Glutamate-mediated inhibition of oxidative phosphorylation in cultured retinal cells

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Abstract

Glutamate is an excitotoxin responsible for causing neuronal damage associated with mitochondria dysfunction. We have analyzed the relationship between the mitochondrial respiratory rate, the membrane potential ($\Delta\Psi$) and the activity of mitochondrial complexes in retinal cells in culture, used as neuronal models. Glutamate (10 µM–10 mM) dose-dependently decreased the O_2 consumption and the membrane potential. A linear correlation was found between these parameters, suggesting that the mitochondrial respiratory function was affected. Exposure to glutamate (100 µM) for 10 min, in the absence of Mg^{2+}, inhibited the activity of complex I (26.3%), complexes II/III (22.2%) and complex IV (26.7%). MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine hydrogen maleate), a non-competitive antagonist of the NMDA (N-methyl-D-aspartate) receptors, completely reversed the effect exerted by 100 µM glutamate at the level of complexes I, II/III and IV. These results suggest that NMDA receptor-mediated inhibition of mitochondrial respiratory chain complexes may be responsible for the alteration in the respiratory rate of chick retinal cells submitted to glutamate. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Glutamate; Mitochondria; NMDA receptor; Retinal cells

1. Introduction

Glutamate has a fundamental role in normal brain function, in the physiological processes of learning and memory, and also in many pathological processes, such as trauma or ischemia, and in a number of neurodegenerative disorders, such as Alzheimer's disease, Amyotrophic lateral sclerosis, Parkinson's disease and Huntington's disease. Excitotoxic neuronal injury depends on the interaction of glutamate with the receptors located principally on postsynaptic neurons. The ionotropic glutamate receptors comprise the NMDA and the non-NMDA receptors, which include the α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) and the kainate receptors subtype (Vizi et al., 1997; Tapia et al., 1999). Upon activation, these receptors are responsible for the deregulation of the ionic fluxes across the cell membrane, leading to increases in intracellular $[Ca^{2+}]$ and $[Na^+]$. Generation of reactive oxygen species (ROS), namely nitric oxide (NO') (Gunasekar et al., 1995) and superoxide radicals (O_2^-) (Lafon-Cazal et al., 1993) have been shown to require the activation of the NMDA receptors and are suggested to be highly involved in

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the progression of neurodegeneration. NO· can easily react with the O_2^- generated by the mitochondria and produce $ONOO^-$ (peroxynitrite), mediating the formation of the hydroxyl radical (HO·), one of the most deleterious radicals in living systems.

At the level of the mitochondria respiratory chain, O_2^- is formed by complexes I and III under normal conditions (Turrens, 1997). After exposure to glutamate, mitochondria has been shown to play an important role in buffering cytoplasmic Ca^{2+} (Budd and Nicholls, 1996; Wang and Thayer, 1996) and production of ROS was observed (Dugan et al., 1995; Bindokas et al., 1996; Castilho et al., 1999). Furthermore, glutamate was shown to affect the mitochondrial membrane potential (Ankarcrona et al., 1995) and to reduce the energy production (Budd and Nicholls, 1996; Ankarcrona et al., 1995). Previous studies reported an impaired oxygen consumption after exposure to glutamate (Atlante et al., 1996; Almeida et al., 1998), suggesting that an impairment in energy metabolism is involved in excitotoxicity.

In this work, experimental studies were performed to establish whether glutamate was responsible for a reduction of mitochondrial function in cultured retinal cells. These cells have been shown to be good models for neuropathological conditions associated with excitotoxicity, particularly because the retina is a CNS structure, glutamate is used as a major excitatory input and, similarly to other neuronal cell types, the retinal cells are energy-dependent and are very sensitive to oxidative damage. We found that low glutamate concentrations highly decreased mitochondrial respiration and the membrane potential, which seems to occur as a consequence of a reduction in the activity of the respiratory chain complexes (I, II/III and IV), in a process mediated by the activation of the NMDA receptors.

2. Experimental procedures

2.1. Materials

Basal medium of Eagle (Earle's salts-BME) was purchased from Sigma (USA), trypsin from GIBCO (UK) and fetal calf serum from BioChrom KG (Berlin, Germany). MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine hydrogen maleate) was a kind gift of Merck Sharp and Dohme (West Point, PA, USA). Ubiquinone-1 was a kind gift from Eisai Co., Tokyo, Japan. All other chemicals were of the purest grade available from regular commercial sources.

