[Ca^{2+}]_i$  (Fig. 3A). Similar results were obtained in analogous experiments with another inhibitor of mitochondrial respiration, antimycin A (0.5  $\mu$ M), used in combination with the mitochondrial ATPase inhibitor, oligomycin (2.5  $\mu$ g/ml) [15,17].

Evidently, mitochondria of cerebellar granule cells at rest lack releasable  $Ca^{2+}$  pool [17]. Exposure of nerve cells to 100  $\mu$ M GLU in  $Mg^{2+}$ -free, 10  $\mu$ M glycine solution usually increased the fura-2 fluorescence ratio (340/380 nm) from its basal level (0.8–0.9) to the plateau value (2.5–3.5) (see Figs. 3 and 4). The addition of  $CN^-$  to the GLU-containing medium in a number of cases caused an additional increase in  $[Ca^{2+}]_i$ , whose amplitude varied between cells, apparently depending on the extent of MD produced by GLU itself. Thus, in Fig. 3A repeated short-term  $CN^-$  trials induced high-amplitude reversible  $[Ca^{2+}]_i$  responses. To explain these  $CN^-$  effects, it is necessary to assume that GLU alone produced in this cell only a relatively small MD (A pattern) which did not abolish mitochondrial  $Ca^{2+}$  uptake. Therefore, the  $CN^-$  pulses were able to cause additional pronounced  $V_{mit}$  reduction. Indeed, these particular changes in Rh 123 fluorescence were observed in parallel experiments performed on the same day with cells from this culture. The result of one of these experiments ( $n = 9$ ) is presented in Fig. 2B. We believe that the large and rapid  $[Ca^{2+}]_i$  responses to  $CN^-$  application in Fig. 3A resulted from both acute cessation of electrogenic  $Ca^{2+}$  uptake in the face of enhanced GLU-induced  $Ca^{2+}$  influx, and the fast unloading of the mitochondrial  $Ca^{2+}$  store. Of special interest is the reversibility of  $CN^-$ -induced  $Ca^{2+}$  responses. Note also that, in Fig. 2A, the wash-out of GLU from the cell led to rapid partial  $[Ca^{2+}]_i$  recovery mediated apparently by mitochondrial  $Ca^{2+}$  uptake (see also [18]). Similarly high  $[Ca^{2+}]_i$  responses to  $CN^-$  application during prolonged GLU treatment were observed in 8 analogous experiments, and all these cells exhibited partial  $[Ca^{2+}]_i$  recovery in the post-glutamate period. Application of  $CN^-$  against the background of this low post-glutamate  $Ca^{2+}$  plateau usually elicited a new reversible  $[Ca^{2+}]_i$  increase; the amplitude of the latter varied widely from one cell to another. In other neurons ( $n = 14$ ),  $CN^-$  application during GLU challenge either induced only minor additional  $[Ca^{2+}]_i$  elevation or had no effect at all. These neurons exhibited a high  $[Ca^{2+}]_i$  plateau in the post-glutamate period. Application of  $CN^-$  (alone or in combination with

