Dominant role of mitochondria in protection against a delayed neuronal Ca^{2+} overload induced by endogenous excitatory amino acids following a glutamate pulse

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Abstract The objective of this study was to evaluate the contribution of mitochondria to the clearance of Ca^{2+} loads induced by glutamate or 25 mM K^+ pulses. The mitochondrial Ca^{2+} uptake was suppressed by application of 0.5 μM antimycin A or 3–5 mM NaCN in combination with 2.5 μg/ml oligomycin. In most cells such treatments both in the presence and in the absence of external Na^+ failed to abolish the early fast phase of $[Ca^{2+}]_i$ recovery following a 1-min 100 μM glutamate pulse. However, the late slow phase of $[Ca^{2+}]_i$ recovery in the presence of mitochondrial poisons was transformed into a delayed $[Ca^{2+}]_i$ elevation culminating in the neuronal Ca^{2+} overload. Suppression of the Na^+/Ca^{2+} exchange caused by glutamate-induced $[Na^+]_i$ elevation promoted the development of delayed Ca^{2+} increase. Under identical conditions, the high $[Ca^{2+}]_i$ transient induced by 25 mM K^+ was never accompanied by a delayed Ca^{2+} elevation. The glutamate-induced delayed Ca^{2+} increase could be readily abolished by the removal of external Ca^{2+} or by application in the post-glutamate period of the antagonist of NMDA receptors, 100–200 μM AP-5. The results obtained suggest that mitochondria play a dominant role in the protection against the neuronal Ca^{2+} overload induced by endogenous excitatory amino acids released in response to a short-term glutamate challenge.

Key words: Mitochondrial Ca^{2+} uptake; Ca^{2+} overload; Endogenous excitatory amino acid; Cultured neuron

1. Introduction

The role of mitochondria in the clearance of Ca^{2+} loads from mammalian central neurons following a short-term activation of receptor-operated and voltage-sensitive channels has not yet been elucidated. White and Reynolds [1] found that application of the mitochondrial uncoupler FCCP to cultured cortical neurons immediately after termination of the GLU challenge caused a marked delay in $[Ca^{2+}]_i$ recovery. This effect was interpreted as a result of FCCP-induced inhibition of the mitochondrial Ca^{2+} uptake during the recovery period. There is, however, another explanation of the above effect of FCCP: the deceleration of $[Ca^{2+}]_i$ recovery caused by FCCP may result from a release of Ca^{2+} accumulated in the mitochondria during the GLU pulse. To choose between these two possibilities, we suppressed the mitochondrial Ca^{2+} uptake not only following the GLU pulse but also before and during its application. This was achieved by a combined application of antimycin (ANT), an inhibitor of mitochondrial electron transport, and oligomycin (OLIG), which blocks the mitochondrial ATPase. The latter was used in order to prevent the rapid hydrolysis of cytoplasmic ATP by the reversal of ATP synthase [2]. The changes in the $[Ca^{2+}]_i$ dynamics were monitored following a short-duration GLU or a high K^+ challenge. It has first been established that the mitochondrial Ca^{2+} uptake is not a prerequisite for the early fast $[Ca^{2+}]_i$ recovery following a 1-min GLU pulse. However, mitochondria protect nerve cells against a delayed Ca^{2+} overload resulting from release of endogenous excitatory amino acids (EAA) following a GLU challenge.

2. Materials and methods

Dissociated cerebellar granule cell cultures were prepared from the cerebella of 7–8-day-old Wistar rats using the procedure described in [3]. The cultures were incubated in a medium with 25 mM K^+ throughout the whole cultivation period. The experiments were carried out on 7–8-day cell cultures. $[Ca^{2+}]_i$ was measured in individual neurons loaded with the Ca^{2+}-sensitive fluorescent probe, fura-2/AM, as described earlier [4]. The experimental chamber was mounted on a Nikon inverted-stage microscope connected to a 'Spex' (NJ, USA) spectrofluorimeter equipped with a beam splitter, two excitation monochromators and a dual mirror chopping mechanism with a specialized optical configuration to allow rapid alternation (100 Hz) between two fura-2 excitation wavelengths, 340 and 380 nm. The excitation bandwidths were set at 3.5 nm. The emitted fluorescence was filtered through a cut-off filter at 505 nm. $[Ca^{2+}]_i$ was measured by the ratio of fura-2 fluorescence excited at 340 nm to that excited at 380 nm and calibrated according to the procedure described in [5]. The control HEPES-buffered salt solution (HBSS) contained (mM): 145 NaCl, 5 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 20 HEPES, 5 glucose, pH 7.4. Sucrose was added to bring the osmolarity up to 320 mosmol.