2.2. Retinal cell cultures

Primary cultures of retinal cells were prepared from 8-day-old chick embryos as described previously (Rego et al., 1996). The cells were plated at a density of 2.09×10^6 cells/cm^2 for the measurement of oxygen consumption and membrane potential, or at a density of 0.44×10^6 cell/cm^2 for determining the activities of mitochondrial complexes. The cells were plated on poly-L-lysine (0.1 mg/ml) coated 100 mm Petri dishes, avoiding the growing of a large proportion of Müller cells. For $[Ca^{2+}]_i$ fluorescence determinations, the retinal cells were plated at a density of 0.64×10^6 cell/cm^2 on poly-L-lysine coated 10 mm glass coverslips. The cells were cultured for 5 to 6 days in BME medium buffered with 25 mM Hepes and 10 mM $NaHCO_3$, and supplemented with 5% fetal calf serum (heat inactivated), penicillin (100 units/ml) and streptomycin (100 μg/ml), and maintained at 37°C in an atmosphere of 95% air and 5% CO_2. Similar retinal cultures were described to be highly enriched in amacrine neuron-like cells, but also containing neurons resembling bipolar cells, and some few glial cells (Huba and Hofmann, 1990).

Before the experiments, the culture medium was removed and the retinal cells were washed twice with Na^+ saline solution, containing (in mM): NaCl 140.0, KCl 5.0, $CaCl_2$ 1.5, $MgCl_2$ 1.0, NaH_2PO_4 1.0, glucose 5.6 and Hepes 20.0, at pH 7.4 (37°C), and pre-equilibrated in this solution for 10–15 min, at 37°C, in a waterbath. For the $[Ca^{2+}]_i$ measurements, the cells were incubated with 100 μM glutamate in Na^+ solution, at pH 7.4 (37°C). For the measurement of the activity of mitochondrial complexes, the cells were incubated for 10 min in the absence (control) or in the presence of glutamate (100 μM), in Na^+ medium without Mg^{2+}. When present, MK-801 (5 μM) was incubated 5–10 min prior and during the glutamate exposure, in the absence of Mg^{2+} to ensure maximal opening of the NMDA receptor-associated channel. For the measurement of O_2 consumption or the membrane potential, the retinal cells were dissociated with 0.01% trypsin in Na^+ saline solution, for 15 min, at 37°C. The cells were recovered and centrifuged twice to remove the medium containing the trypsin, and further resuspended in Na^+ solution.

2.3. Measurement of oxygen consumption

The consumption of oxygen was measured polarographically by using an oxygen electrode (Hansatech type), in Na^+ saline solution containing 5.6 mM glucose (0.4 ml, final volume), before (control) and after glutamate (10 μM–10 mM) addition, at 37°C. Continuous stirring allowed O_2 to diffuse freely, although not causing damage to the cell suspension.

Respiration rates were calculated assuming an oxygen concentration of 454 nAt O/ml in the experimental medium at 37°C. Incubation with 1 mM KCN totally inhibited the respiration rate. The rate of oxygen uptake, obtained as a tangent at the initial part of the progress curve, was calculated as nanoatoms O/min/mg protein, and expressed as a percentage of control.

2.4. Measurement of membrane potential ($\Delta\Psi$)

The membrane potential was estimated by calculating transmembrane distribution of TPP$^+$ (tetraphenylphosphonium) with a TPP$^+$ selective electrode. TPP$^+$ (4 μM) uptake was followed by a decrease of TPP$^+$ concentration in Na$^+$ saline solution (0.8 ml, final volume), at 37°C, after incubation of the retinal cells (about 0.25 mg protein) with 10 μM–10 mM glutamate, under magnetic stirring. The mitochondrial $\Delta\Psi$ was determined as relative changes in potential rather than absolute values, and estimated from the following equation (Kamo et al., 1979):

$$\Delta\Psi(mV) = 59 \ \log(v/V) - 59 \ \log(10^{\Delta E/59} - 1),$$

where v, V and ΔE are the mitochondrial volume, the volume of incubation medium and the deflection of electrode potential from the baseline, respectively. This equation was derived assuming that TPP$^+$ distribution between the mitochondria and the medium follows the Nernst equation and that the law of mass conservation is applicable. The results were expressed as a percentage of control, in the absence of glutamate.

