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Arachidonic Acid Enhances Intracellular $[Ca^{2+}]_i$ Increase and Mitochondrial Depolarization Induced by Glutamate in Cerebellar Granule Cells

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Abstract—Maturation of primary neuronal cultures is accompanied by an increase in the proportion of cells that exhibit biphasic increase in free cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) followed by synchronic decrease in electrical potential difference across the inner mitochondrial membrane ($\Delta\Psi_m$) in response to stimulation of glutamate receptors. In the present study we have examined whether the appearance of the second phase of $[Ca^{2+}]_i$ change can be attributed to arachidonic acid (AA) release in response to the effect of glutamate (Glu) on neurons. Using primary culture of rat cerebellar granule cells we have investigated the effect of AA (1-20 μM) on $[Ca^{2+}]_i$, $\Delta\Psi_m$, and [ATP] and changes in these parameters induced by neurotoxic concentrations of Glu (100 μM, 10-40 min). At ≤10 μM, AA caused insignificant decrease in $\Delta\Psi_m$ without any influence on $[Ca^{2+}]_i$. The mitochondrial ATPase inhibitor oligomycin enhanced AA-induced decrease in $\Delta\Psi_m$; this suggests that AA may inhibit mitochondrial respiration. Addition of AA during the treatment with Glu resulted in more pronounced augmentation of $[Ca^{2+}]_i$ and the decrease in $\Delta\Psi_m$ than the changes in these parameters observed during independent action of AA; removal of Glu did not abolish these changes. An inhibitor of the cyclooxygenase and lipoxygenase pathways of AA metabolism, 5,8,11,14-eicosatetraynoic acid, increased the proportion of neurons characterized by Glu-induced biphasic increase in $[Ca^{2+}]_i$ and the decrease in $\Delta\Psi_m$. Palmitic acid (30 μM) did not increase the percentage of neurons exhibiting biphasic response to Glu. Co-administration of AA and Glu caused 2-3 times more pronounced decrease in ATP concentrations than that observed during the independent effect of AA and Glu. The data suggest that AA may influence the functional state of mitochondria, and these changes may promote biphasic $[Ca^{2+}]_i$ and $\Delta\Psi_m$ responses of neurons to the neurotoxic effect of Glu.

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Key words: neurons, glutamate, calcium, mitochondrial potential, arachidonic acid

Maturation of neurons in culture is accompanied by significant changes in their responses to stimulation of glutamate receptors [1, 2]. In a culture of young neurons (6-9 DIV) glutamate (Glu) application (usually 100 μM Glu for 15-20 min) causes transient increase in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) and weak mitochondrial depolarization (MD) (see for review [2, 3]) in most neurons. After Glu removal $[Ca^{2+}]_i$ and mitochondrial potential ($\Delta\Psi_m$) return to the basal level. In most mature neurons in culture, Glu causes biphasic responses and higher $[Ca^{2+}]_i$ values and MD are preserved after Glu removal [1, 4]. Study of neuron populations [5] and individual neurons [6] revealed a correlation between cell inability to restore low $[Ca^{2+}]_i$ in the post-glutamate period and their subsequent death. Studies of the relationship between the secondary increase in $[Ca^{2+}]_i$ and strong MD of mature neurons and their energy status revealed that Glu-induced biphasic effects of $[Ca^{2+}]_i$ and MD typical for

Abbreviations: AA) arachidonic acid; $[Ca^{2+}]_i$) intracellular concentration of free Ca^{2+}; CGC) primary culture of rat cerebellar granule cells; DIV) days *in vitro*; DNP) dinitrophenol; $\Delta\Psi_m$) electrical potential difference across the inner mitochondrial membrane; ETYA) 5,8,11,14-eicosatetraynoic acid; FCCP) carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MD) mitochondrial depolarization; Oligo) oligomycin; PA) palmitic acid.

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mature cells could be induced in young neurons by removal of glucose from the medium [7, 8].