Fura-2/AM was purchased from Molecular Probes (USA). All the other chemicals were from Sigma Chemical Company (USA).

3. Results and discussion

Fig. 1A demonstrates the typical biphasic time course of $[Ca^{2+}]_i$ recovery following a 1-min GLU (100 μM) pulse to the untreated cerebellar granule cell. A subsequent combined treatment of the resting cell by 0.5 μM ANT and 2.5 μg/ml OLIG did not change the baseline $[Ca^{2+}]_i$ thus supporting the view that under normal conditions mitochondria lack a releasable Ca^{2+} pool ($n = 25$). The first fast phase of $[Ca^{2+}]_i$ recovery following the GLU pulse remained practically unchanged.

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Abbreviations: $[Ca^{2+}]_i$ and $[Na^+]_i$, the cytosolic Ca^{2+} and Na^+ concentrations, respectively; Fura-2/AM, acetoxymethyl ester of fura-2; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NMDA, *N*-methyl-D-aspartate; GLU, glutamate; AP-5, 2-amino-5-phosphonopentanoic acid; NMDG, *N*-methyl-D-glucamine; HBSS, HEPES-buffered salt solution; ANT, antimycin A; OLIG, oligomycin; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; DCE, delayed $[Ca^{2+}]_i$ elevation; EAA, excitatory amino acids

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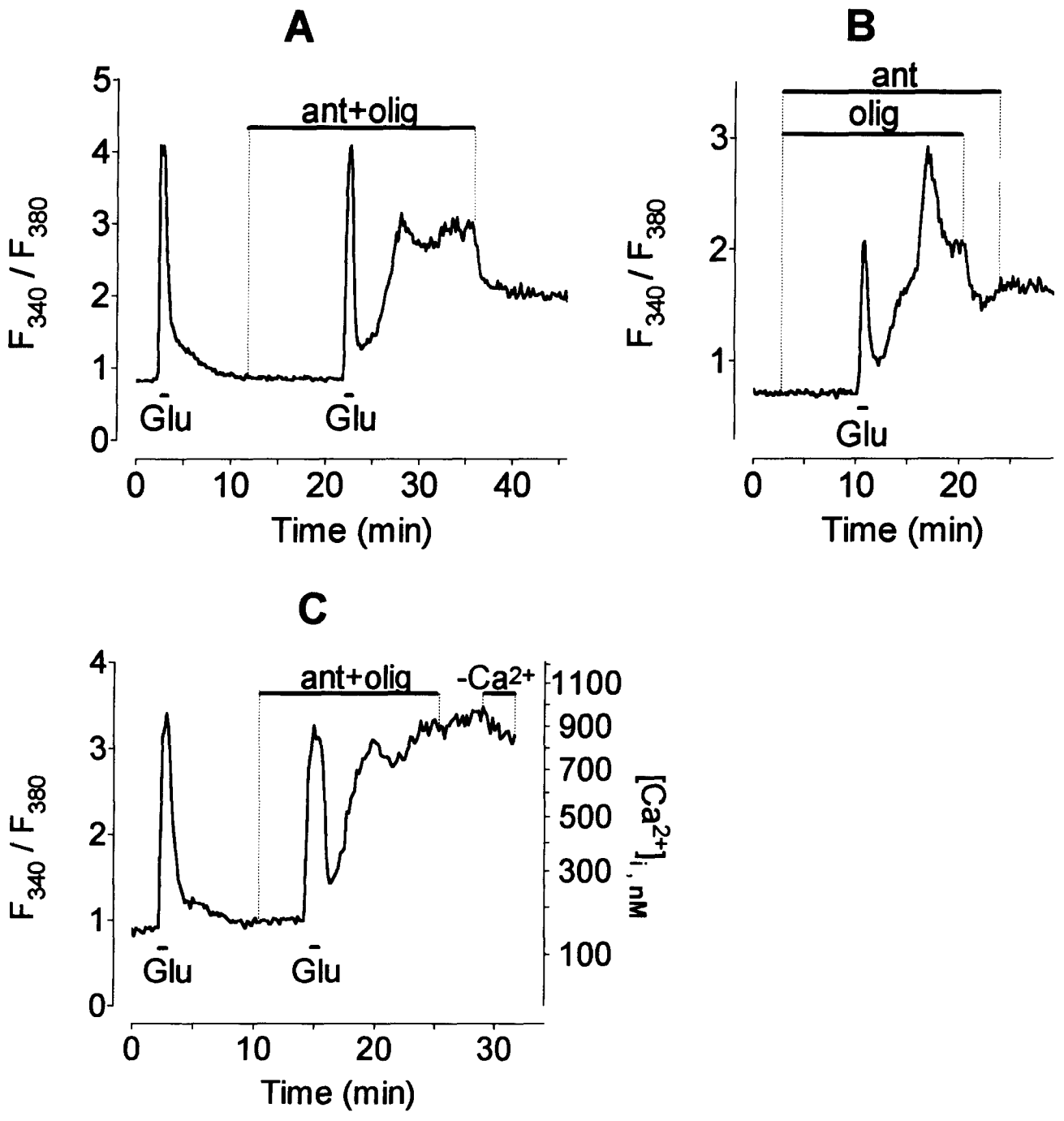

