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Glutamate regulates the viability of retinal cells in culture

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Abstract

In this study, we show that glutamate regulates the viability of cultured retinal cells upon transient glucose deprivation. At low concentrations (10–100 μM) glutamate decreased MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction to about 50% of control and decreased intracellular ATP levels (about 4-fold) after transient glucose removal. Under these conditions, the decrease in MTT reduction was associated with the activation of NMDA (*N*-methyl-D-aspartate) receptors. Upon exposure to high (10 mM) glutamate and transient glucose deprivation, the intracellular levels of glutamate increased. High glutamate significantly counteracted the decrease in MTT reduction and ATP production observed in the presence of low glutamate concentrations. AOAA (aminooxyacetic acid), a non-specific inhibitor of mitochondrial transaminases, enhanced the intracellular glutamate levels, but did not largely affect glutamate-mediated changes in MTT reduction or ATP production. Furthermore, the intracellular levels of pyruvate were not significantly altered, suggesting that changes in ATP production were not due to an increase in glycolysis. Thus, the recovery from glucose deprivation seems to be facilitated in retinal neuronal cells that had been exposed to high glutamate, in comparison with low glutamate, suggesting a role for high glutamate and glucose in maintaining retinal cell function following conditions of glucose scarcity.

Keywords: Excitotoxicity; Glutamate; Mitochondria; NMDA receptors; Retinal cells

1. Introduction

Excitotoxicity and the dysfunction of mitochondrial energy metabolism have been assigned to the etiology of neuronal death in many neurodegenerative disorders (e.g. Beal, 1992). During the pathogenesis of acute ischemic neuronal damage, under conditions of oxygen and/or glucose deprivation, neuronal vulnerability to glutamate excitotoxicity increases. Hypoxia or ischemia conditions were previously reported to induce the release of excitatory amino acids in the isolated retina (Zeevalk & Nicklas, 1990) or in cultured retinal cells (Rego, Santos, & Oliveira, 1996), and inhibition of glycolysis was shown to reduce the energy levels in the retinal cells (Rego, Santos, & Oliveira, 1996, 1997).

Glutamate neurotoxicity, associated with the activation of the NMDA receptors, has been shown to involve the intracellular overload of Ca^{2+} (Mattson, Lovell, Furukawa, & Markesbery, 1995; Ferreira, Duarte, & Carvalho, 1996), which is regulated by the mitochondria (Budd & Nicholls, 1996; Wang & Thayer, 1996). Glutamate receptor activation was also shown to induce mitochondrial depolarization and production of (mitochondrial) reactive oxygen species (Lafon-Cazal, Pietri, Culcasi, & Bockaert, 1993; Dugan et al., 1995; Gunasekar, Kanthasamy, Borowitz, & Isom, 1995; White & Reynolds, 1996; Keelan, Vergun, & Duchen, 1999). Thus, both, the release of glutamate and the generation of reactive oxygen species, were shown to potentiate neuronal damage during ischemia (Pellegrini-Giampietro, Cherici, Alesiani, Carla, & Moroni, 1990).

The study of neuronal changes mediated by glutamate seems to be particularly relevant in conditions of

Abbreviations: AOAA, Aminooxyacetic acid; Asp, Aspartate; BME, Basal medium of eagle; Glu, Glutamate; LDH, Lactate dehydrogenase; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[a,d]-cyclohepten-5,10-imine maleate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMDA, *N*-methyl-D-aspartate.

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recovery and/or adaptation from hypoglycemia episodes, previously reported to induce the impairment of cerebral function (Nehlig, 1997). Glutamate was shown to be metabolized by the trichloroacetic acid cycle in the mitochondria of astrocytes (McKenna, Tildon, Stevenson, & Huang, 1996a) and converted to deaminated products, like lactate, an effective substrate for energy metabolism in synaptic terminals (Schurr, West, & Rigor, 1988), suggesting that the conversion of glutamate to energy-yielding substrates in neurons could be beneficial under conditions of low glucose levels.

Thus, in this study, we have examined the effect of transient glucose deprivation on glutamate-mediated changes in cell viability and ATP production in chick retinal cells in culture. The contribution of NMDA receptor activation was also analysed. The effect of AOAA, a non-specific inhibitor of mitochondrial transaminases, was used as a tool to determine alterations associated with glutamate transamination. We show that, depending whether low or high glutamate concentrations are present during glucose deprivation, a neurotoxic or a neuroprotective effect is observed upon exposure to glucose. These observations may be important to understand a partial recovery of retinal cell function near the penumbra area after an ischemic lesion. Under these conditions, access to glucose together with high levels of glutamate may help to counterbalance the demand in energy after injury and temporarily preserve neuronal survival.

2. Methods

2.1. Materials

Basal Medium of Eagle (BME) was purchased from Sigma (USA), trypsin from GIBCO (UK) and fetal calf serum from BioChrom KG (Berlin, Germany). (+)-5-Methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]

× cyclohepten-5,10-imine maleate (MK-801) was from Merck Sharp and Dohme (West Point, PA, USA). All other chemicals used were purchased from Sigma (USA).