It was demonstrated previously that decrease in glucose concentration and prolongation of incubation in glucose free medium caused Glu-induced Ca^{2+}-dependent arachidonic acid (AA) release in neuron culture or brain slices [9-12]. In neurons, AA is formed during Glu-induced activation of various phospholipase A_2 (PLA2) subtypes [13]. Rat cerebellar granule cells express Ca^{2+}-dependent PLA2-β [14]. In ischemic brain, marked amounts of palmitic (PA) and stearic acids have been detected in addition to AA [15]. Palmitic and stearic acids can form a Ca^{2+}-permeable pore in phospholipid membranes [16, 17] and cyclosporin A-insensitive pore in the inner mitochondrial membrane [18, 19], and AA accelerates this pore formation [20].

Free fatty acids, especially in combination with Ca^{2+} influx into cells, can significantly influence mitochondrial functioning. They increase ion conductance of mitochondrial membranes [21-24], block respiratory chain Complex I [25, 26], and induce formation of the permeability transition pore [27].

In the present study we have investigated whether exogenous AA and PA can increase the proportion of neurons responding to Glu with biphasic increase in $[Ca^{2+}]_i$ and decrease in $\Delta\Psi_m$.

MATERIALS AND METHODS

Procedures for preparation of primary cultures of rat cerebellar granule cells have been described previously [28]. Buffers containing Glu, AA, PA, and also inhibitors of respiratory chain and H^+-ATPase were prepared just before use. Intracellular concentrations of free Ca^{2+} ($[Ca^{2+}]_i$) were measured using low affinity fluorescent Ca^{2+} indicator fura-2FF (TefLabs, USA). It allows changes of micromolar $[Ca^{2+}]_i$ to be detected. The indicator was administered as the acetoxymethyl ether (fura-2FF/AM; 6 μM, 50 min, 23-25°C), mixed with the non-ionic detergent Pluronic F-127 (0.02%; Molecular Probes, USA). Changes of $[Ca^{2+}]_i$ were expressed as ratio of fura-2FF fluorescence intensity obtained under excitation at 340 and 380 nm (F340/F380).

Changes of $\Delta\Psi_m$ were evaluated using the potential-sensitive fluorescent probe rhodamine 123 (rh123, 3-4 μg/ml, 15-17 min), which stained the cells after loading with the Ca^{2+} indicator. The measurements were carried out at 25-28°C in buffer containing 145 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM D-glucose, 20 mM Hepes; pH value of 7.4 was adjusted with 1 M NaOH. For Glu washing, we used calcium-free solution containing 2 mM $MgCl_2$ and 0.1 mM EGTA. For demonstration of neuronal mitochondrial Ca^{2+} accumulation during Glu action and also for detection of rh123 signal magnitude corresponding to maximal MD, the protonophore FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) (1 μM), was added at the end of experiment. The maximal fura-2FF signal was calibrated by adding ionomycin (1 μM) followed by increase in Ca^{2+} concentration in the buffer up to 10 mM. Solutions containing AA and PA were prepared 1-2 min before use: stock solution of these acids in ethanol were added as rapid injections from micropipettes or microsyringes into a glass vial with buffer under constant stirring with a magnetic stirrer. AA (10 mM ethanol solution) was kindly supplied by V. V. Bezuglov. Other chemicals were purchased from Sigma (USA).

The system for signal registration from individual cells included a xenon lamp of 175 W (Sutter Instrument, USA), the inverted fluorescent microscope Axiovert-200 (Zeiss, Germany) with 20x/0.75 quartz lens, 12-byte CCD camera SnapCool-fx (Roper Scientific, USA). The light from the lamp passed through interference filters of 340 and 380 nm for fura-2FF and 486 nm for rh123, respectively; these filters were placed into a filter wheel, which moved them each 50-100 msec. For all filters the bandpass half-width was ~10 nm. The exciting light of these wavelengths passed via a liquid light guide into the input port to the microscope and then to a dichroic mirror with threshold wavelength of 485-505 nm. Fluorescence of fura-2FF and rh123 passed through interference filters of 505-535 and 515-565 nm, respectively. The emission light filters were placed into a second filter wheel positioned just before the CCD-camera; they were synchronously moved together with excitation light filters by the filter wheel drive mechanism LB 10-2S (Sutter Instrument). Camera images were treated in the real time mode using MetaFluor and MetaFluor Analyst programs (Universal Imaging Corporation, USA).

