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Blockade of mitochondrial Ca^{2+} uptake by mitochondrial inhibitors amplifies the glutamate-induced calcium response in cultured cerebellar granule cells

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Abstract The objective of this study was to evaluate the role of mitochondrial Ca^{2+} uptake (MCU) in modulation (shaping) of the glutamate (Glu)-induced changes in neuronal cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$). In order to block MCU, nerve cells were treated with mitochondrial inhibitors (MI) inducing collapse of the mitochondrial potential ($\Delta\Psi_m$). Measurements of changes in $[Ca^{2+}]_i$ were performed using either the low-affinity (fura-2FF) or high-affinity (fura-2) Ca^{2+} indicators. Loading of nerve cells with rhodamine 123 made it possible to monitor changes in $\Delta\Psi_m$. In the first series of experiments it was shown that blockade of MCU in fura-2FF-loaded cells with a cocktail of rotenone (2 μM)+oligomycin (2.5 μg/ml) greatly (2.53 ± 0.4 times, $n = 61$) increased the $[Ca^{2+}]_i$ response to a 1-min Glu (100 μM) pulse. In fura-2-loaded cells, this increase was small (less than 1.3 times) or absent. In the second series of experiments, cocktails of rotenone+oligomycin or FCCP (1 μM)+oligomycin were applied during a prolonged Glu application. This produced strong mitochondrial depolarisation and an additional $[Ca^{2+}]_i$ increase. In most cells the latter could be reversed or prevented by a removal of external Ca^{2+}. The MI-induced additional $[Ca^{2+}]_i$ increase was especially pronounced in cells loaded with fura-2FF. In some neurones a removal of external Ca^{2+} did not produce a decrease in $[Ca^{2+}]_i$ during combined Glu+MI application, suggesting an impairment of $[Ca^{2+}]_i$ extrusion mechanisms of these cells. The conclusion is drawn that MCU makes a considerable contribution to regulation of $[Ca^{2+}]_i$ responses caused by Ca^{2+} influx via Glu-activated ionic channels. The reasons for a quantitative difference between $[Ca^{2+}]_i$ responses observed in fura-2- and fura-2FF-loaded neurones are discussed.

Key words: Glutamate; Intracellular Ca^{2+}; Mitochondrial inhibitor; Mitochondrial depolarisation; Cultured neuron

1. Introduction

Mitochondria are able to sequester and accumulate an essentially unlimited amount of cytoplasmic Ca^{2+} which forms a complex with phosphate in the mitochondrial matrix (for reviews see [1–3]). This Ca^{2+} uptake is known to be mediated by the mitochondrial uniporter and is driven by the high inside-negative membrane potential (V_{mit}) of energized mitochondria [2,3]. Since the discovery of mitochondrial Ca^{2+} uptake (MCU), many attempts have been made to estimate its contribution to neuronal $[Ca^{2+}]_i$ homeostasis under physiological and pathological conditions. To this aim, the effects of MCU inhibition on baseline $[Ca^{2+}]_i$ and stimulus-induced $[Ca^{2+}]_i$ responses were studied. In the absence of a selective membrane-permeant blocker of the uniporter the investigators usually utilised some mitochondrial inhibitors (MI) inducing a collapse of V_{mit}. Among those, there were the uncoupler FCCP and the respiratory chain blockers antimycin (Ant) or rotenone (Rot) in combination with the mitochondrial ATP-ase inhibitor oligomycin (Oli) [4–8]. The latter was used to prevent fast ATP depletion caused by reversed operation of the mitochondrial ATP synthase. Taking into account the extraordinarily high Ca^{2+} buffering capacity of mitochondria, one could expect that the blockade of MCU by MI will enhance the Glu-induced $[Ca^{2+}]_i$ response. However, experimental testing of this prediction by different research groups gave contradictory results. Thus, some authors failed to establish a marked effect of MCU blockade on the size of the Glu-induced $[Ca^{2+}]_i$ transient [4]; others found a prominent increase in $[Ca^{2+}]_i$ response in the presence of MI [7,8]; finally, it was reported [5,6] that Rot+Oli or Ant+Oli pretreatment of nerve cells may greatly suppress the Glu-induced $[Ca^{2+}]_i$ transient. In the present work, we tried to clarify the origin of some of these contradictions in experiments with cultured mammalian central neurones loaded either with the high-affinity Ca^{2+} indicator fura-2AM ($K_d = 0.26$ μM) or its low-affinity analogue fura-2FF ($K_d = 5$ μM). The latter was used because fura-2 had been reported to underestimate the maximal stimulus-induced $[Ca^{2+}]_i$ elevation [9,10]. In our experiments, two major experimental paradigms were used: (i) application of FCCP or Rot in combination with Oli before and during a 1-min Glu test pulse; and (ii) application of these MI during prolonged Glu exposure.