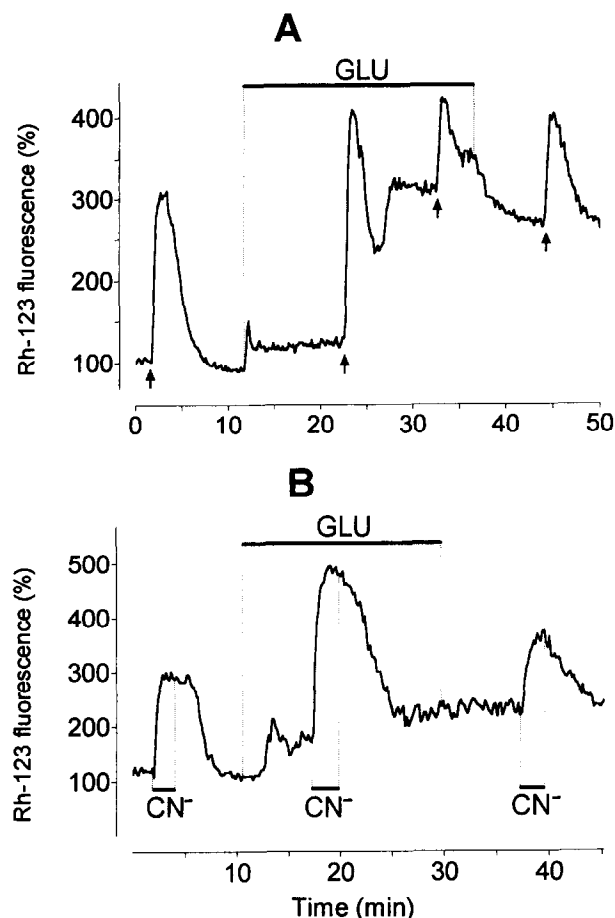


Fig. 2. Changes in Rh 123 fluorescence produced by 0.5  $\mu$ M FCCCP (arrows) and 3 mM NaCN ( $\text{CN}^-$ ) pulses during and after a prolonged GLU (100  $\mu$ M) challenge. Pulse durations for FCCCP and  $\text{CN}^-$  were 30 s and 3 min, respectively.

oligomycin) in this period did not produce an additional increase in  $[\text{Ca}^{2+}]_i$  (Fig. 3B). Qualitatively similar results were obtained in experiments with the combined application of 0.5  $\mu$ M antimycin and 2.5  $\mu$ g/ml oligomycin ( $n=7$ ) or 0.25–1  $\mu$ M FCCP ( $n=13$ ). When applied during the period of the high plateau following a GLU challenge, FCCP either failed to produce any changes in  $[\text{Ca}^{2+}]_i$  or elicited only a very small elevation (see Fig. 3B).

All the data presented above suggest that the post-glutamate  $[\text{Ca}^{2+}]_i$  overload following prolonged GLU application is causally linked to the concomitant MD. To verify this hypothesis, we performed a series of experiments on a group of cells co-loaded with both fluorescent probes, Rh 123 and fura-2 ( $n=19$ ). In each of these cells, Rh 123 fluorescence was monitored continuously before, during and after GLU challenge; in contrast, the fura-2 fluorescence was measured only before and just after the cessation of the 15 min GLU application. It was found that in all the cells, in which prolonged GLU treatment induced an A pattern of MD (see Fig. 1A), the fura-2 fluorescence ratio (reflecting  $[\text{Ca}^{2+}]_i$ ) was increased in the post-glutamate period only 1.1–1.4-fold ( $n=10$ ). In contrast, in all the cells which exhibited high and prolonged MD (B and C patterns), fura-2 measurements revealed a high  $[\text{Ca}^{2+}]_i$  plateau following a 15 min GLU challenge: the 340/380 nm fluorescence ratio was increased 3–4-fold with respect

to its basal value ( $0.84 \pm 0.187$ ,  $n=19$ ). It is necessary to emphasize that short-term (3 min) GLU application failed to produce a high  $[\text{Ca}^{2+}]_i$  plateau in the post-glutamate period even in those cells which exhibited high-amplitude MD (not illustrated).

To evaluate quantitatively the correlation between the GLU-induced increase in Rh 123 fluorescence and  $[\text{Ca}^{2+}]_i$  (340/380 nm ratio) in the post-glutamate period, we performed a linear regression analysis of the data obtained (Fig. 4). The correlation turned out to be unexpectedly high: the determination coefficient,  $r^2$ , was  $0.88 \pm 0.03$  ( $n=19$ ), suggesting that 88% of the variations in the post-glutamate  $[\text{Ca}^{2+}]_i$  could be attributed to prolonged MD.

#### 4. Discussion

This paper describes for the first time the major patterns of GLU-induced MD (see Fig. 1A–C) in cerebellar granule cells. What is the origin of these variations?