Intracellular ATP levels were assayed by luciferin–luciferase chemiluminescence following the supplier's protocol (Sigma), using the Anthos Lucy-1 microplate luminometer (Anthos Labtech Instruments, Austria) in 96-well plates. Each experimental point represents a mean of three plates. Measurements were carried out using 7 DIV cultures grown on different days.

RESULTS

Effects of arachidonic and palmitic acids on Glu-induced changes of Ca^{2+} and $\Delta\Psi_m$. Changes in $[Ca^{2+}]_i$ and $\Delta\Psi_m$ observed during the treatment of primary culture of rat cerebellar granule cells (CGC) with 100 μM Glu (15-25 min) can be subdivided into two types (Fig. 1, a and d). The major population of neurons responds to Glu application with rapid increase in $[Ca^{2+}]_i$ followed by the decrease to some steady-state level exceeding the resting cell $[Ca^{2+}]_i$ (Fig. 1a). Removal of Glu by washing in a calcium free buffer restored the initial level of $[Ca^{2+}]_i$ and $\Delta\Psi_m$. However, in a small proportion of neurons the pri-

mary transitory changes of $[Ca^{2+}]_i$ and $\Delta\Psi_m$ were then followed by a second phase of increase in $[Ca^{2+}]_i$ accompanied by synchronous strong MD. The magnitude of the secondary $[Ca^{2+}]_i$ increase varied depending on the Ca^{2+} indicator employed and the type of neuronal culture from 10 to 50 μM [29-31].

In young cultures (6-9 DIV) the proportion of neurons exhibiting biphasic increase in $[Ca^{2+}]_i$ and MD usually does not exceed 10-15% (n = 584). In 66% of neurons (47 of 71 cells, five cultures) demonstrating the biphasic type of response to Glu, high values of $[Ca^{2+}]_i$ and MD were observed during 15-25 min after Glu removal (post-glutamate plateau). The remaining 34% of neurons restored (completely or partially) $[Ca^{2+}]_i$ during 15-25 min of the post-glutamate period. Addition of FCCP (1 μM, 3-5 min) after Glu removal by washing with calcium-free buffer caused total mitochondrial depolarization (Fig. 1d). The neurons exhibiting the second phase of $[Ca^{2+}]_i$ increase followed by normalization of low $[Ca^{2+}]_i$ in the post-glutamate period were characterized by strong increase in $[Ca^{2+}]_i$ immediately after FCCP addition. Delay or lack of $[Ca^{2+}]_i$ increase was found only in those neurons which did not exhibit the second Glu-induced phases of $[Ca^{2+}]_i$ and $\Delta\Psi_m$.

Addition of AA (final concentration of 20 μM) to CGC caused the increase in $[Ca^{2+}]_i$ and MD which terminated by $\Delta\Psi_m$ collapse and $[Ca^{2+}]_i$ plateau after 20-25 min (not shown). So in subsequent experiments we employed AA concentrations (≤10 μM) which did not induce changes in $[Ca^{2+}]_i$ in the absence of Glu (Fig. 1, b and c). In 87% of neurons (75 of 86 cells, two cell cultures) subsequent addition of 100 μM Glu in the presence of AA caused rapid increase in $[Ca^{2+}]_i$ and strong MD (Fig. 1, b and e). In most neurons there was no clearly detectable intermediate decrease in $[Ca^{2+}]_i$, which was usually observed between the end of the first phase and the beginning of the second one (Fig. 1, a and d). Washing of cells subjected to combined treatment with Glu and 10 μM AA for 15-20 min did not restore $[Ca^{2+}]_i$ and $\Delta\Psi_m$ to the level observed in the resting neurons. Such loss of $[Ca^{2+}]_i$ and $\Delta\Psi_m$ restoring capacity in the post-glutamate period is typical for "old" neurons (⩾14 DIV) [2].