The results obtained clearly demonstrate that the blockade of MCU associated with mitochondrial depolarisation may dramatically enhance the neuronal $[Ca^{2+}]_i$ response to both short-term and long-term Glu challenges.

2. Materials and methods

Primary cerebellar granule cell cultures were prepared from the

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Abbreviations: $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; fura-2/AM, acetoxymethyl ester of fura-2; fura-2FF/AM, acetoxymethyl ester of fura-2FF; ARA-C, cytosine arabinoside; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Glu, glutamate; Mem, memantine; MEM, minimum essential medium; MI, mitochondrial inhibitors; FCCP, carbonyl cyanide *p*-(tri-fluoromethoxy) phenylhydrazone; Rot, rotenone; Oli, oligomycin; HBSS, HEPES-buffered salt solution; V_{mit}, mitochondrial potential; MCU, mitochondrial calcium uptake; MD, mitochondrial depolarisation

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cerebella of 7–8-day-old Wistar rats as described previously [11]. The cells were plated on poly-L-lysine-coated 22-mm glass coverslips in MEM containing: 10% fetal bovine serum, 10 mM HEPES, 12 mM $NaHCO_3$, 2 mM L-glutamine, 0.2 ED/ml insulin, 0.6% glucose, 25 mM KCl and 2.5 μM ARA-C (36.5°C, 5% CO_2) and were examined on day 7–9 in culture. $[Ca^{2+}]_i$ was measured in neurones loaded with fluorescent probes, the high-affinity indicator fura-2/AM or the low-affinity probe fura-2FF/AM, for 1 h in the same medium. For mitochondrial potential (V_{mit}) measurements, the cells were incubated for 15 min in a solution containing rhodamine (Rh) 123 (2 μM), washed three times with a control HEPES-buffered salt solution and placed into the experimental chamber. The chamber was mounted onto a Nikon inverted-stage microscope connected to a spectrofluorimetric system (SPEX, NJ, USA) equipped with a photon counting system or an imaging system including a CCD camera (IPC Videoscan Co. Ltd, Japan) and a PC-based computer. This permitted us to record the fluorescence emissions of the indicators (510 nm for fura-2 and fura-2FF and 535 nm for Rh 123) evoked by excitation through monochromators (340/380 nm for Ca^{2+}-sensitive dyes or 485 nm for Rh 123) in individual neurones [4].

The buffers for $[Ca^{2+}]_i$ calibration and the formula for actual $[Ca^{2+}]_i$ calculation were used as described in [10]. We used $K_d = 0.26$ μM for fura-2 and 5 μM for fura-2FF [12]. The HBSS contained (mM): 145 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 20 HEPES, 5 glucose, pH 7.4. In Na^+-free solution NaCl was replaced with 140 mM LiCl (pH was adjusted with LiOH). Fura-2FF (Teflabs USA) was kindly supplied by Donner Babcock. Fura-2AM was purchased from Molecular Probes. All the other chemicals were from Sigma or Fisher Chemical (USA). Results are expressed as the mean ± S.E.M.

3. Results

3.1. Effects of mitochondrial inhibitors on the Glu-induced $[Ca^{2+}]_i$ transient

In almost all the neurones loaded with fura-2 or fura-2FF, a 5–10-min application of Rot (2 μM)+Oli (2.5 mg/ml) did not affect the basal $[Ca^{2+}]_i$. This finding supported the notion [4,5,13] that in cultured cerebellar granule cells MCU either does not participate in the maintenance of low baseline $[Ca^{2+}]_i$ or can be effectively replaced by compensatory activation of other homeostatic mechanisms. Fig. 1 demonstrates the effects of MI on $[Ca^{2+}]_i$ transients induced by a 1-min Glu (100 μM Glu in Mg^{2+}-free, 10 μM glycine-containing solution) application. In nerve cells loaded with fura-2FF, the Rot+Oli application caused a pronounced (2.53 ± 0.4 times, $n = 61$) increase in the size of the Glu-induced $[Ca^{2+}]_i$ response (Fig. 1A,C). In contrast, in fura-2-loaded cells (Fig. 1B,D) the same MI treatment either did not change the size of the $[Ca^{2+}]_i$ transient or slightly increased it (by no more than 1.25 times, $n = 15$).