2.2. Primary culture of retinal cells

Cultures of retinal cells were obtained from 8-day-old chick (White Leghorn) embryos, as described previously (Agostinho, Duarte, Carvalho, & Oliveira, 1994; Agostinho, Duarte, & Oliveira, 1996). Briefly, the retinas were dissociated with trypsin for 15 min, at 37°C. After centrifugation, the digested tissue was resuspended in BME, buffered with 25 mM HEPES and 10 mM $NaHCO_3$, and supplemented with 5% fetal calf serum (heat inactivated), penicillin (100 units ml^{-1}) and streptomycin (100 μg ml^{-1}). The cells were plated on poly-L-lysine (0.1 mg ml^{-1}) pre-coated 12-multiwell Costar plates at a density of 0.4×10^6 cells cm^{-2}. The cells were cultured at 37°C in an atmosphere of 95% air and 5% CO_2, for 5 days. Similar cultures of retinal cells were described to be highly enriched in amacrine-like neurons, and also neurons resembling bipolar cells and few glial cells (Huba & Hofmann, 1990; Hofmann & Möckel, 1991; Agostinho et al., 1996).

2.3. Induction of glucose deprivation in cultured retinal cells exposed to glutamate

The BME culture medium was removed and the cells were pre-incubated, for 10 min, in a sodium saline solution, at 37°C, 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. Glucose deprivation was induced by pre-incubating the retinal cells in the absence of glucose, for 30 min, followed by exposure to a glucose-free medium, for 2 h (37°C), in the absence or in the presence of different glutamate concentrations (10 μM–10 mM). Exposure to extracellular glutamate ranged from 10 μM, a physiological level, to 10 mM, a putative maximal concentration, occurring after cell disruption, close to the damaged focus (Kempski & Volk, 1994). It was verified that 10 mM glutamate did not affect the pH of the incubation medium. Retinal cells not submitted to glucose deprivation were incubated in the presence of glucose during 30 min, plus 2 h, in the absence or in the presence of glutamate. Control retinal cells were incubated in the absence of glutamate.

In some experiments, the retinal cells were incubated for 30 min plus 2 h in the absence of Mg^{2+} or in a saline solution without Mg^{2+} but containing 5 μM MK-801, the non-competitive antagonist of the NMDA receptors. In order to increase its efficiency, MK-801 was incubated for 30 min before the incubation with glutamate. When present, AOAA (2 mM) was added 5 min before and during the incubation with glutamate, for 2 h.

At the end of the incubation for 2 h, the medium was changed and the cells were incubated in sodium solution containing glucose but in the absence of glutamate, for 3 h, at 37°C (post-incubation period), in order to turn necrotic the irreversibly damaged neurons and allow reversibly damaged cells to recover from glucose deprivation, as shown by the schematic representation of the experimental protocol (Fig. 1). A post-incubation with glucose preceded by the incubation in glucose-free medium is defined as a 'transient glucose deprivation'.

2.4. Measurement of LDH leakage and MTT reduction

LDH (lactate dehydrogenase) leakage to the extracellular medium, a measurement of plasma membrane

integrity, was measured after 2 h exposure to glutamate with or without glucose or after 3 h post-incubation in the presence of glucose. Briefly, the extracellular medium was collected and the cells were lysed with 10 mM Hepes, pH 7.4, scraped and harvested. Cell debris were removed after centrifugation at 15 200 *g*, for 2 min (0–4°C). LDH activity was measured spectrophotometrically by following the conversion of NADH–NAD^+ at 340 nm, according to the method described by Bergmeyer and Brent (1974). The cuvettes contained 0.5 ml of 9.76 mM pyruvate, 100 μl of sample and 2.5 ml of 2.4 mM NADH, used to initiate the reaction. Pyruvate and NADH solutions were prepared in Tris/NaCl buffer, containing 81.3 mM Tris and 203.3 mM NaCl, pH 7.2, at 30°C. The absorbance was measured against a blank containing Tris/NaCl buffer instead of pyruvate. LDH leakage was expressed as a percentage of total activity, by using the millimolar absorption coefficient of NADH (0.63 l $mmol^{-1}$ mm^{-1}).

Measurement of cell viability by the MTT colorimetric assay is based on the reduction of MTT to the insoluble intracellular formazan (Mosmann, 1983), which depends on the activity of intracellular dehydrogenases and is independent of changes in the integrity of the plasma membrane. MTT reduction is usually an early indicator of cell dysfunction. Incubation with MTT (0.5 mg ml^{-1} prepared in sodium saline solution, in the dark) was carried out during glutamate exposure, for 2 h, or in medium containing glucose, during 3 h post-incubation, at 37°C. At the end of the incubation with MTT, an equal volume of acid–isopropanol (0.04 M HCl in isopropanol) was added and mixed thoroughly to dissolve the crystals of formazan. Then, the mixture was collected from the wells and the extent of MTT reduction was measured spectrophotometrically at 570 nm. The results were expressed as a percentage of control cells, thoroughly incubated in the presence of glucose, in the absence of glutamate.