For evaluation whether AA influences $[Ca^{2+}]_i$ and $\Delta\Psi_m$ in Glu-pretreated cells characterized by already increased $[Ca^{2+}]_i$, we added AA 10 min after the onset of the Glu effect. In such mode of experiment AA application caused sharp increase in $[Ca^{2+}]_i$ and strong MD (Fig. 1, c and f). Subsequent exposure of cells to Glu and AA

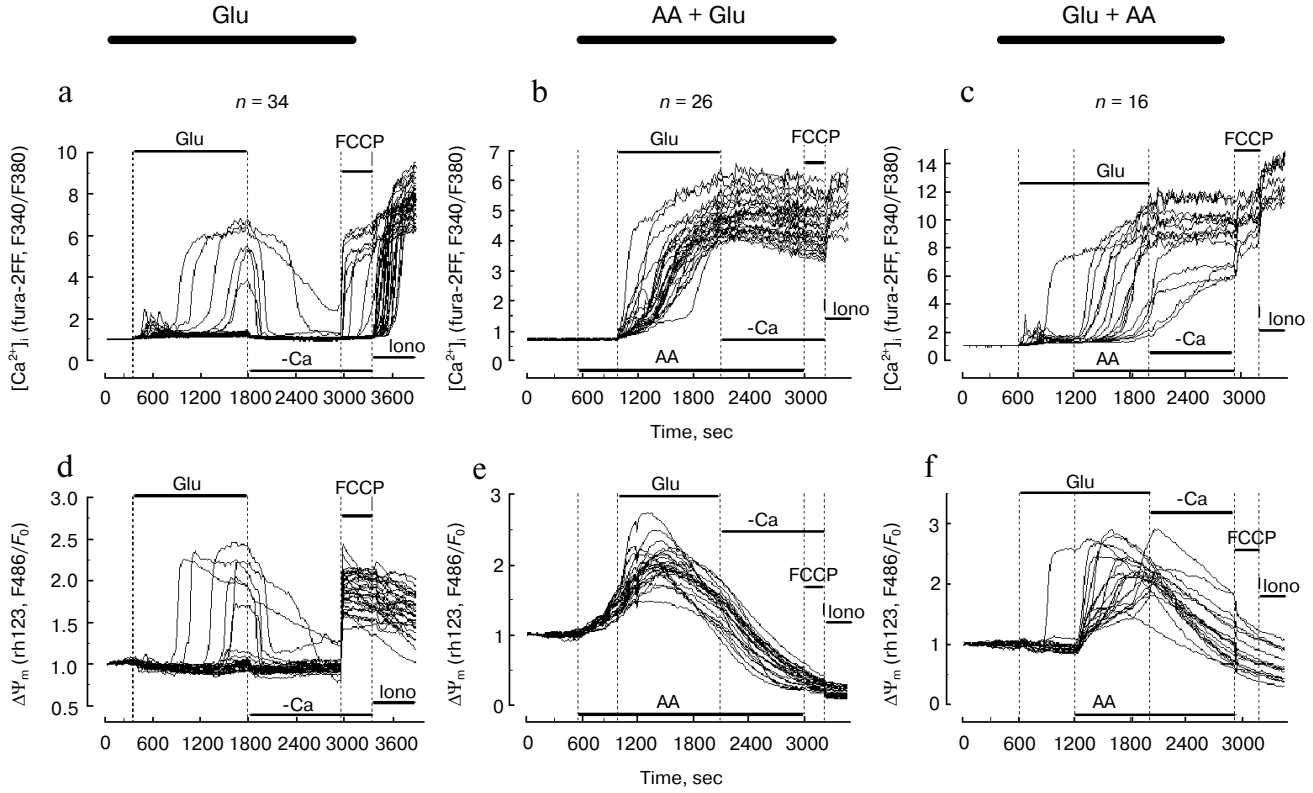

Fig. 1. Effect of arachidonic acid (AA) on changes in $[Ca^{2+}]_i$ (a-c) and $\Delta\Psi_m$ (d-f) induced by glutamate (100 μM; $-Mg^{2+}$ + 10 μM glycine) in primary culture of rat cerebellar granule cells. Changes in $[Ca^{2+}]_i$ are given as the ratio of fluorescence intensity of fura-2FF during excitation at 340 and 380 nm (F340/F380); $\Delta\Psi_m$ changes are given as the ratio of rh123 fluorescence intensity during excitation at 486 nm to the fluorescence value in the beginning of experiment (F486/Fo). AA concentration was 10 μM.

for 10 min resulted in appearance of $[Ca^{2+}]_i$ plateau in almost all neurons. Removal of Glu by cell washing did not abolish this $[Ca^{2+}]_i$ plateau. Moreover, the neurons retaining low $[Ca^{2+}]_i$ level during the period of concerted action of Glu and AA were characterized by $[Ca^{2+}]_i$ increase in the post-glutamate period.

To evaluate the specificity of the effect of AA in modulation of Glu-induced $[Ca^{2+}]_i$ and $\Delta\Psi_m$ neuronal responses, we have used palmitic acid (PA). Selection of PA as the reference compound for AA is explained by the fact that both acids predominate in the spectrum of fatty acids formed during Glu-induced toxicity of neurons in the central nervous system [15]. PA can form a Ca^{2+} permeable pore in phospholipid membranes and in mitochondrial membranes as well [16-20]. However, although PA concentration was three times higher than that of AA, treatment of neurons with PA using the protocol employed for AA caused insignificant changes of $[Ca^{2+}]_i$ and $\Delta\Psi_m$ in response to Glu compared with corresponding control (Glu without PA). Lack of PA effect on $\Delta\Psi_m$ and $[Ca^{2+}]_i$ may be attributed to poor insertion of this acid