2.5. Measurement of mitochondrial complexes activity

After incubation, the retinal cells were collected by gentle scraping in 0.25 M sucrose/5 mM Hepes, pH 7.4 (0–4°C), to obtain a cell suspension. The suspension of retinal cells was rapidly frozen and kept at −80°C. Just before the measurements, the cellular suspension was homogenised. Measurement of complexes activity was performed in sucrose medium, containing (in mM): sucrose 130, KCl 50, MgCl$_2$ 5, KH$_2$PO$_4$ 5 and Hepes 5, at pH 7.4 (30°C). The enzymatic activities were assayed at 30°C.

2.5.1. Complex I (NADH-ubiquinone oxidoreductase)

The decrease in absorbance of NADH at 340 nm was measured by following the method described by Ragan et al. (1987), in a medium supplemented with 1 mM KCN, 150 μM NADH, 1.25 mg/mg free fatty acid BSA and retina homogenates (corresponding to about 50 μg protein) in 1 ml final volume. After a pre-incubation for 5 min, at 30°C, the reaction was initiated with 50 μM ubiquinone-1. Following 5 min (30°C), 10 μM rotenone was added to define the change in absorbance due to complex I activity. Rotenone inhibited complex I by about 90%. NADH oxidation upon addition of ubiquinone-1 was linear up to 15 min. The results were calculated as nmol/min/mg protein and expressed as a percentage of control, in the absence of glutamate.

2.5.2. Complex II/III (succinate dehydrogenase plus succinate-cytochrome-c-reductase)

The activity of complex II/III was measured in sucrose medium supplemented with 8 μM rotenone, 1 mM KCN, 54 μM oxidized cytochrome c and retina homogenates, in a modified method as described by King (1967). After pre-incubation for 5 min (30°C), the reaction was initiated by the addition of 5 mM succinate. Appearance of reduced cytochrome c was measured spectrophotometrically at 550 nm. 0.1 μg antimycin A completely inhibited enzyme activity. The results were calculated as nmol/min/mg protein and expressed as a percentage of control, in the absence of glutamate.

2.5.3. Complex IV (cytochrome oxidase)

The activity of complex IV was measured polarographically, by following the method described previously (Brautigan et al., 1978). The retina homogenates were resuspended in sucrose medium, supplemented with 2 μM rotenone, 0.25 μg/ml antimycin A, 10 μM cytochrome c, 0.75 mg/ml Triton X-100. The measurement of complex IV was initiated upon the addition of 5 mM ascorbate plus 0.25 mM TMPD (N,N,N',N'-tetramethyl-p-phenylenediamine). Incubation with 1 mM KCN inhibited complex IV activity. The results were calculated as nanoatoms O/min/mg protein and expressed as a percentage of control, in the absence of glutamate.

2.6. $[Ca^{2+}]_i$ measurements

The $[Ca^{2+}]_i$ in cultured retinal cells was measured by using the probe indo-1/AM (3 μM), as described previously (Rego et al., 1996), except that after loading, fluorescence was measured in control (non-treated) retinal cells, and 100 μM glutamate was added to the cuvette.