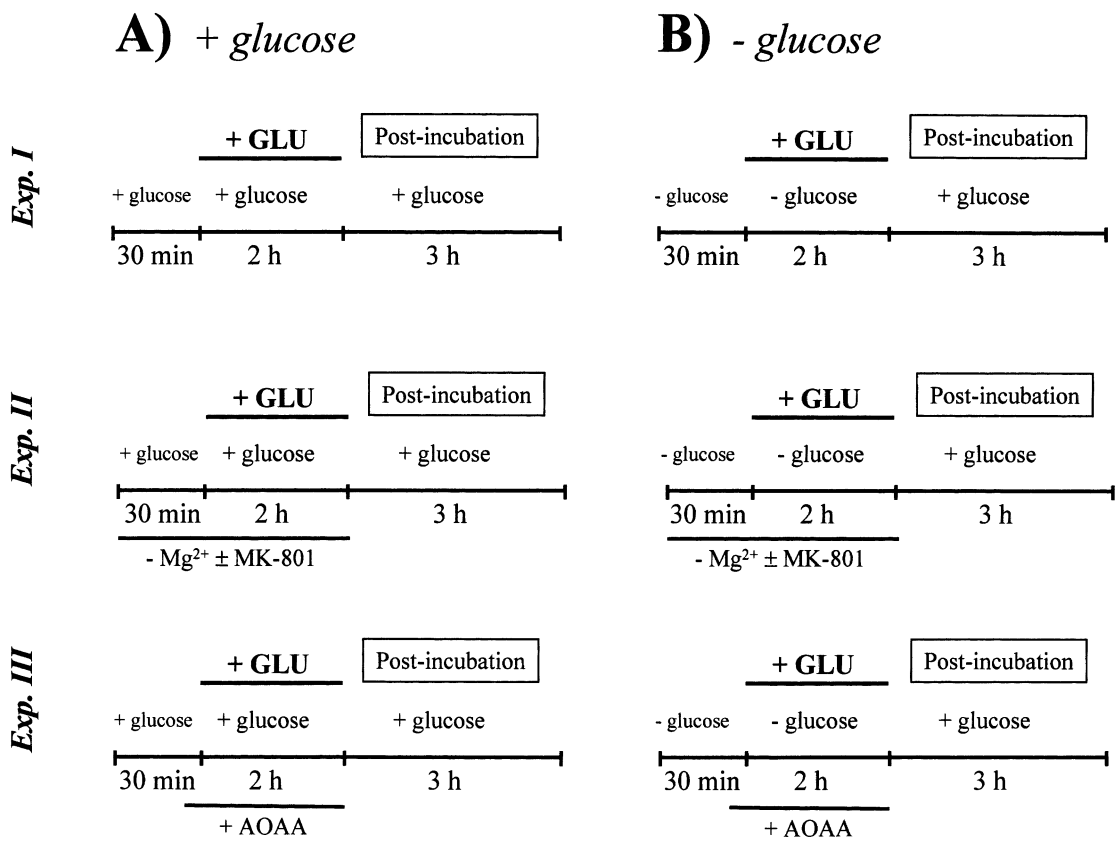

Fig. 1. Schematic representation of the experimental protocol. In A, the cells were incubated with glucose throughout the experiment (+ glucose). Under these conditions, the cells were pre-incubated with sodium solution containing glucose for 30 min and then exposed to glutamate for 2 h. In B, the cells were pre-incubated with glucose-free medium for 30 min to allow the cells to adapt to the saline solution. Then, the cells were exposed to glutamate for 2 h in glucose-free medium (− glucose). At the end of this incubation, the cells (A or B) were incubated with medium containing glucose, but without glutamate for 3 h (post-incubation). In experiments type II, the cells were incubated in the absence of Mg^{2+} or in medium without Mg^{2+} containing MK-801 (30 min before and during 2 h with glutamate). In experiments type III, AOAA was added 5 min before and during the incubation with glutamate (2 h). Throughout the text, 'transient glucose deprivation' refers to experimental condition B. Control cells were incubated in the absence of glutamate.

2.5. Analysis of excitatory amino acids

At the end of the post-incubation period, the intracellular levels of aspartate and glutamate were measured after adding 0.3 ml of 1 M NaOH to the cells, in ice. The lysed cells were scraped off from the wells and collected. Determination of total protein content in NaOH extracts was performed by the method described by Sedmak and Grossero (1977). The samples were centrifuged at 15 800 *g*, for 2 min, to eliminate cell debris, and the supernatants were frozen at −80°C, just before amino acid analysis. The amino acids were measured after pre-column derivatization and separation by reverse phase Gilson-ASTED HPLC system. A Spherisorb ODS column (150 mm × 4.6 mm i.d., 5 μm, C18, Rainin Microsorb) was used, and the samples were eluted at a flow rate of 2.5 ml min^{-1}, for about 45 min, using a ternary solvent system, consisting of: solvent A-37.5 mM sodium phosphate, 50 mM propionic acid, 7% acetonitrile and 3% dimethyl sulfoxide, pH 6.2; solvent B-40% acetonitrile, 33% methanol and 7% dimethyl sulfoxide; and solvent C-62.5 mM sodium phosphate, 50 mM propionic acid, 7% acetonitrile and 3% dimethyl sulphoxide, pH 5.5. The amino acids were detected as fluorescent derivatives after pre-column derivatization with *O*-phthaldialdehyde/2-mercaptoethanol, at 340 nm excitation and 410 nm emission, with a Gilson fluorescent detector, model 121. The amount of amino acids was determined against the peak areas of an external standard and expressed as nmol per mg of protein.