into cell membranes and poor PA penetration into cells. For example, PA addition to phospholipid vesicles in Ca^{2+} containing buffers resulted in Ca^{2+} binding to PA; this prevented PA insertion into the membranes [19]. So, we have examined the PA effect on neurons in Ca^{2+}-free buffer. Incubation of CGC with PA (30 μM for 10 min) in the Ca^{2+}-free buffer did not influence either $[Ca^{2+}]_i$ or $\Delta\Psi_m$ (data not shown). However, it should be taken into consideration that mitochondrial pore formation by PA requires the presence of Ca^{2+} in the mitochondrial matrix [18, 19]. So we also repeated PA addition to CGC after the treatment with Glu (when mitochondria contain Ca^{2+} [2]). PA was added during Glu removal by washing in the Ca^{2+}-free buffer; however, in these experiments PA also did not cause MD and release of Ca^{2+} from mitochondria into the cytosol. Subsequent addition of FCCP (1 μM) to neurons in the Ca^{2+}-free buffer was accompanied by sharp MD in all neurons and rapid increase in $[Ca^{2+}]_i$ in most of them. This suggests that PA does not influence $[Ca^{2+}]_i$ and $\Delta\Psi_m$ even when mitochondrial matrix is loaded with Ca^{2+}.

In cells AA can undergo oxidative metabolism [32], so we have investigated the effect of lipoxy-/cyclooxygenase inhibition of the changes in $[Ca^{2+}]_i$ and $\Delta\Psi_m$ induced by Glu in the presence of AA. In these experiments, we used relatively low concentration of AA (3 μM), which caused insignificant changes in cell responses to the treatment with Glu compared to corresponding control (data not shown). 5,8,11,14-Eicosatetraynoic acid (ETYA) (30 μM), the inhibitor of lipoxy-/cyclooxygenase metabolism of AA, did not influence neuronal changes in $[Ca^{2+}]_i$ and $\Delta\Psi_m$ induced by the treatment with Glu. Combined application of AA and ETYA significantly increased the proportion of neurons exhibiting the second phase of $[Ca^{2+}]_i$ increase and strong MD. This suggests that AA rather than putative products of its metabolism (at least in the lipoxy-/cyclooxygenase oxidation pathway) modulate neuronal responses to the treatment with Glu.