2.7. Statistical procedures

All data are expressed as the means $\pm$ SEM of the indicated number of independent experiments, run in duplicate or triplicate. Statistical comparisons were performed by the unpaired two-tailed Student's t-test for the comparison of the means of two Gaussian populations or by the one-way ANOVA Tukey post-test for multiple comparisons.
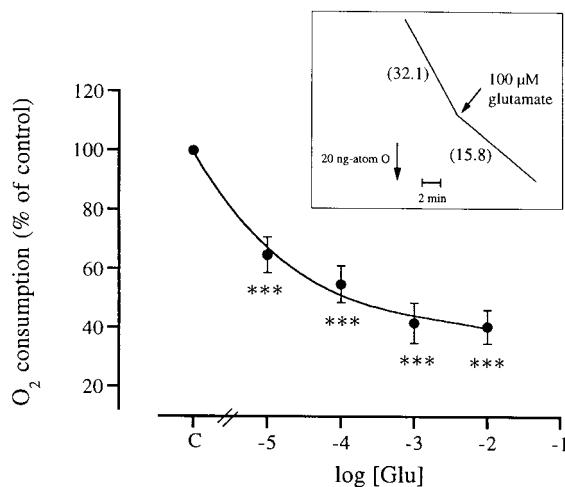
Fig. 1. Effect of glutamate on the consumption of O_2 in dissociated retinal cells. The O_2 consumption was measured polarographically before (control, C) and after glutamate (10 µM–10 mM Glu) addition, in Na^+ saline solution containing 5.6 mM glucose. The rate of oxygen uptake was expressed as a percentage of control, in the absence of glutamate. Insert shows the polarographic trace of a representative experiment before and after addition of 100 µM glutamate; the numbers in brackets represent rates of respiration in ng.atom O/mg protein/min. Data are the means $\pm$ SEM of seven to ten measurements, by using four to five suspensions of retinal cells, obtained from independent preparations. Statistical significance: ***$P < 0.001$ as compared to the control.
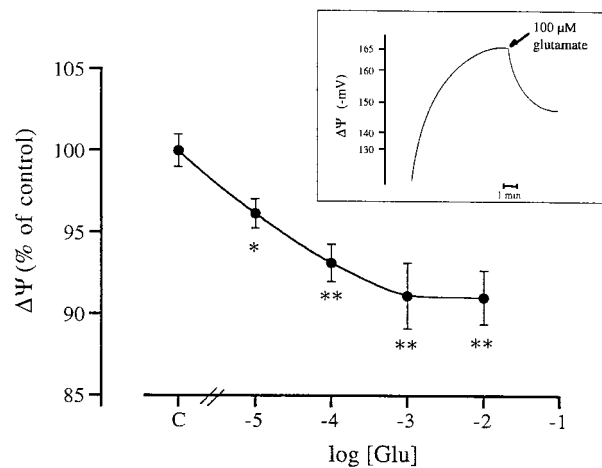

Fig. 2. Effect of glutamate on the membrane potential ($\Delta\Psi$) of retinal cells. The membrane potential ($\Delta\Psi$) was monitored by following the accumulation of TPP^+ by retinal cells submitted to glutamate (10 µM–10 mM), by using a TPP^+ selective electrode. Insert shows the $\Delta\Psi$ after addition of 100 µM glutamate, estimated according to Kamo et al. (1979). Data, expressed as a percentage of control (C), in the absence of glutamate, are the means $\pm$ SEM from six to seven determinations, by using three independent retinal cell suspensions. Statistical significance: *$P < 0.05$ or **$P < 0.01$ as compared to the control.

3. Results

3.1. Glutamate inhibits the rate of mitochondrial respiration in cultured retinal cells

Initial experiments were performed to measure the rate of O_2 consumption in the retinal cells. In control retinal cells, not submitted to glutamate exposure, cellular respiration was of 32.07 ± 2.07 nanoatoms/min/mg protein. Mitochondrial respiration was shown to be responsible for the observed effects, because cyanide, an inhibitor of mitochondrial cytochrome oxidase activity, completely inhibited O_2 consumption (not shown).

Addition of L-glutamate (10 µM–10 mM) induced a dose-dependent inhibition of O_2 consumption in retinal cells (Fig. 1). The inhibition of O_2 consumption occurred right upon exposure to glutamate (Fig. 1, insert). Major decreases in mitochondrial respiration were observed in the presence of low glutamate concentrations (Fig. 1), because the O_2 consumption decreased in the presence of 10 or 100 µM glutamate, respectively, to 64.87 ± 6.06 or $54.94 \pm 6.27\%$ of control, in the absence of glutamate ($P < 0.001$). In the presence of 10 mM glutamate O_2 consumption was reduced to $40.60 \pm 5.79\%$ (Fig. 1). The stereoisomer D-glutamate, used at the same concentrations, did not

elicit the reduction in the consumption of O_2 (not shown).