Fig. 1. Effect of a combined antimycin (ant) and oligomycin (olig) treatment of the cell on $[Ca^{2+}]_i$ dynamics following a 1-min GLU pulse. A,B,C: Examples of some variations in the shape of the delayed $[Ca^{2+}]_i$ elevation (DCE) following 1-min Glu pulses. In A and B, DCE was partially reversible; in C, DCE was irreversible. Horizontal bars indicate the times of application of GLU (100 μM, in a Mg^{2+}-free 10 μM glycine-containing solution); ant (0.5 μM) and olig (2.5 μg/ml); $-Ca^{2+}$: a nominally Ca^{2+}-free solution containing ant+olig.

By contrast, the late slow phase of $[Ca^{2+}]_i$ recovery was transformed into a delayed $[Ca^{2+}]_i$ elevation (DCE). This effect was partly reversible: a wash-out of the cell with HBSS decreased $[Ca^{2+}]_i$ to a new quasi-plateau level. Qualitatively similar results were obtained in 19 analogous experiments with ANT+OLIG and in five experiments with NaCN (5 mM)+OLIG (2.5 μg/ml). Quantitative differences between the cells mainly concerned the rate, magnitude and reversibility of DCE. Some examples of these variations in the shape of DCE are presented in Fig. 1B,C. In only three out of 25 cells DCE was absent: instead the cell exhibited a very low post-glutamate Ca^{2+} plateau (not illustrated). All these differences in DCE testify to a variable degree of $[Ca^{2+}]_i$ dependence on mitochondrial Ca^{2+} uptake following a GLU challenge.

In the experiments presented in Fig. 1, GLU was used at a high concentration (100 μM) which imposed a large Ca^{2+} load. To decrease this load we reduced the GLU concentration to 3 μM and shortened the pulse duration to 30 s. In Fig. 2A, this GLU pulse was applied at first before then in the period of ANT+OLIG application. In the latter case the amplitude of the GLU-induced $[Ca^{2+}]_i$ transient was increased by about 30% relative to its initial value. This increase of the