2.6. Analysis of ATP

After incubation of retinal cell, as indicated in figure legends, the cells were immediately chilled in ice and extracted with 0.3 M perchloric acid (0.3 ml). The cell extract was centrifuged at 15 800 *g*, for 5 min, at 0–4°C. The pellets were analysed for total protein (Sedmak & Grossero, 1977) after solubilisation in 1 M NaOH, whereas the supernatants were neutralised with 10 M KOH in 5 M Tris. After neutralisation, the samples were centrifuged at 15 800 *g*, for 10 min, at 0 − 4°C, and stored at −80°C, just before HPLC injection. The supernatants were assayed for ATP analysis by reverse phase HPLC (Stocchi, Cucchiarini, Chiarantini, Palma, & Crescentini, 1985) in a System Beckman Gold equipped with a 126 Binary Pump Model and a 166 Variable UV detector, controlled by a computer, and by using a Lichrospher 100 RP-18 (5 μm) column from Merck (Darmstadt, Germany). Adenine nucleotides were eluted at a flow rate of 1.2 ml min^{-1} with 100 mM KH_2PO_4, at pH 7.4, and 1% methanol, and detected at 254 nm. Data were expressed as nmol ATP per mg protein.

2.7. Analysis of intracellular pyruvate

Retinal cells were incubated for 2 h with glutamate (100 μM or 10 mM), in the presence or in the absence of glucose. The cells were further incubated in the presence of glucose for 3 h (post-incubation). Then, the cells were washed twice with PBS (0–4°C), scraped and extracted with 8% perchloric acid. The samples were centrifuged at 10 000 rpm for 2 min (Eppendorf Centrifuge) to remove cell debris. The resulting supernatant was used to determine the intracellular content in pyruvate, by the SIGMA Pyruvate Diagnostics kit.

2.8. Statistical analysis

Data were expressed as the means ± SEM of the indicated number of experiments. 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 (a P value < 0.05 was considered significant).

3. Results

3.1. Influence of transient glucose deprivation on glutamate-mediated changes in retinal cell viability

In this study we have analysed the viability of retinal cell by the MTT method upon glutamate exposure under glucose deprivation conditions (Fig. 2, dark circles). As determined in a previous study (Rego, Areias, Santos, & Oliveira, 1998), retinal cells incubated with glucose-free medium, in the absence of glutamate, for 2 h, showed a large decrease in MTT reduction (about 25% of control), not significantly altered upon incubation with glutamate (Fig. 2A). In contrast, after a post-incubation with glucose-containing medium (3 h), the cells maintained the capacity to reduce MTT (Fig. 2B, dark circles). LDH leakage, a measurement of plasma membrane integrity and cell damage, was not significantly altered in cells incubated in the presence or in the absence of glucose (Table 1). The capacity to restore MTT reduction after the post-incubation, without significant changes in plasma membrane integrity, suggests a recovery process.

Glutamate (10 μM–10 mM) was shown to significantly decrease MTT reduction in cells incubated in the presence of glucose (Fig. 2B, open circles), indicating some toxic effect induced by glutamate. In cells submitted to post-incubation after transient glucose deprivation, exposure to 10 μM glutamate induced a sharp decrease in MTT reduction (Fig. 2B, dark circles) and a slight increase in LDH leakage (Table 1). However, within the range > 10 μM–10 mM, in cells submitted to