3.2. Glutamate decreases the membrane potential ($\Delta\Psi$)

Exposure to glutamate (10 µM–10 mM), induced a dose-dependent decrease in $\Delta\Psi$ (Fig. 2). Similarly as determined by the measurement of O_2 consumption, major decreases in $\Delta\Psi$ were observed in the presence of low concentrations of glutamate (10 or 100 µM), with values that decreased from $\Delta\Psi = -166.35 \pm 1.67$ mV in controls, to $\Delta\Psi = -159.97 \pm 1.50$ mV ($P < 0.05$) or $\Delta\Psi = -154.95 \pm 1.92$ mV ($P < 0.01$), in the presence of 10 or 100 µM glutamate, respectively. The $\Delta\Psi$ was not significantly affected upon addition of a high concentration of glutamate (10 mM) as compared to 1 mM glutamate (Fig. 2). Addition of a depolarizing concentration of KCl (50 mM), before glutamate exposure, did not affect significantly the values of $\Delta\Psi$ (not shown), suggesting that changes in $\Delta\Psi$ occurring after glutamate addition were mostly a function of changes in mitochondrial membrane potential. These data also suggested that the trypsinization may have affected the plasma membrane potential of cultured retinal cells. In order to correlate changes in the respiratory rate with alterations in membrane potential, these two parameters were plotted in Fig. 3. We observed a linear correlation (r value of 0.97) between the respiratory rate and the $\Delta\Psi$ in retinal cells exposed to increasing
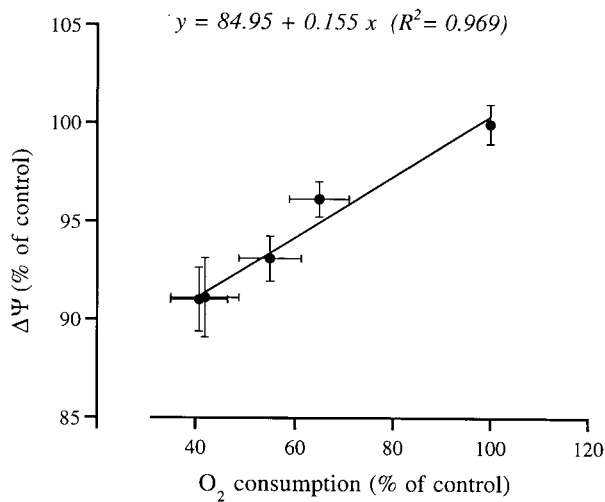
Fig. 3. Correlation between O_2 consumption and the membrane potential ($\Delta\Psi$). Data are the means $\pm$ SEM, as plotted in Figs. 1 and 2.

glutamate concentrations (Fig. 3), suggesting that changes in membrane potential may reflect changes at the level of the mitochondrial membrane.

3.3. NMDA receptors mediate the reduction in the activity of mitochondrial complexes after glutamate exposure

Because changes in the consumption of O_2 upon exposure to glutamate may result from the dysfunction of mitochondrial complexes, affecting the redox component(s) of the mitochondrial respiratory chain, we measured the activities of complex I (NADH-ubiquinone oxidoreductase), complex II/III (succinate dehydrogenase plus succinate-cytochrome-c-reductase) and complex IV (cytochrome oxidase) in homogenates obtained from retinal cells exposed to 100 μM glutamate (Fig. 4). The effect of MK-801, a non-competitive antagonist of the NMDA subtype of glutamate receptors, was also analyzed.

Exposure to glutamate in Mg^{2+}-free medium reduced significantly the activities of complex I (26.3% inhibition, $P < 0.01$), complex II/III (22.2% inhibition, $P < 0.05$) and complex IV (26.7% inhibition, $P < 0.01$). The reduction in the activity of mitochondrial complexes (I, II/III or IV) was completely reversed when the cells were incubated with glutamate (100 μM) in the presence of MK-801 (5 μM), as observed in Fig. 4, indicating that the inhibitory effects were mediated through the activation of the NMDA receptors. When measuring the activity of complex II/III, glutamate plus MK-801 enhanced the activity observed in the control.