The results of our experiments with fura-2FF-loaded cells proved to be in good agreement with those obtained in hippocampal [7] and forebrain [8] neurones. In the latter study the low-affinity indicator Magfura-2 was used. It was found that mitochondrial depolarisation induced by FCCP, Rot+Oli or dinitrophenol caused a considerable increase in the Glu (100 μM)-induced Magfura-2 signal. However, Magfura-2 is not

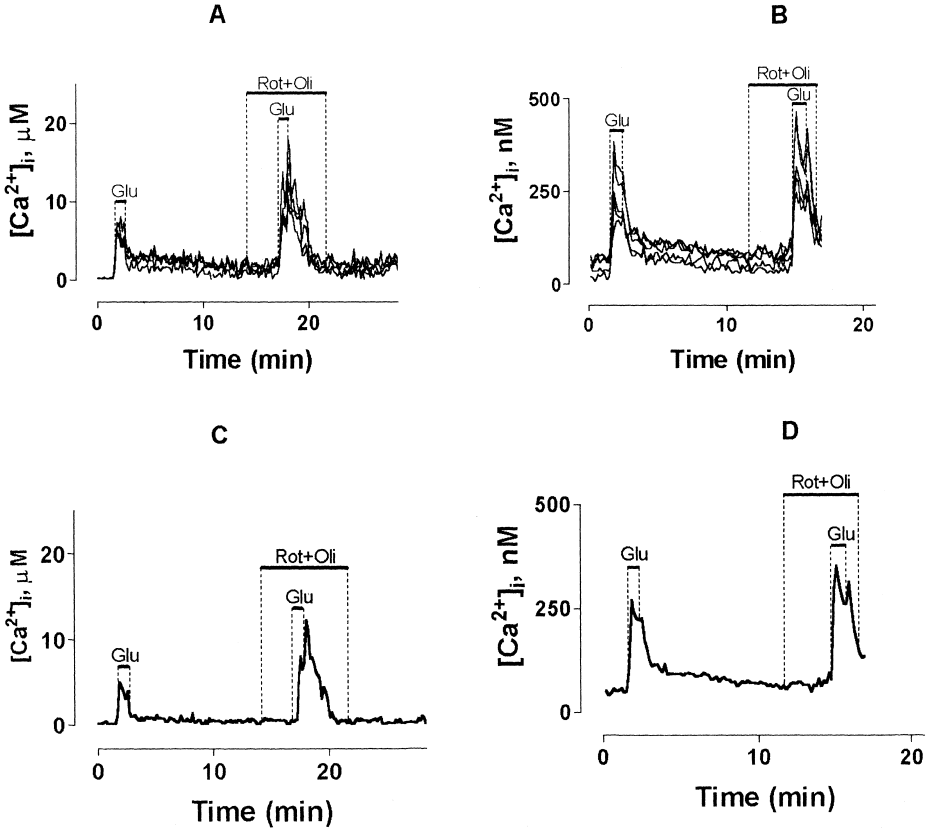

Fig. 1. Effect of mitochondrial depolarisation induced by a Rot+Oli cocktail on the size of the $[Ca^{2+}]_i$ transient induced by a Glu pulse in cerebellar granule cells loaded with fura-2FF (A and C) and fura-2 (B and D). A and B: Records from five and six individual neurones on the same coverslips are presented to illustrate the similarity of $[Ca^{2+}]_i$ responses in different cells. C and D: Mean data from all neurones shown in A and B, respectively. The neurones were stimulated for 1 min with 100 μM Glu in a Mg^{2+}-free, 10 μM glycine-containing solution. Rot+Oli: a cocktail of 2 μM rotenone and 2.5 μg/ml oligomycin.

ideally suited for measurements of $[Ca^{2+}]_i$ signals, since this indicator is also sensitive to Glu-induced changes in $[Mg^{2+}]_i$ [14]. In contrast, fura-2FF, used in the present study, is characterised by high Ca^{2+} selectivity and thus provided more reliable information about the changes in the Glu-evoked $[Ca^{2+}]_i$ transient caused by MCU inhibition.