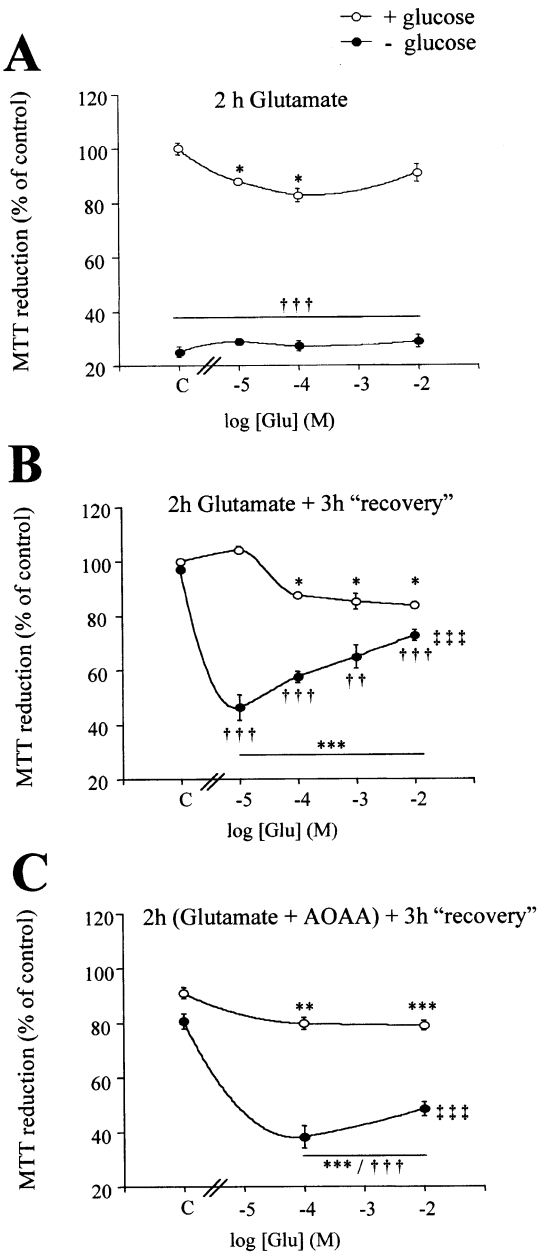

Fig. 2. Glutamate-mediated dose-dependent changes in MTT reduction before and after the post-incubation period. Influence of AOAA. Retinal cells were pre-incubated for 30 min and then exposed to 10 μM-10 mM glutamate (Glu) for 2 h in the absence (− glucose, dark circles) or in the presence of glucose (+ glucose, open circles) (A). Then, the cells were incubated in medium containing glucose for 3 h (post-incubation) (B). Some cells were also exposed to AOAA (2 mM), 5 min before and during glutamate exposure (2 h) and further incubated in the presence of glucose (3 h post-incubation) (C). Control cells (C) were incubated in the absence of glutamate. Data, expressed as a percentage of control in the presence of glucose, are the means ± SEM of triplicates from 3 (A), 7–12 (B) or 5 (C) experiments. Statistical significance: $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$ as compared to the respective control, $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ or $^{\dagger\dagger\dagger}P < 0.001$ as compared to the same glutamate concentration, in the presence of glucose. $^{\ddagger}P < 0.05$ or $^{\ddagger\ddagger\ddagger}P < 0.001$ as compared to 10, 30 or 100 μM glutamate in cells incubated with glutamate in the absence of glucose.

transient glucose deprivation, a dose-dependent increase in MTT reduction was observed (Fig. 2B, dark circles): 10 μM glutamate decreased MTT reduction to 46.4 ± 4.7% of control, whereas 10 mM glutamate decreased MTT reduction to 72.8 ± 2.0% of control ($P < 0.001$, as compared to 10 or 100 μM glutamate). Under these conditions, 10 mM glutamate increased significantly the release of LDH (15.33 ± 1.56% of total), indicating some degree of plasma membrane damage.

3.2. Involvement of NMDA receptor activation on glutamate-mediated changes in MTT reduction

Activation of NMDA receptors has been shown to be responsible for cell damaging effects induced by glutamate in the retina (Zeevalk & Nicklas, 1990; Williams, Murphy, Glowinski, & Prémont, 1995; Ferreira et al., 1996). In this study, we analysed the contribution of NMDA receptors on the capacity of retinal cells to reduce MTT, by incubating the retinal cells in the absence of Mg^{2+}, to allow the opening of the NMDA channel, or in Mg^{2+}-free medium containing

Table 1
Effect of post-incubation on glutamate-mediated changes in LDH leakage [a]

	LDH leakage (% of total)	
	+ glucose	− glucose
(A) Incubation (2 h)		
Control	3.45 ± 0.32	4.85 ± 0.50
10 μM Glu	4.62 ± 0.56	6.99 ± 0.65
100 μM Glu	4.01 ± 0.38	7.50 ± 0.68 [c,e]
10 mM Glu	4.93 ± 0.37	7.46 ± 0.65 [c,e]
(B) Incubation 2 h + post-incubation 3 h		
Control	1.10 ± 0.19	5.02 ± 1.94
10 μM Glu	0.85 ± 0.14	9.17 ± 2.65 [d]
100 μM Glu	3.44 ± 1.48	13.16 ± 2.82 [d]
10 mM Glu	6.20 ± 1.46 [b]	15.33 ± 1.56 [c,e]

[a] Retinal cells were pre-incubated in the presence (+glucose) or in the absence of glucose (−glucose) for 30 min. Then, the cells were exposed to 10 μM–10 mM glutamate (Glu) in the presence or in the absence of glucose, respectively, for 2 h (A). Other cells were also exposed to glutamate for 2 h and further incubated in the presence of glucose for 3 h (post-incubation) (B). In control conditions, the cells were not exposed to Glu. LDH leakage was determined by collecting the incubation medium after 2 h (A) or 3 h (B) incubation. Data are the means ± SEM of seven to twelve experiments, run in triplicate or duplicate.

[b] $P < 0.05$.

[c] $P < 0.01$ as compared to the respective control.

[d] $P < 0.05$.

[e] $P < 0.01$ as compared to similar glutamate concentrations in the presence of glucose.