Data from Fig. 4 indicated that the inhibition of the mitochondrial respiratory chain by glutamate was due to the activation of the NMDA receptors in retinal
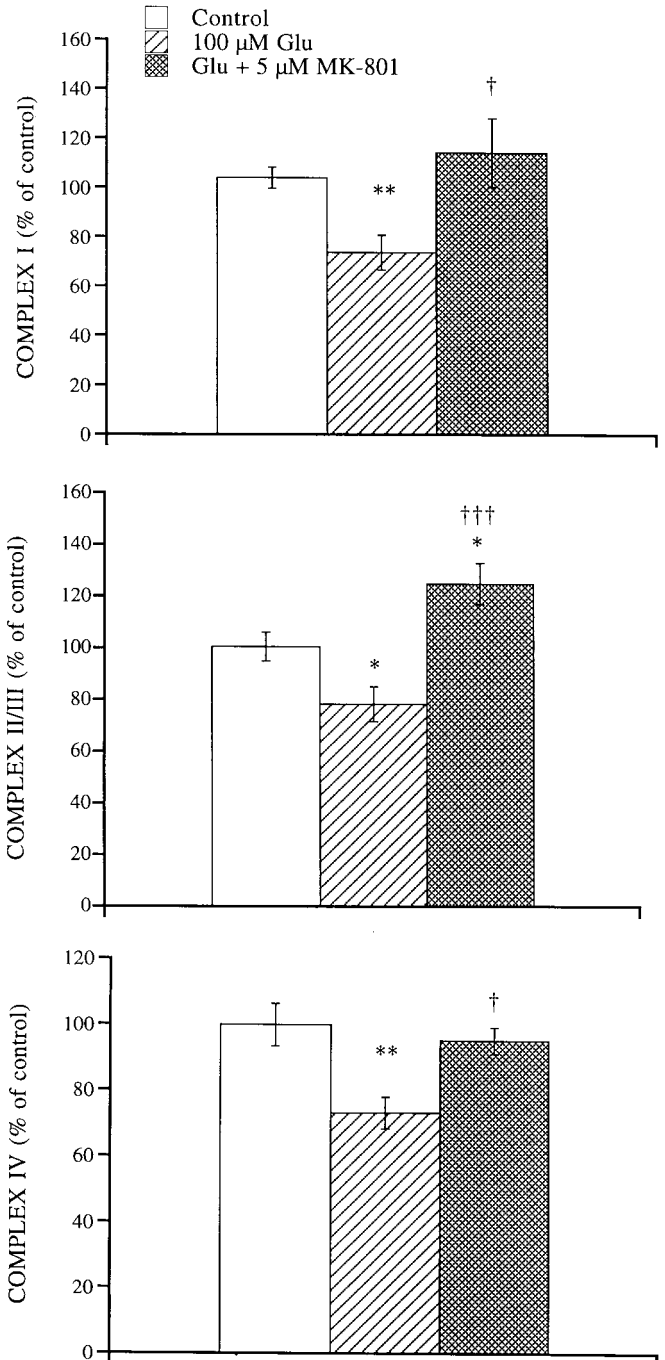

Fig. 4. Effect of glutamate on the activities of mitochondrial respiratory complexes (I, II/III and IV). Retinal cells were incubated with 100 μM glutamate (Glu), for 10 min, at 37°C. Some cells submitted to glutamate were also exposed to 5 μM MK-801 in Mg^{2+}-free medium (Glu + 5 μM MK-801). The activities of mitochondrial complexes I (NADH-ubiquinone oxidoreductase), II/III (succinate dehydrogenase plus succinate-cytochrome-c-reductase) and IV (cytochrome oxidase) were measured in sucrose medium, at 30°C. Data are the means $\pm$ SEM from the number of experiments indicated in the figure. Statistical significance: $^*P < 0.05$ or $^{**}P < 0.01$ as compared to control, in the absence of glutamate; $P < 0.05$ or $P < 0.001$ in comparison with cells exposed to 100 μM Glu.
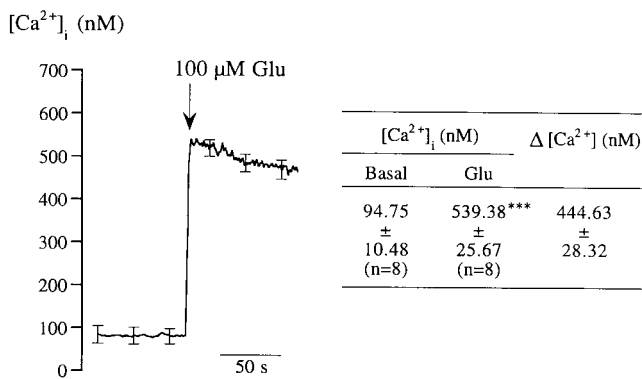
[Ca^{2+}]$_i$ (nM)