3.2. *Effect of mitochondrial inhibitors on $[Ca^{2+}]_i$ during prolonged Glu exposure*

In young (6–8 days in culture) cerebellar granule cells loaded with Rh 123, prolonged (10–15 min) Glu application usually induced only relatively small mitochondrial depolarisation (MD) [15]. The latter, however, could be greatly enhanced by addition of MI (FCCP or Rot+Oli) to a Glu-containing solution after the beginning of Glu action (Fig. 2A,B). Removal of external Ca^{2+} did not affect MI-induced MD. Note also that MD remained at a stable level after the termination of a prolonged Glu challenge.

As shown above (Fig. 1), the application of Rot+Oli to the resting cell usually did not induce any noticeable changes in $[Ca^{2+}]_i$. In contrast, application of these MI to the cell during Glu exposure produced an additional increase in $[Ca^{2+}]_i$ in most cells. This effect was observed in neurones loaded both with fura-2FF (Fig. 3A,C) and with fura-2 (Fig. 3B,D). It is noteworthy that in all fura-2FF-loaded cells ($n = 19$ cells) the magnitude of the additional $[Ca^{2+}]_i$ increase evoked by MI exceeded the magnitude of the initial $[Ca^{2+}]_i$ elevation, elicited by Glu. Qualitatively similar results were obtained in analogous experiments with FCCP (0.25 μM) or FCCP (0.25 μM)+Oli (2.5 μg/ml). In contrast, addition of Oli alone to the Glu-containing solution failed to affect the $[Ca^{2+}]_i$ level (not illustrated).

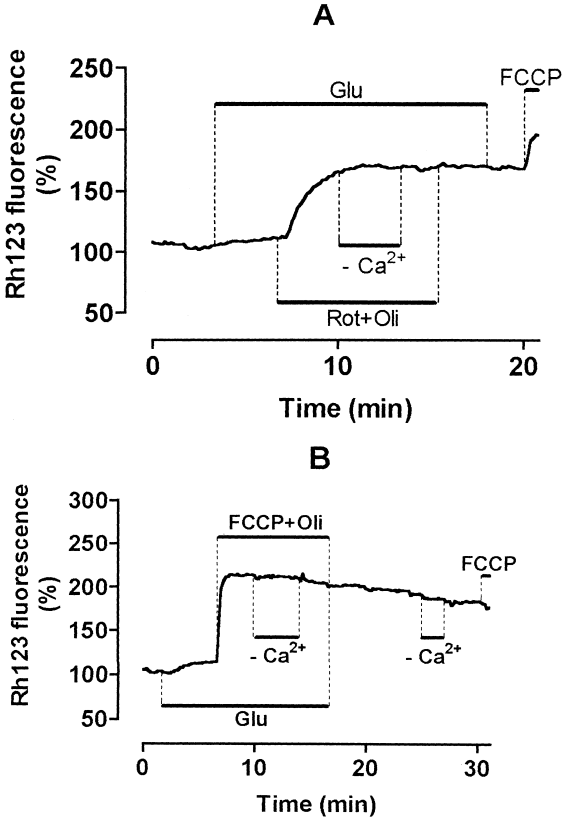

Fig. 2. Changes in the mitochondrial potential in Rh 123-loaded cells induced by Glu (100 μM) and subsequent addition of Rot+Oli (A) or FCCP+Oli (B) to Glu-containing solution. ($-Ca^{2+}$) is a temporary replacement of an external Ca^{2+}-containing solution by a nominally Ca^{2+}-free EGTA (50 μM)-containing medium. The initial Rh 123 fluorescence was taken as 100%. An increase in the Rh 123 fluorescence indicates depolarisation of the inner mitochondrial membrane. The concentrations of Rot and Oli are the same as in Fig. 1.

A secondary increase in $[Ca^{2+}]_i$ caused by addition of FCCP [7,13,16] or CN [15] to the Glu-containing solution was observed earlier in experiments with cerebellar granule cells [13,15,16] and hippocampal neurones [7]. In the latter work the results of direct measurements of ATP in the cultures during a short-term (5 min) Glu and FCCP coapplication led the authors to conclude that brief application of FCCP can be used as a tool to study MCU without significantly altering the cellular ATP level. This conclusion agrees with our finding that blockade of mitochondrial ATPase by Oli during Glu exposure does not affect the $[Ca^{2+}]_i$ level apparently because of the fact that in cultured neurones glycolysis makes a considerable contribution to ATP synthesis [7].