Comparison of the effect of AA and mitochondrial poisons on $\Delta\Psi_m$. Since combined effects of Glu and AA (irrespectively to the order of their addition) share similarity with the combined effect of Glu with protonophore or mitochondrial respiration inhibitor [28], we compared the effect of AA on $\Delta\Psi_m$ with the effects of protonophores DNP or FCCP, and mitochondrial respiration inhibitor cyanide (Fig. 2). Depolarization of CGC induced by AA (10 μM) and registered by changes in rh123 fluorescence (Fig. 2a) was relatively small but comparable with depolarization induced by 3 mM NaCN (Fig. 2, a and c) or low concentration of DNP (8 μM) (Fig. 2b). Higher dose of DNP (200 μM) and FCCP (1 μM) inducing total MD caused 3-4 times more pronounced increase in rh123 fluorescence than AA. Combined addition of AA and F_1F_0-ATP synthase/ATPase inhibitor oligomycin (Oligo, 2.5 μg/ml) resulted in sharp depolarization, which was comparable to MD caused by FCCP (Fig. 2a). Addition

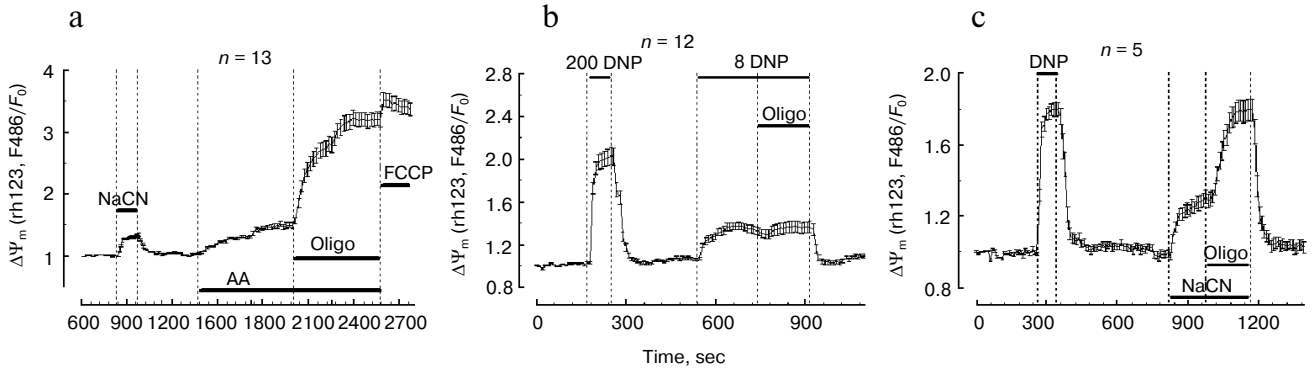

Fig. 2. Changes in $\Delta\Psi_m$ in primary culture of rat cerebellar granule cells treated with the protonophores DNP (200 and 8 μM) and FCCP (1 μM), AA (10 μM), Oligo (2.5 μg/ml), and mitochondrial respiration inhibitor cyanide (NaCN, 3 mM).

of Oligo alone causes hyperpolarization of neuronal mitochondria due to blockade of proton flow through ATP synthase [3]. In contrast to AA-induced depolarization, the partial depolarization induced by low concentration of DNP was insensitive to Oligo (Fig. 2b). Combined addition of cyanide with Oligo (Fig. 2c) caused total MD as in the case of combined treatment with AA plus Oligo (Fig. 2a). The data of Fig. 2 suggest that the effect of AA on the mitochondrial potential of neurons may be attributed to inhibition of mitochondrial respiration rather than protonophore properties of this acid. However, use of low concentrations of NaCN (0.5 mM) and DNP (8 μM) revealed that combination of the mitochondrial respiration inhibitor and the protonophore can cause stronger MD than the arithmetic sum of the individual responses to these agents (data not shown). Such synergistic action was also found in the case of cyanide plus AA and also AA plus low DNP concentration.