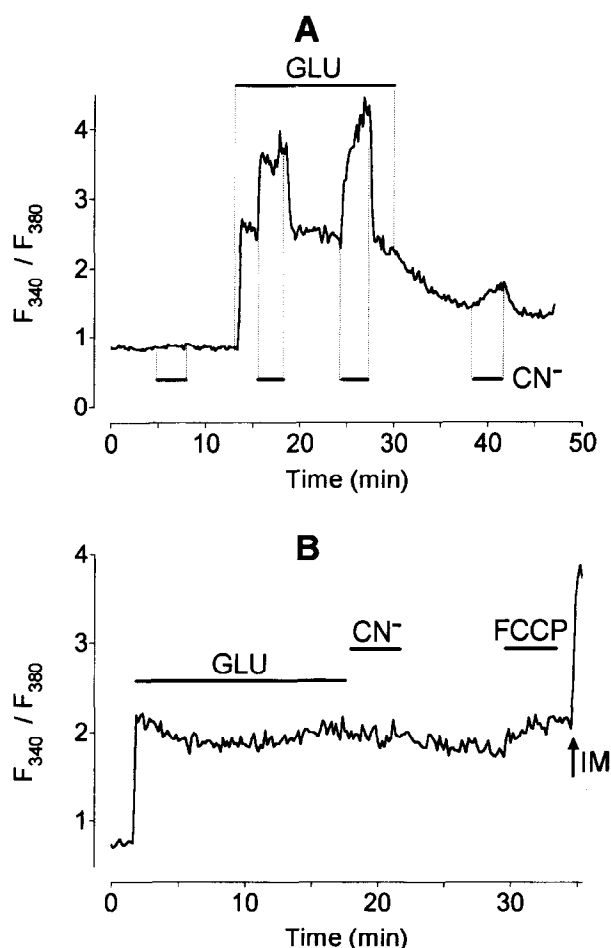


Fig. 3. (A) Changes in  $[\text{Ca}^{2+}]_i$  caused by 3 mM NaCN pulses ( $\text{CN}^-$ ) before, during and after a 15 min treatment with 100  $\mu$ M GLU. Judging from the effects of  $\text{CN}^-$  in this cell, GLU alone failed to induce pronounced mitochondrial depolarization. (B) Effects of application of  $\text{CN}^-$  (3 mM NaCN) and FCCCP (1  $\mu$ M) on  $[\text{Ca}^{2+}]_i$  in the period of post-glutamate high  $\text{Ca}^{2+}$  plateau. The large increase in  $[\text{Ca}^{2+}]_i$  induced by 5  $\mu$ M ionomycin (IM) shows that the inability of  $\text{CN}^-$  and FCCCP to induce appreciable changes in  $[\text{Ca}^{2+}]_i$  cannot be explained by the saturation of fura-2 but apparently resulted from the GLU-induced collapse of the mitochondrial potential.

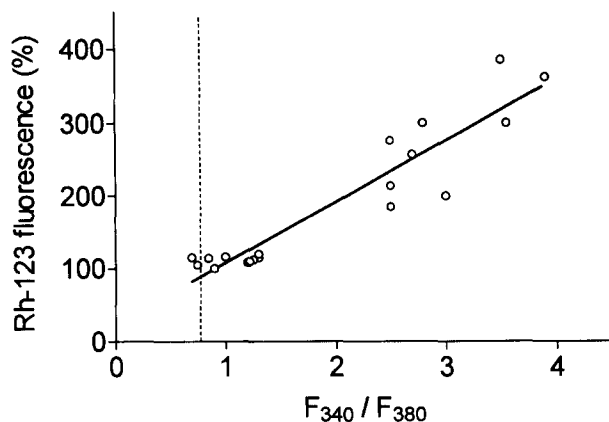


Fig. 4. Correlation between GLU-induced mitochondrial depolarization and the post-glutamate  $[Ca^{2+}]_i$  increase in individual cerebellar granule cells. Each symbol corresponds to a single cell. (Abscissa) Maximal increase in Rh 123 fluorescence during the GLU challenge. (Ordinate) Fura-2 fluorescence ratio. The dotted vertical line shows the average baseline 340/380 nm ratio:  $0.84 \pm 0.87$  (mean  $\pm$  S.D.,  $n = 19$ ). The calculated determination coefficient  $r^2 = 0.878272$ .