In the previous studies, the contribution of the Ca^{2+} influx from the external medium and the Ca^{2+} efflux from depolarised mitochondria to the MI-induced $[Ca^{2+}]_i$ increase was not explored. To clarify this problem in the present work we examined the changes in $[Ca^{2+}]_i$ induced by a removal of Ca^{2+} from the external Glu+MI-containing solution. In most experiments ($n = 54/61$) the replacement of external Ca^{2+} by 50–100 μM EGTA produced a clear-cut decrease in $[Ca^{2+}]_i$ as shown in Fig. 3. This finding indicates that combined Glu+MI application did not abolish the ability of cells to extrude Ca^{2+} into the external medium. A subsequent return to the Ca^{2+}-containing medium renewed the $[Ca^{2+}]_i$ increase (Fig. 3A–D). This secondary $[Ca^{2+}]_i$ elevation clearly demonstrates that the MI-induced $[Ca^{2+}]_i$ elevation during prolonged Glu exposure was mainly due to the acute blockade of the high-capacity mitochondrial Ca^{2+} sink in the face of a continuous Ca^{2+} influx via Glu-activated channels of the neuronal membrane. In order to estimate the contribution of Ca^{2+} release from mitochondria during MI-induced depolarisation, experiments were performed according to the protocol shown in Fig. 4A. It can be seen that application of FCCP+Oli in a Ca^{2+}-free Glu-containing medium produced only a relatively small additional $[Ca^{2+}]_i$ elevation which suddenly greatly increased upon readdition of Ca^{2+} to the external solution. Following the Glu challenge $[Ca^{2+}]_i$ remained at a plateau level in spite of the presence of the NMDA channel blocker memantine (Mem) in the washout solution. This indicates that a sustained increase in $[Ca^{2+}]_i$ in the post-glutamate period cannot be explained by the release of endogenous Glu [4,18]. Qualitatively similar results were obtained in analogous experiments with Rot+Oli application. The magnitude of the MI-evoked $[Ca^{2+}]_i$ increase in Ca^{2+}-containing Glu solution greatly exceeded that produced by MI in Ca^{2+}-free solution. These data strongly support the above conclusion that the MI-induced increase in $[Ca^{2+}]_i$ during Glu exposure is mainly determined by the Ca^{2+} influx from external medium.

In some experiments analogous to those presented in Fig. 3, we replaced Na^+ by Li^+ in the external solutions. Such a replacement is known to inhibit Ca^{2+} extrusion via the plasma membrane Na^+/Ca^{2+} exchange [17]. Nevertheless, a removal of Ca^{2+} from the Glu+MI-containing solution produced a relatively fast decrease in $[Ca^{2+}]_i$ (Fig. 4B). Evidently, in the