Effect of AA on intracellular ATP level in primary culture of rat cerebellar granule cells. Previously we [28] and others [33] have demonstrated that uncouplers and inhibitors of mitochondrial respiration decreased intracellular ATP content in CGC. Combination of these agents with Glu caused more pronounced decrease in ATP content. So for additional experimental validation of the hypothesis that AA can act as an uncoupler and inhibitor of mitochondrial respiration in neurons, we investigated the effect of AA alone and in combination with Glu on the level of intracellular ATP.

Figure 3 shows results of one experiment on ATP assay in CGC treated with Glu and AA alone and with their combination. Glu (100 μM, 30 min) decreased ATP level by 30-50% compared to control, whereas combined action of Glu with AA decreased ATP content to 15-20% of control. This value is at least two times lower than it would be in the case of the additive actions of these agents.

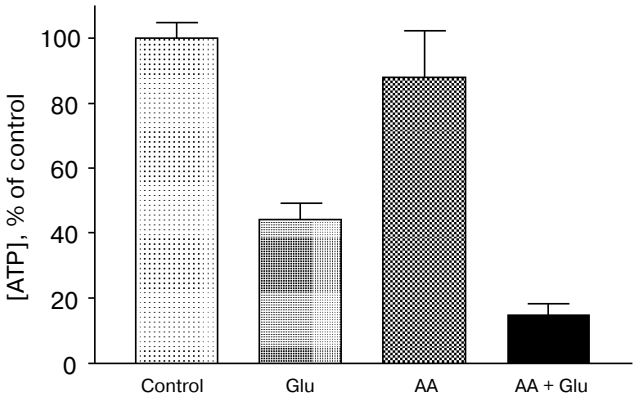

Fig. 3. Effects of Glu (100 μM), AA (10 μM), and their combination on the intracellular ATP content ([ATP]) (% of control). Cells were incubated with Glu and AA at room temperature for 30 min; ATP content was determined by the method of luciferin–luciferase chemiluminescence.

DISCUSSION

The significant increase in the proportion of neurons characterized by large augmentation of $[Ca^{2+}]_i$ during combined action of Glu and AA compared with the effect of Glu alone can be attributed to several reasons. First, a whole-cell clamp study on the isolated neurons revealed that AA (10 μM) caused about two-fold potentiation of current through NMDA receptors [34, 35] even at saturating agonist concentrations [35]. Potentiation appeared as a result of channel opening without changes in conductivity, and it was not mediated by putative products of AA oxidation. Under our experimental conditions the effect of AA on the Glu-induced increase of $[Ca^{2+}]_i$ was more pronounced in the presence of ETYA (30 μM), the inhibitor of lipoxy-/cyclooxygenase pathways of AA oxidation. This is consistent with results of electrophysiological measurements on the role of AA rather than products of its metabolism [35].

Lack of an effect of PA on $[Ca^{2+}]_i$ and $\Delta\Psi_m$ suggests that the AA effect cannot be attributed to its detergent properties. The critical micelle concentration of PA is approximately one order of magnitude less than that of AA and the partition coefficient for distribution between membrane and aqueous phase is even higher in PA compared with AA [36]. So if the AA effects on cells were related with micelle formation and their detergent action on cell membranes, PA effects would be more pronounced than those of AA. However, in reality we did not observed prevalence of PA over AA effects.