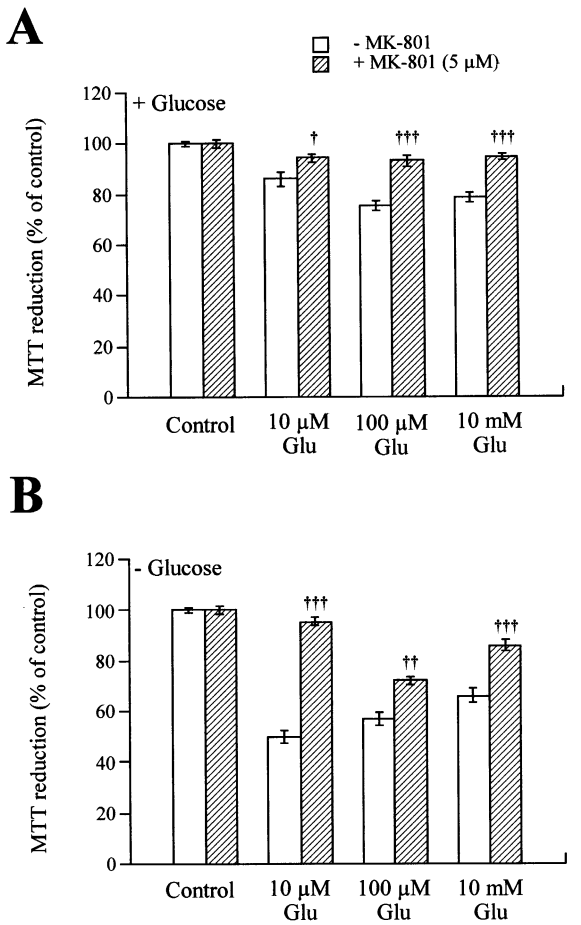

Fig. 3. Involvement of NMDA receptor activation on the reduction of MTT induced by glutamate upon glucose deprivation. Retinal cells were incubated for 30 min plus 2 h in the presence (A) or in the absence (B) of glucose. Glutamate (10 μM-10 mM Glu) was incubated during 2 h. Then, the cells were exposed to incubation medium containing glucose, for 3 h. All incubations where carried out without Mg^{2+} (− MK-801) or without Mg^{2+} but containing 5 μM MK-801 (+ MK-801). Data were determined as a percentage of control, in the absence of glutamate and are the means ± SEM of triplicates from three to four experiments. Statistical significance: $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ or $^{\dagger\dagger\dagger}P < 0.001$ as compared to experimental conditions in the absence of MK-801.

MK-801, in order to block the NMDA receptor-associated channel (Fig. 3).

In retinal cells incubated in the presence of glucose (Fig. 3A), exposure to glutamate in the absence of Mg^{2+} decreased MTT reduction, whereas MK-801 (5 μM) significantly reverted this effect. The involvement of NMDA receptor activation was also analysed after transient glucose deprivation (Fig. 3B). Upon exposure to glutamate, MTT reduction was largely decreased, similarly to data shown in Fig. 2B. Incubation with 5 μM MK-801 completely protected against the large decrement in MTT reduction observed in the presence of 10 μM glutamate (Fig. 3B).

3.3. Effect of extracellular glutamate on the intracellular levels of aspartate and glutamate

The intracellular levels of the excitatory amino acids, aspartate and glutamate, were also determined in retinal cells exposed to increasing glutamate concentrations, in the presence or in the absence of glucose. Increasing extracellular glutamate (10 μM–10 mM) increased significantly the intracellular glutamate levels, as determined in cells submitted to the post-incubation period (Fig. 4). In the presence of 10 mM glutamate, the intracellular levels increased to about 1013 nmol glutamate mg^{-1} protein, in cells incubated either in the presence or in the absence of glucose (Fig. 4A). We have also observed an increase in the intracellular levels of aspartate (Fig. 4B), which is in agreement with the activity of aspartate aminotransferase. Glutamate-mediated increase in intracellular aspartate was almost maximal in the presence of 100 μM glutamate (95.6 ± 17.3 nmol aspartate mg^{-1} protein in the presence of glucose), which may be due to substrate saturation of

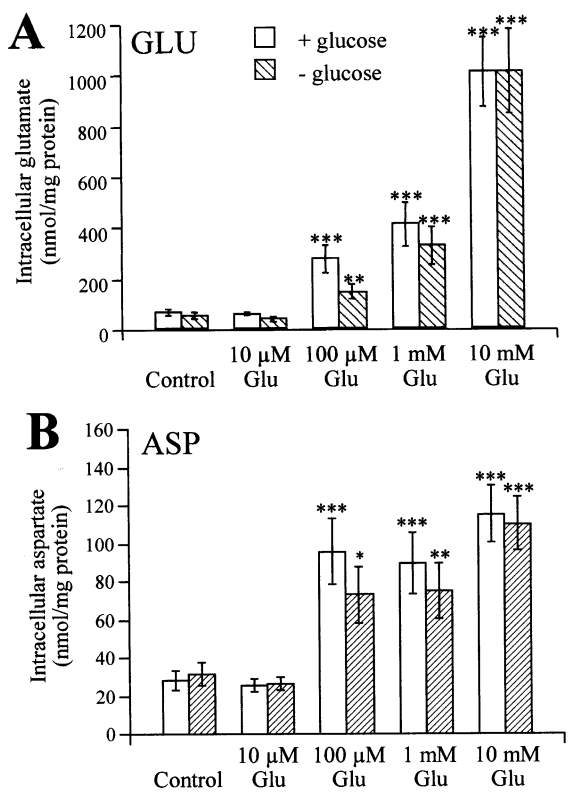

Fig. 4. Intracellular levels of glutamate and aspartate upon exposure to extracellular glutamate. The cells were incubated for 30 min plus 2 h in the presence or in the absence of glucose. Glutamate (10 μM–10 mM Glu) was exposed during the 2h-incubation. Then, the cells were incubated for 3 h in the presence of glucose (post-incubation). The content in intracellular excitatory amino acids, Glu and aspartate (Asp) were analysed by reverse phase HPLC. Data are the means ± SEM of four to six experiments run in triplicates. Statistical significance: $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$ as compared to the respective control, in the absence of Glu.

aspartate aminotransferase ($K_m = 0.43$ mM) in the presence of higher glutamate concentrations.

3.4. Influence of transient glucose deprivation on intracellular ATP after glutamate exposure

An increase in intracellular glutamate levels (Fig. 4A) and a higher capacity to reduce MTT (Fig. 2B), induced by high glutamate upon transient glucose deprivation, highly suggested an increase in retinal cell function. In order to evaluate the changes in retinal cells viability associated with glutamate exposure, we determined the production of intracellular ATP. Fig. 5 shows that exposure to glutamate (10 μM–10 mM), in the presence of glucose, does not significantly affect the levels of ATP, as compared to the control, in cells incubated in the absence of glutamate.