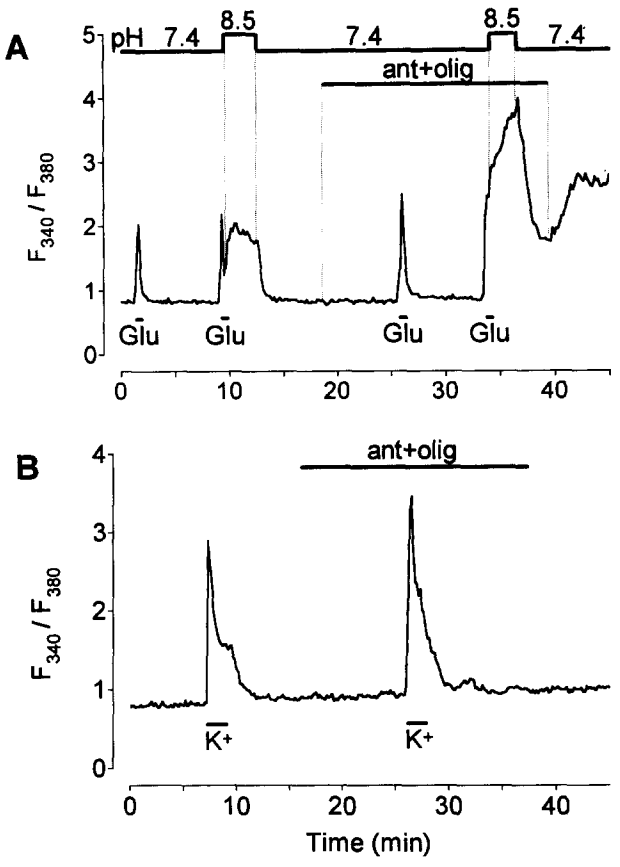

Fig. 2. Dependence of DCE on the magnitude (A) and origin (B) of the Ca^{2+} load. A: Small Ca^{2+} load induced by 3 μM GLU in the presence of ant+olig at pH_o 7.4 failed to induce DCE; pH_o elevation to 8.5 just following a 3 μM GLU pulse dramatically increased the GLU-induced Ca^{2+} load which triggered DCE. Note the effect of ant+olig on the high-pH_o-induced changes in $[Ca^{2+}]_i$ dynamics in the post-glutamate period. B: DCE did not develop following a 25 mM K^+ pulse in the presence of ant+olig in the solution.

Ca^{2+} load, however, did not suffice to trigger DCE. The latter appeared only after a dramatic increase in the magnitude and duration of the $[Ca^{2+}]_i$ response to the next GLU application caused by elevation of the external pH (pH_o) from 7.4 to 8.5 in the post-glutamate period. Earlier we have shown that such an effect of alkaline pH_o on $[Ca^{2+}]_i$ dynamics results mainly from inhibition of the plasmalemmal Ca^{2+}/H^+ pump [4]. Now we can see that blockade of the mitochondrial Ca^{2+} uptake by ANT+OLIG greatly enhances this effect of alkaline pH_o on $[Ca^{2+}]_i$ response (compare effects of pH_o elevation following 3 μM GLU pulses applied before and during the action of ANT+OLIG). Similar results were obtained in three other analogous experiments.

Fig. 2B compares the $[Ca^{2+}]_i$ responses elicited by a 25 mM K^+ pulse before and during the ANT and OLIG action. It can be seen that the ANT+OLIG-induced inhibition of mitochondrial Ca^{2+} uptake increased the amplitude of the $[Ca^{2+}]_i$ response but had little effect on its time course. The $[Ca^{2+}]_i$ transients in this case were never followed by DCE ($n = 12$).

Fig. 3A,B shows that removal of Na^+ from the ANT+OLIG solution (NMDG replacement of Na^+) neither prevented the initial fast $[Ca^{2+}]_i$ decrease following a GLU (100 μM) or 25 mM K^+ pulse nor precluded the DCE development following the GLU-induced $[Ca^{2+}]_i$ transient ($n = 7$). The latter observation indicates that DCE cannot be explained by Na^+/Ca^{2+} exchange reversal, since the removal of external Na^+ (i) decreases the resting $[Na^+]_i$ (to below of 1 mM [5,6]) and (ii) prevents the Na^+ influx and the $[Na^+]_i$ increase during a GLU pulse. Therefore after a return of the cell from a Na^+-free to a Na^+-containing solution the driving force for the Na^+/Ca^{2+} exchange becomes greatly increased; this explains the fast $[Ca^{2+}]_i$ recovery to its resting level despite the inhibition of the mitochondrial Ca^{2+} uptake by ANT and OLIG (see Fig. 3A,B). It is evident that in the experiments shown in Figs. 1 and 2, the plasmalemmal Na^+/Ca^{2+} exchange could not prevent the DCE development just because of the $[Na^+]_i$ increase during the GLU pulse [5,7].

Removal of Ca^{2+} from an ANT+OLIG-containing solution shortly after the termination of the GLU challenge stopped DCE and caused $[Ca^{2+}]_i$ to fall rapidly towards its resting level (Fig. 4A, $n = 5$). These results suggested that DCE might be due to an increase in Ca^{2+} permeability of the neuronal membrane. To reveal a possible involvement of endogenous EAA (aspartate, glutamate) in the mechanisms of DCE, we used the competitive NMDA receptor antagonist AP-5 (100 μM) and the blocker of open NMDA channels memantine (50 μM). Fig. 4B shows that application of 100 μM AP-5 3 min after the termination of the GLU challenge abruptly stopped the delayed $[Ca^{2+}]_i$ increase and induced its fast decrease.