Fig. 5. Intracellular Ca^{2+} concentration ([Ca^{2+}]$_i$) after glutamate exposure. [Ca^{2+}]$_i$ was measured by using the fluorescent probe indo-1/AM, as described previously (Rego et al, 1996). The retinal cells were exposed to 100 μM glutamate. Data represent the means ± SEM from eight independent experiments. Δ[Ca^{2+}]$_i$ of 444.63 ± 28.32 nM was determined by calculating the difference between data obtained after glutamate exposure and the basal, before glutamate exposure.

cells in culture. Because many of the effects mediated by this receptor are due to increases in [Ca^{2+}]$_i$ we also measured whether glutamate increased intracellular Ca^{2+}, by using the fluorescent probe indo-1. Fig. 5 shows the increase in [Ca^{2+}]$_i$ from 94.75 ± 10.48 nM, under non-stimulated conditions (basal), to 539.38 ± 25.67 nM after addition of 100 μM glutamate.

4. Discussion

In this study, we give evidence that glutamate reduces the mitochondrial respiratory rate due to the inhibition of complex I (NADH-ubiquinone oxido-reductase), complexes II/III (succinate dehydrogenase plus succinate-cytochrome-c-reductase) and complex IV (cytochrome oxidase) by about 20%, in a process mediated by the activation of the NMDA receptors. These observations seem to be the basis for explaining glutamate-mediated inhibition of O$_2$ consumption. Our results also suggest that glutamate mediated the changes in the mitochondrial membrane potential in cultured retinal cells, because a linear correlation between the loss of membrane potential and the reduction of mitochondrial respiration was observed. By using the fluorescent probe JC-1, glutamate was suggested to mediate significative changes in the mitochondrial membrane potential (Ankarcrona et al., 1995; Stout et al., 1998). Isaev et al. (1996) have also observed that glutamate collapsed the mitochondrial membrane potential, since the neuronal cells lost the ability to accumulate rhodamine 123, a membrane potential-dependent probe.

Likely as observed for the alteration in membrane

potential (Fig. 2, insert), changes in mitochondrial respiration occurred after addition of glutamate (Fig. 1, insert). Thus, the decrease in mitochondrial respiratory chain activity (Fig. 4) may help to explain the decrease in O$_2$ consumption (Fig. 1) and the possible collapse of mitochondrial membrane potential (Figs. 2 and 3) upon a short exposure to glutamate. Our work seems to be supported by Ankarcrona et al. (1995), showing that glutamate induced the early deregulation of mitochondrial function, through the decrease in cellular energy charge and the collapse in mitochondrial membrane potential. According to this study, a proportion of retinal neurons could have died in the early acute phase of injury as a result of NMDA-receptor activation and partial inhibition of mitochondrial complexes activity (Fig. 4). Recently, inhibition of complex II/III and IV activities was observed in the surviving cortical neurons 24 h following glutamate exposure, although no changes in the activity of complex I were observed (Almeida et al., 1998). In that study, a decrease in mitochondrial complexes activity was suggested to be mediated by NMDA-receptor stimulation, leading to the increase in NOS activity and the formation of ONOO$^-$, closely associated with the depletion of GSH (Almeida et al., 1998). Furthermore, thiol oxidation, a concomitant loss of mitochondrial complex I and a decreased activity of complex II and II/III after exposure of brain slices to an agonist of AMPA receptors, indicated that oxidative stress is a primary downstream effect after glutamate receptor activation, leading to mitochondrial dysfunction (Sriram et al., 1998). Interestingly, Sriram et al. (1998) also showed an inhibition of complex I after exposure of brain slices to 1 mM glutamate. In addition, Atlante et al. (1996) have previously observed that glutamate (100 μM) rapidly uncoupled oxidative phosphorylation in primary cultures of cerebellar granule neurons.