A large decrement (about 4-fold) in the intracellular levels of ATP was observed upon incubation in the absence of glucose and exposure to 10 μM glutamate, before (Fig. 5A) and after the post-incubation period (Fig. 5B). Increasing the extracellular concentration of glutamate, in cells submitted to transient glucose deprivation followed by the post-incubation, dose-dependently increased intracellular ATP production (Fig. 5B): cells incubated with 10 mM glutamate showed intracellular ATP levels of 28.0 ± 6.3 nmol ATP mg^{-1} protein, significantly different ($P < 0.05$) as compared to cells incubated with 10 μM or 100 μM glutamate (8.7 ± 3.4 nmol ATP mg^{-1} protein). In contrast, in retinal cells incubated in the absence of glucose for 2 h, without post-incubation, exposure to increasing glutamate concentrations did not significantly changes the production of ATP (Fig. 5A).

Interestingly, changes in ATP production upon glutamate exposure and transient glucose deprivation (Fig. 5B) were very similar to changes in the capacity of retinal cells to reduce the MTT (Fig. 2B).

3.5. Effect of AOAA on retinal cell viability and ATP production

Because glutamate utilization by the mitochondria could be in the basis of a small decrement in MTT reduction (Fig. 2B) and a small ATP decrement (Fig. 5A) in the presence of high intracellular glutamate levels (Fig. 4A) upon transient glucose deprivation, we have also examined the effect of AOAA, a non-specific inhibitor of transaminases. AOAA was previously shown to be a potent inhibitor of aspartate, alanine and GABA aminotransferases. Treatment with AOAA (2 mM) significantly increased (about 1.8-fold) the intracellular levels of glutamate upon post-incubation in retinal cells incubated with 10 mM glutamate, in the presence or in the absence of glucose (Table 2). These data suggest that AOAA effectively reduces the metabolisation of glutamate by

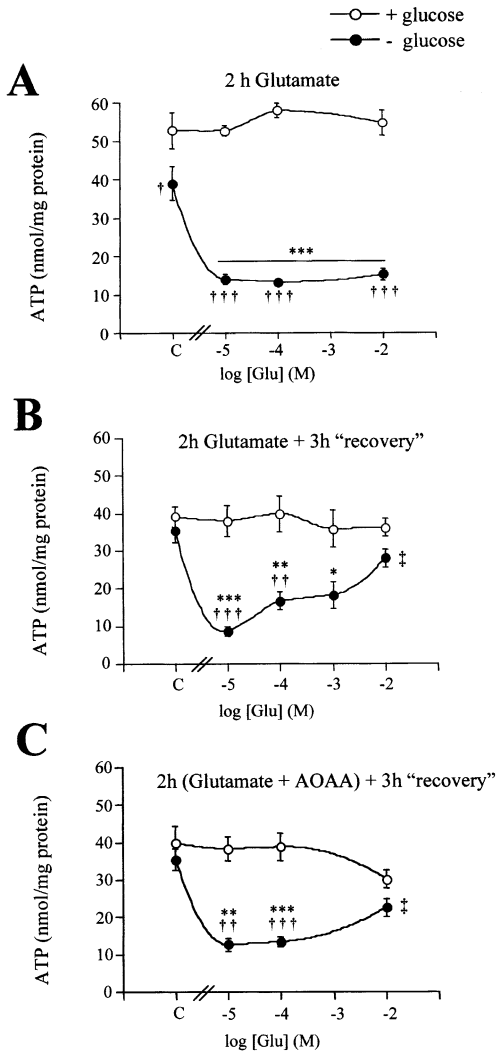

Fig. 5. Glutamate-mediated changes in intracellular ATP levels upon transient glucose deprivation. Influence of AOAA. Retinal cells were pre-incubated for 30 min and then exposed to 10 μM–10 mM glutamate (Glu) for 2 h in the absence (-glucose, dark circles) or in the presence of glucose (+ glucose, open circles) (A). Then, the cells were incubated in medium containing glucose for 3 h (post-incubation) (B). Some cells were also exposed to AOAA (2 mM) 5 min before and during glutamate exposure (2 h), and further incubated in the presence of glucose without glutamate (3 h post-incubation) (C). Intracellular ATP levels were measured by reverse phase HPLC after an acidic extraction. The results are the means ± SEM of duplicates or triplicates from five to seven experiments. Statistical significance: $^{**}P < 0.01$ or $^{***}P < 0.001$ as compared to the respective control; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ or $^{\dagger\dagger\dagger}P < 0.001$ as compared to data obtained in the presence of glucose; $^{\ddagger}P < 0.05$ as compared to data obtained upon incubation with 10 μM glutamate, in the absence of glucose.

transaminases in retinal cells exposed to high glutamate concentrations plus post-incubation.

Thus, we have also analysed the influence of AOAA on glutamate-mediated changes in MTT reduction (Fig. 2C) and ATP production (Fig. 5C) following the post-incubation period. MTT reduction was slightly decreased upon incubation with AOAA in control cells deprived of

glucose. In retinal cells treated with AOAA, MTT reduction was decreased upon incubation with 100 μM glutamate in the absence of glucose (about 1.6-fold). Although at a smaller extent, high glutamate still counteracted the decrease in MTT reduction observed in the presence of low glutamate (Fig. 2C, dark circles). In addition, incubation with low glutamate concentrations upon transient glucose deprivation largely decreased ATP levels in retinal cells incubated in the presence of AOAA (about 10 nmol ATP mg^{-1} protein), whereas high glutamate did not significantly reduced intracellular ATP (22.6 ± 6.8 nmol ATP mg^{-1} protein) (Fig. 5C, dark circles). These data show that intracellular ATP are not significantly affected by AOAA.