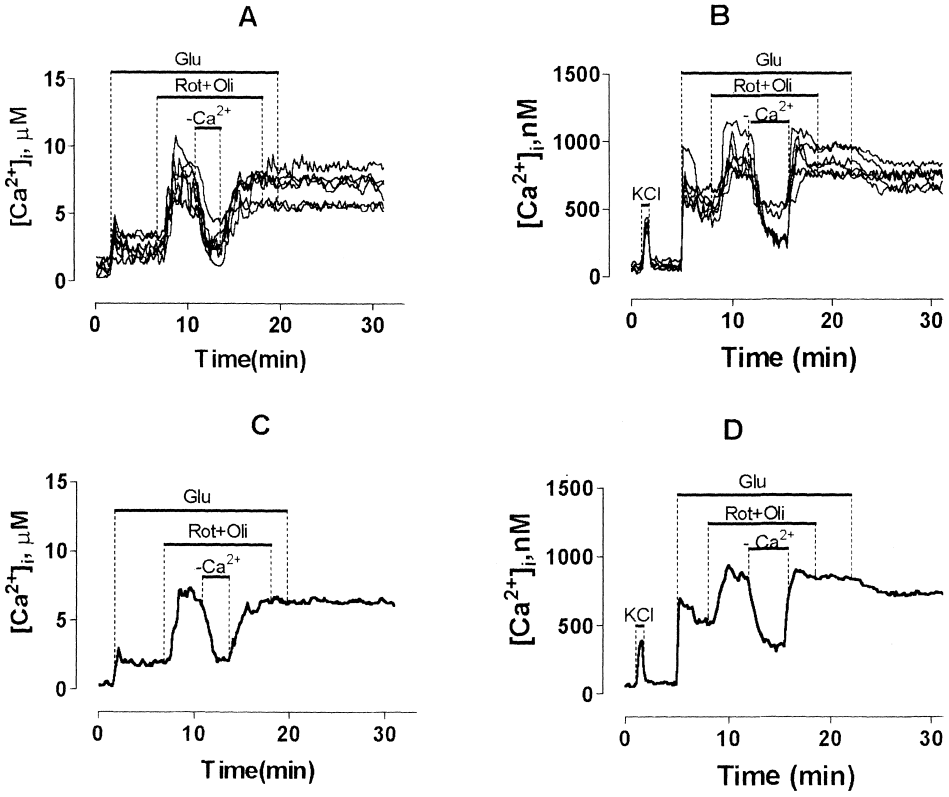

Fig. 3. Rot+Oli elicits an additional $[Ca^{2+}]_i$ increase during prolonged Glu (100 μM) treatment of cerebellar granule cells loaded with fura-2FF (A and C) or fura-2 (B and D). $-Ca^{2+}$ indicates the replacement of a Ca^{2+}-containing external solution by a Ca^{2+}-free one (with 50 μM EGTA). A and B: Records from seven and eight individual neurones on the same coverslips. C and D: Mean data from all neurones shown in A and B, respectively.

absence of external Na^+, the Ca^{2+}/H^+ pump of the neuronal membrane alone was able to clear up the Ca^{2+} load caused by combined Glu+MI application.

As indicated above, a reversible decrease in $[Ca^{2+}]_i$ in response to $[Ca^{2+}]_i$ removal during Glu+MI coapplication was not observed in all neurones. In some experiments ($n = 7/61$) Ca^{2+} removal failed to decrease $[Ca^{2+}]_i$ which remained at a high plateau level even after the subsequent termination of the Glu challenge (Fig. 4C). Evidently in these cells prolonged Glu+MI treatment induced a stable profound inactivation of both Ca^{2+} influx and Ca^{2+} efflux systems of the neuronal membrane.

In several experiments of this series, we also used hippocampal neurones loaded with fura-2FF. Earlier [18] it has been shown that in mature hippocampal neurones prolonged (10–15 min) Glu application produces a profound submaximal MD associated with a high $[Ca^{2+}]_i$ plateau in the post-glutamate period. In this study, we found that in such neurones FCCP or Rot+Oli application failed to induce an additional $[Ca^{2+}]_i$ increase. Removal of Ca^{2+} from the external solution during Glu or Glu+FCCP application caused only a small, if any, decrease in $[Ca^{2+}]_i$ (not illustrated) thus indicating the deterioration of the Ca^{2+} extrusion systems of the neuronal membrane.

4. Discussion

As mentioned in Section 1, there is no consensus in the literature about the effect of MI-induced mitochondrial depolarisation on the $[Ca^{2+}]_i$ transient induced by a Glu pulse. The data obtained in the present work show that potentiation of the Glu-induced $[Ca^{2+}]_i$ response by MI pretreatment can best be revealed by using the low-affinity $[Ca^{2+}]_i$ indicator fura-2FF (Figs. 1 and 3). What is the reason for such a dependence of the effect of MI on Ca^{2+} affinity for the indicator used? The most probable explanation is that fura-2 underestimates the average increase in $[Ca^{2+}]_i$ due to saturation of the indicator in those microdomains ('hot spots') in which local cytoplasmic $[Ca^{2+}]_i$ concentrations can rise to 30–100 μM [8–10]. Fura-2FF does not undergo an appreciable saturation in these high-Ca^{2+} domains and may therefore allow one to estimate the average neuronal $[Ca^{2+}]_i$ more accurately than does fura-2. The most probable location of these hot spots is thought to be the inner openings of NMDA and voltage-operated Ca^{2+} channels [9,10], mitochondrial intermembrane space [19] as well as distinct areas between the mitochondria and the neuronal membrane.