The second reason for impairment of deregulation of Ca^{2+} homeostasis in CGC culture may be related to direct action of AA on mitochondria functioning. AA is a protonophore, and protonophore-induced strong MD preventing or at least significantly reducing Ca^{2+} accumulating capacity of mitochondria significantly increased Glu-induced increase in $[Ca^{2+}]_i$ [2, 3]. Studies on rat brain submitochondrial particles revealed that AA can inhibit Complex I of the respiratory chain [25]. Respiration inhibitors causing small MD may also promote collapse of potential after Glu addition or cause augmentation of Glu-induced increase in $[Ca^{2+}]_i$ [2]. In both cases a $[Ca^{2+}]_i$ plateau was preserved in most cells for 10-15 min of the post-glutamate period until removal of a protonophore or respiration blocker [28]. This so-called immediate $[Ca^{2+}]_i$ deregulation can be induced by ATP deficit caused by conformational transition of ATP synthase into ATPase [37]. Thus, strong MD (Fig. 1, e and f) and significant increase in $[Ca^{2+}]_i$ caused by the combined action of Glu and AA (Fig. 1, b and c) can be attributed to both protonophore effect of AA and its inhibition of the respiratory chain. Dual action of AA on respiration of isolated rat brain mitochondria has been demonstrated earlier [38, 39]. A reasonable question arises: whether inhibition predominates or one should take into consideration both mechanisms of depolarization in the case of

a neuronal culture? At concentrations ⩽10 μM AA alone can cause small MD, which is significantly increased by addition of Oligo (Fig. 2a). The same effect was observed during application of Oligo with cyanide (Fig. 2c). However, the combination of Oligo plus low concentration of DNP was ineffective (Fig. 2b). It is possible that incubation of CGC culture with 10 μM AA for 10 min is sufficient for conformational transition of mitochondrial ATP synthase into the ATPase, required for maintenance of $\Delta\Psi_m$ by using ATP. The conformational transition of ATP synthase may occur during the increase in $\Delta\Psi_m$ up to −120 mV [40], or during inhibition of respiratory chain enzymes. Relatively small increase in rh123 fluorescence during the action of AA alone (Figs. 1f and 2a) indicates that conformational transition of ATP synthase into ATPase does not occur due to sharp decrease in $\Delta\Psi_m$.

The effect of AA on intracellular ATP (Fig. 3) was comparable to previously reported independent effects of cyanide or DNP [28]. Combination of AA and Oligo had minor influence on ATP level (data not shown). Perhaps, in the absence of stimulation of neurons by Glu and with functionally competent glycolysis a short-term mitochondrial dysfunction (for 10-15 min) insignificantly alters the level of intracellular ATP.

The third reason underlying impairments of calcium homeostasis in neurons during concerted action of AA and Glu may consist in formation of a low selectivity non-classic permeability transition pore in the inner mitochondrial membrane. In contrast to classic mitochondrial permeability transition pore the latter is characterized by: a) insensitivity to cyclosporin A; b) Sr^{2+} may be as effective as Ca^{2+} in formation of this non-classic pore but it cannot induce the classic pore. Among saturated fatty acids, palmitic and stearic acids were the most potent inducers of the non-classic pore [18, 19]. Unsaturated free fatty acids were one order of magnitude slower in formation of the non-classic pore of low selectivity. However, if mitochondria accumulated significant amounts of Ca^{2+} (or Sr^{2+}) unsaturated fatty acids would also induce the non-classic pore [20]. Moreover, in contrast to other unsaturated fatty acids, AA potentiated PA effect under co-application of these acids. Under our conditions, PA concentrations exceeding those of AA did not cause any potentiation effect on Glu-induced Ca^{2+} entry and MD. So, it seems unlikely that this mechanism dominates at least in CGC.

Glu-induced augmentation of free fatty acids [10, 11] may thus play a dual role. On one hand, free fatty acids may feed the tricarboxylic acid cycle and thus compensate for pyruvate deficiency, for example in the case of glucose deprivation. On the other hand, AA may inhibit the mitochondrial respiratory chain and at higher concentrations it may also increase proton leak via the inner mitochondrial membrane [41, 42] and decrease ATP synthesizing capacity of mitochondria and their ability to accumulate Ca^{2+} excess from cytosol.

Results of the present study suggest that free fatty acids, especially AA, may be an important factor responsible for appearance of the second phase of $[Ca^{2+}]_i$ increase and strong MD during stimulation of neuronal culture with glutamate.

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