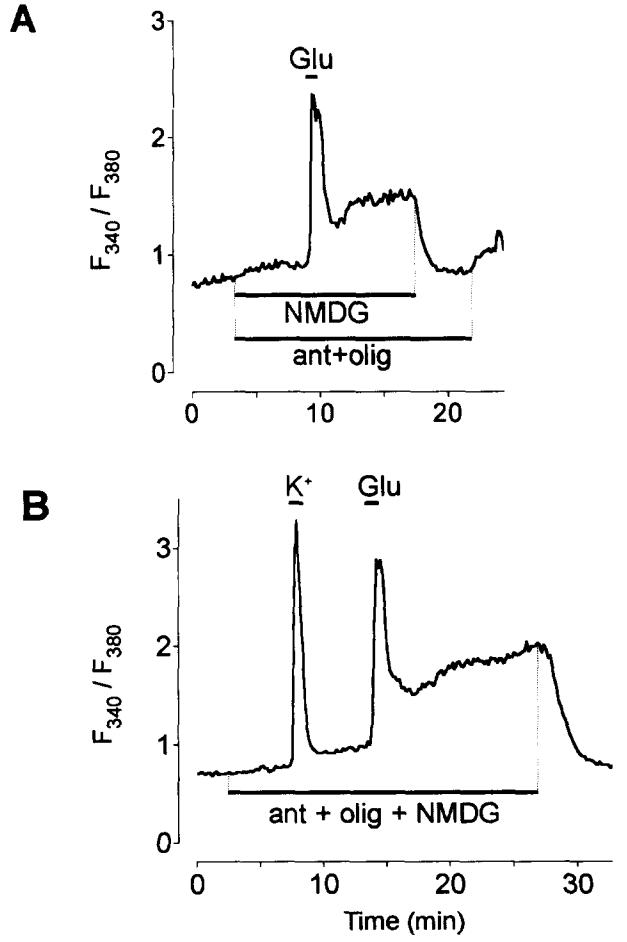

Fig. 3. Effect of external Na^+ removal from an ant+olig-containing solution on $[Ca^{2+}]_i$ recovery following 1-min GLU (A) or GLU and 25 mM K^+ pulses (B). Na^+ was replaced equimolarly by NMDG. Note a fast and complete $[Ca^{2+}]_i$ recovery after a return of the cell to a Na^+-containing medium.

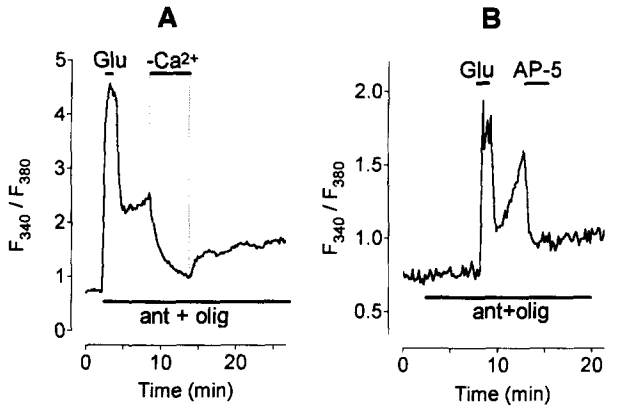

Fig. 4. Role of external Ca^{2+} and endogenous excitatory amino acid in the mechanism of DCE. A: Removal of Ca^{2+} from an ant+olig-containing solution abolished DCE. $-Ca^{2+}$ indicates the replacement of a Ca^{2+}-containing medium by a nominally Ca^{2+}-free one. B: Blockade of NMDA receptors by 100 μM AP-5 abolished DCE.

Similar blockade of DCE was induced by memantine (not illustrated). These results strongly indicate the involvement of NMDA receptors in the mechanism of DCE. Evidently, the latter results from a secondary activation via NMDA channels by endogenous excitatory amino acids (EAA) released in response to the GLU-induced Ca^{2+} influx [8–11]. This finding clarified many of our observations including a variable time course of DCE (see Figs. 1 and 3), its Ca^{2+} dependence (see Fig. 4A), and a partial DCE reversal caused by wash-out of the cell (see Fig. 1A).