The relatively small MD (A pattern, see Fig. 1A) induced by GLU in a subpopulation of cerebellar granule cells resembles that observed by Loew et al. [19] who studied the effects of high  $K^+$  (135 mM) and bradykinin on  $V_{mit}$  in NIE-115 neuroblastoma cells. The most probable explanation for such MD is the electrogenic  $Ca^{2+}$  influx into mitochondria via the uniporter. In contrast, an abrupt GLU-induced collapse of  $V_{mit}$  (B and C patterns of MD, see Fig. 1) observed in other neurons was probably due to the opening of the 'permeability transition pore' in the inner mitochondrial membrane [2,20]. There exist a number of factors which promote this pore opening [20,21]. Among these factors, of particular importance are the 'reactive oxygen species' (ROS) [20] generated by mitochondria during GLU challenge [22–24]. It is tempting to suppose that the second step step of MD (see Fig. 1B) as well as the fast one-step collapse of  $V_{mit}$  (see Fig. 1C) produced by GLU were triggered by ROS generated in response to excessive  $Ca^{2+}$  entry into the mitochondrial matrix. It is also quite probable that the neurons exhibiting the A pattern of MD were protected against the GLU excitotoxic action by an intrinsic antioxidant defence system [25].

Another major finding of this work is a high correlation between the GLU-induced MD and the post-glutamate neuronal  $Ca^{2+}$  overload (see Fig. 4). A sustained increase in  $[Ca^{2+}]_i$  in the post-glutamate period was observed only in those neurons in which prolonged GLU treatment induced strong MD. In these cells, the application of  $CN^-$  or FCCP in the post-glutamate period failed to produce a further increase in  $[Ca^{2+}]_i$  (see Fig. 3B). What might be the reason for such dependence of  $[Ca^{2+}]_i$  dynamics on the functional state of mitochondria? It is well known that the collapse of  $V_{mit}$  has a dual effect on neuronal  $Ca^{2+}$  transport: (i) inhibition of the electrogenic  $Ca^{2+}$  uptake and mitochondrial buffering; and (ii) reversal of mitochondrial ATP synthase [17], which leads to the rapid hydrolysis of cytoplasmic ATP. It is evident that the depletion of neuronal ATP (and the corresponding increase in ADP/ATP ratio) should suppress the ATP-fuelled  $Na^+/K^+$  and  $Ca^{2+}/H^+$  pumps, and diminish, in addition, the activity of the plasmalemmal  $Na^+/Ca^{2+}$  exchanger. Since these effects develop slowly in time it becomes clear why a

short-term collapse of  $V_{mit}$  fails to produce a high  $[Ca^{2+}]_i$  plateau in the post-glutamate period. However, cessation of the electrophoretic  $Ca^{2+}$  uptake by mitochondria after the collapse of  $V_{mit}$  should also make a considerable contribution to the delayed destabilization of  $Ca^{2+}$  homeostasis, since it has been established that under physiological conditions the amount of  $Ca^{2+}$  accumulated by the mitochondria without any deterioration of their bioenergetic properties is essentially unlimited,  $Ca^{2+}$  forming a complex with phosphate [26]. It is therefore tempting to assume that a sustained increase in  $[Ca^{2+}]_i$  following toxic GLU challenge results from the inhibition of both the mitochondrial  $Ca^{2+}$ -buffering and ATP-dependent  $[Ca^{2+}]_i$  regulating systems.

*Note:* Since the submission of this manuscript, the reports of Reynolds and White [27] and Schinder [28] have been published strongly supporting the view that mitochondrial deenergization is a primary event in GLU neurotoxicity. The authors, however, did not analyse the correlation between GLU-induced changes in MD and the post-glutamate  $Ca^{2+}$  overload presented in our paper.

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