Exposure of cultured retinal cells to glutamate (100 μM) increased the [Ca^{2+}]$_i$ by about 5-fold (Fig. 5) and the uptake of Ca^{2+} by about 6-fold after incubation for 2 h (Rego et al., 1998), indicating that the intracellular Ca^{2+} levels are largely increased even after a prolonged exposure to glutamate. Although the energy charge or the plasma membrane integrity of retinal cells was not significantly affected upon exposure to glutamate for 2 h, a 20% reduction of cellular redox status was observed (Rego et al., 1998), as determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, a proposed index used to evaluate mitochondrial function (Mosmann, 1983). In the retinal cells, exposure to glutamate or agonists of NMDA and AMPA/kainate receptors were shown to mediate cytotoxic effects, which were mediated by Ca^{2+} influx occurring principally through the receptor-associated channels (Ferreira et al., 1996). Furthermore, in chick telence-

phali neurons, exposure to glutamate produced a concentration-dependent cytotoxicity after 30 min, which was accompanied by lipid peroxidation, in a process partly mediated by the NMDA receptors (Jacobsson and Fowler, 1999).

The intracellular mechanism through which glutamate exerts the inhibition of mitochondrial respiratory chain activity after NMDA receptor activation in retinal cells may have involved the alteration of Ca^{2+} homeostasis. A transient increase in $[Ca^{2+}]_i$ suggests a relevant role of intracellular Ca^{2+} sequestering mechanisms (Fig. 5). Under this perspective, mitochondria has been considered to be a target for glutamate neurotoxicity, because it plays an important role in buffering intracellular Ca^{2+} after glutamate exposure (Budd and Nicholls, 1996; Wang and Thayer, 1996). Moreover, a Ca^{2+}-dependent disturbance of mitochondrial membrane potential, associated with ultrastructural alterations, was demonstrated to depend upon the activation of NMDA receptors (Isaev et al., 1996). Additionally, the entry of Ca^{2+} through the NMDA and the non-NMDA receptors has been shown to mediate the formation of ROS (Lafon-Cazal et al., 1993; Bindokas et al., 1996). Previously, van de Water et al. (1994) showed that intracellular hydroperoxides mediated the dissipation of the mitochondrial membrane potential, which could have occurred as a consequence of increased $[Ca^{2+}]_i$, accompanied by an increase in mitochondrial Ca^{2+} concentration. Activation of NMDA glutamate receptors were shown to induce the formation of hydroperoxides and ROS generated by the mitochondria, as determined with the fluorescent probes hydroethidine (Bindokas et al., 1996; Castilho et al., 1999) or dihydrorhodamine 123 (Dugan et al., 1995). Formation of O_2^- by the mitochondrial respiratory chain occurs at complex I and at the level of complex III or the ubisemiquinone, the ubiquinone radical (Turrens, 1997). Thus, the formation of O_2^- was implicated as an obligate step in the intracellular events that culminate in cell death after NMDA and kainate receptor activation, as determined by the selective inactivation of aconitase activity, a marker of O_2^- generation (Patel et al., 1996). Excessive formation of mitochondrial O_2^- is metabolized by manganese superoxide dismutase (Mn-SOD), which can regulate membrane lipid peroxidation, $ONOO^-$ formation and mitochondrial dysfunction (Keller et al., 1998). Furthermore, an increased intracellular Ca^{2+} may stimulate mitochondrial nitric oxide synthase (NOS), associated with the inner mitochondrial membrane (Ghafourifar and Richter, 1997), which may have a major relevance in mitochondrial degeneration.

Taking this into account, the formation of ROS upon glutamate-mediated Ca^{2+} rise could be involved in the reduction of the activity of mitochondrial complexes in retinal cells in culture (Fig. 4), causing the inhibition of oxidative phosphorylation (Fig. 1). Accordingly, exposure of isolated mitochondria to the hydroxyl radical ($HO^.$) impaired the activity of complex I (Dykens, 1994), and scavenger antioxidants, such as vitamin E, were shown to reduce excitotoxic cell death in cerebellar granule cultures (Ciani et al., 1996).

In conclusion, our data provide a link between NMDA receptor activation and a reduced activity of mitochondrial respiratory chain complexes upon exposure of chick retinal cells to glutamate. A reduction in oxidative phosphorylation due to glutamate exposure may be particularly relevant after ischemia situations, where massive activation of glutamate receptors have long been considered a key factor for determining the fate of retinal neurons.

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