Our recent studies showed that in cultured cerebellar granule cells the uptake of $^{45}Ca^{2+}$ was increased following a short-duration GLU pulse even in the absence of mitochondrial poisons in the medium [4] and that this increase can be effectively abolished by an addition of 100–200 μM AP-5 to the post-glutamate solution (Fayuk et al., in preparation). However, under normal conditions this enhanced post-glutamate Ca^{2+} influx is effectively buffered by mitochondria and therefore does not noticeably affect the time course of the $[Ca^{2+}]_i$ recovery. This occurs only in the presence of mitochondrial poisons which inhibit both the respiration and mitochondrial ATPase. DCE development is also promoted by inhibition of the Na^+/Ca^{2+} exchange-mediated Ca^{2+} extrusion resulting from intracellular Na^+ accumulation during a GLU pulse (see Fig. 3). It is tempting to suggest that DCE has a regenerative character: Ca^{2+} influx triggers release of EAA, which in turn enhance Ca^{2+} influx and thus $[Ca^{2+}]_i$ elevation. Whether the ANT+OLIG treatment increases the EAA release from the cell is not yet clear. It is only known that mitochondrial glutaminase is involved in GLU synthesis from cytosolic glutamine (see [12]). The fact that DCE develops only after the GLU- but not a high K^+-induced membrane depolarization allows us to speculate that GLU uptake by treated nerve cells may be involved in the mechanism of DCE. An alternative explanation may be that DCE requires specifically Ca^{2+} entry via GLU activated channels. The hypothesis on the 'source specificity' of Ca^{2+} loads was first put forward by Tymianski et al. [13] and later supported by White and Reynolds [1].

4. Conclusion

The present work has two major findings. (1) Mitochondrial Ca^{2+} uptake is not a prerequisite for the initial fast $[Ca^{2+}]_i$ recovery following a GLU pulse. This finding is at variance with White and Reynolds' [1] suggestion and supports our hypothesis [4] that the plasmalemmal Ca^{2+}/H^+ pump makes the major contribution to the initial rapid decrease in $[Ca^{2+}]_i$ after its elevation caused by the GLU pulse. (2) Mitochondria play a major role in protection against a delayed neuronal Ca^{2+} overload resulting from a secondary activation of NMDA receptors by EAA released in response to the GLU pulse.

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References

[1] White, R. and Reynolds, I. (1995) J. Neurosci. 15, 1318–1328.
[2] Budd, S. and Nichols (1996) J. Neurochem. 66, 403–411.
[3] Andreeva, N., Khodorov, B., Stelmashook, E., Cragoe, E. and Victorov, I. (1991) Brain Res. 548, 322–325.
[4] Khodorov, B., Pinelis, V., Vergun, O., Storozhevykh, T., Fayuk, D., Vinskaya, N., Arsenyeva, E., Khaspekov, L., Lyzin, A., Isaev, N., Andreeva, N. and Victorov, I. (1995) FEBS Lett. 371, 249–252.
[5] Kiedrowski, L., Brooker, G., Costa, E. and Wroblewski, J. (1994) Neuron 12, 1–20.
[6] Pinelis, V., Segal, M., Greenberger, V. and Khodorov, B. (1994) Biochem. Mol. Biol. Int. 32, 475–482.
[7] Schwiening, C., Kennedy, H. and Thomas, R. (1993) Proc. R. Soc. Lond. 253, 285–289.
[8] Schouboe, A., Frandsen, A. and Drejer, J. (1989) Neurochem. Res. 14, 871–875.
[9] Gringnon, S., Seager, M. and Couraud, F. (1993) Neurosci. Lett. 155, 87–91.
[10] Rothman, S., Thurston, J. and Hauhart, R. (1987) Neuroscience, 22, 471–480.
[11] Hartley, D. and Choi, D. (1989) J. Pharmacol. Exp. Ther., 250, 752–758.
[12] Nichols, D. and Attwell, D. (1990) Trends Pharmacol. Sci. 11, 462–468.
[13] Tymianski, M., Chariton, M., Carien, P. and Tator, Ch. (1993) J. Neurosci. 13, 2085–2104.