We have to emphasize, however, that neither we nor other authors observed the inhibitory action of Rot+Oli or Ant+Oli on the Glu-induced $[Ca^{2+}]_i$ transient reported by Nicholls' group [5,6]. Originally, Budd and Nicholls [5] tried to explain this observation by assuming a blockade of NMDA channels by high Ca^{2+} accumulated in submembrane microdomains due to inhibition of MCU. However, in subsequent studies [6] the authors failed to reveal any appreciable decrease in the Glu-induced $[Ca^{2+}]_i$ influx under the action of MI on cerebellar granule cells. Therefore the authors advanced a very disputable hypothesis that a decrease in the Glu-induced $[Ca^{2+}]_i$ transient in the presence of MI results from the enhancement of Ca^{2+} extrusion from the cell. The experiments

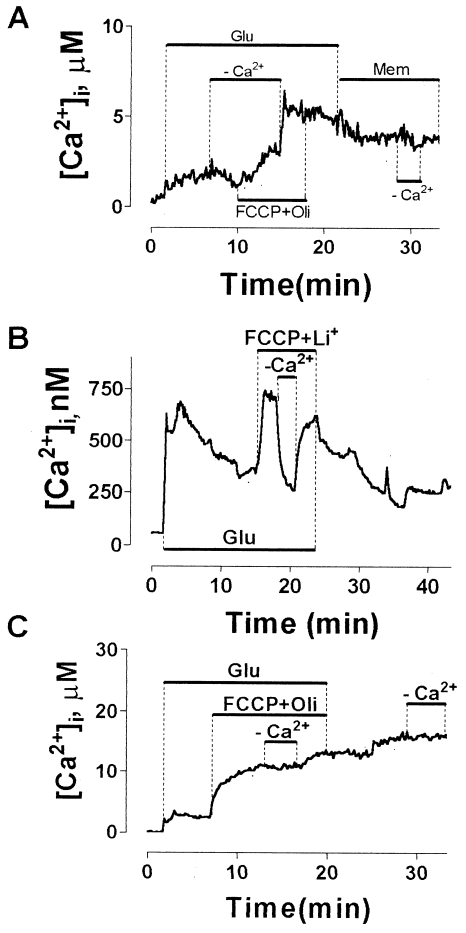

Fig. 4. Effect of FCCP+Oli application on $[Ca^{2+}]_i$ during prolonged Glu exposure of nerve cells in the presence or absence of Ca^{2+} in the external solution. A: A fura-2FF-loaded neurone. A small increase in $[Ca^{2+}]_i$ caused by FCCP+Oli addition to a Ca^{2+}-free (50 μM EGTA) solution was suddenly increased after readdition of Ca^{2+} to the medium. To prevent the secondary activation of NMDA channels by endogenous Glu the blocker of these channels, memantine (Mem) was added to the washout solution after termination of the Glu challenge. B: A fura-2-loaded cell. Inhibition of the plasma membrane Na^+/Ca^{2+} exchange caused by Na^+/Li^+ replacement in a Ca^{2+}-free solution did not prevent a fast $[Ca^{2+}]_i$ decrease during combined Glu and Rot+Oli exposure. C: A fura-2FF-loaded cell. An example of the neurones which lost their ability to decrease high $[Ca^{2+}]_i$ in response to Ca^{2+} removal ($-Ca^{2+}$) during combined FCCP+Oli and Glu application, as well as after termination of the Glu challenge.

with fura-2FF-loaded cells described in this paper (Fig. 1) strongly support the opposite notion [7,8] that inhibition of the MCU greatly amplifies the changes in $[Ca^{2+}]_i$ induced by the Ca^{2+} influx via Glu-activated channels.

The results of the present work may be of interest for understanding the relationships between the Glu-induced changes in V_{mit} and $[Ca^{2+}]_i$ recently established by Vergun et al. [18] in fura-2FF-loaded hippocampal neurones. It has been found that in mature neurones an emergence of the second phase of MD during Glu application is accompanied by a secondary $[Ca^{2+}]_i$ increase. In turn, mitochondrial repolarisation in the post-glutamate period is associated with $[Ca^{2+}]_i$ recovery. It is tempting to suggest that the above synchronous changes in V_{mit} and $[Ca^{2+}]_i$ in fura-2FF-treated neurones are mainly determined by a dependence of local $[Ca^{2+}]_i$ on MCU in some perimitochondrial (submembrane) microdomains.

In conclusion, the data presented in this work provide new evidence in favour of the notion that the uniporter-mediated mitochondrial $[Ca^{2+}]_i$ uptake makes a considerable contribution to modulation (shaping) of the Glu-induced $[Ca^{2+}]_i$ response. A blockade of this uptake by mitochondrial inhibitors greatly increases the $[Ca^{2+}]_i$ elevation induced by Glu.

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