Because AOAA can be used as a tool to examine changes associated with glutamate transamination, as opposed to oxidation, data shown in Fig. 2C and Fig. 5C suggest that increased retinal function in cells exposed to high glutamate and upon incubation with glucose is not dependent on mitochondrial glutamate transamination.

3.6. Influence of transient glucose deprivation on the intracellular levels of pyruvate

Because changes in ATP production could result from altered glycolysis or mitochondrial function, in this study we have also measured the intracellular levels of pyruvate, a final glycolytical product. Table 3 shows that intracellular pyruvate levels are not significantly changed in retinal cells exposed to glutamate, in the presence or in the absence of glucose, followed by the post-incubation period. These data suggest that changes in ATP and MTT reduction observed in the presence of 10 mM glutamate, upon transient glucose deprivation, seem to be independent of an increase in glycolytic function. Our data suggest that high glutamate facilitates glucose-mediated recovery of retinal cell function and ATP production, possibly by being oxidised (oxidative deamination) at the level of the mitochondria.

Table 2
Effect of AOAA on intracellular glutamate levels after exposure to extracellular glutamate in the presence or in the absence of glucose [a]

Incubation (2 h plus 3 h post-incubation)	Intracellular glutamate levels (nmol mg^{-1} protein)	
	−AOAA	+AOAA
+glucose		
Control	70.1 ± 11.0	121.7 ± 26.7
100 μM Glu	280.0 ± 53.5	295.0 ± 64.5
10 mM Glu	1013.5 ± 136.2	1819.5 ± 413.0 [b]
−glucose		
Control	58.8 ± 11.9	103.6 ± 30.5
100 μM Glu	149.8 ± 28.5	196.3 ± 41.5
10 mM Glu	1013.9 ± 164.9	1716.7 ± 370.8 [b]

[a] Retinal cells were incubated with 100 μM or 10 mM glutamate (Glu) for 2 h in the absence (−glucose) or in the presence (+glucose) of glucose, after a 30 min pre-exposure to glucose-free or glucose-containing medium, respectively. Control cells were incubated in the absence of glutamate. AOAA (2 mM) was added 5 min before and during the incubation with glutamate. Then, the cells were washed and incubated in medium containing glucose, for 3 h (post-incubation). The extracellular medium was removed and 0.3 ml of 1 M NaOH added to the cells. After scraping, the cells were centrifuged to remove cell debris. The intracellular content in glutamate was determined by reverse-phase HPLC. Data are the means ± SEM of triplicates from five (−AOAA) or three (+AOAA) experiments.

[b] $P < 0.01$, as compared to data in the absence of AOAA.

Table 3
Intracellular levels of pyruvate upon glutamate exposure and post-incubation [a]

Incubation for 2 h	Pyruvate (nmol mg^{-1} protein)		
	Control	100 μM Glu	10 mM Glu
+glucose	11.75 ± 1.11	12.67 ± 1.29	9.25 ± 1.50
−glucose	13.17 ± 3.23	9.67 ± 1.92	8.49 ± 1.30

[a] The cells were exposed to glutamate (100 μM or 10 mM Glu) for 2 h in the absence or in the presence of glucose. Then, they were incubated in the presence of glucose, but in the absence of Glu, for 3 h (post-incubation). Control retinal cells were incubated in the absence of glutamate. The cells were extracted with 8% perchloric acid and centrifuged to remove cell debris. The resulting supernatant was used to determine the intracellular content in pyruvate, as described in Section 2. Data are the means ± SEM of triplicates from three independent experiments.

4. Discussion

In this study we show that glutamate regulates the viability of retinal cells after transient glucose deprivation. Under these conditions, low glutamate decreased intracellular levels of ATP and cell viability, closely associated with the activation of the NMDA receptors. In contrast, high glutamate was shown to increase cell viability and intracellular ATP, in a process that appears to be independent of increased glutamate transamination or glycolysis stimulation.

4.1. Decreased cell viability and ATP production induced by low glutamate concentrations upon transient glucose deprivation

After transient glucose deprivation, in retinal cells exposed to low glutamate (10–100 μM), MTT reduction decreased significantly, which paralleled the depletion in intracellular ATP, as a result of NMDA receptor activation, similarly to what was reported by Novelli, Reilly, Lysko and Henneberry (1988). Energy depletion occurs when glutamate receptors are overactivated and leads to a severe disturbance of neuronal ion

homeostasis, usually associated with necrotic cell death (Ankarcrona et al., 1995).

In previous studies, MTT reduction was shown to depend on the activity of functional mitochondria (Slater, Sawyer, & Sträuli, 1963; Mosmann, 1983; Keller et al., 1997). In isolated mitochondria, malate plus glutamate and succinate supported MTT reduction (Liu, Peterson, Kimura, & Schubert, 1997). Nevertheless, NADH or NADPH were shown to be better substrates than succinate in regulating MTT reduction (Berridge & Tan, 1993), supporting the observation that the mitochondria have a small influence in MTT reduction (Liu, 1999). Therefore, MTT reduction may involve other subcellular structures, namely endosomes or lysosomes, and more than one enzyme, although representing an early indicator of cell dysfunction (Liu et al., 1997; Patel, Gunasekera, Jen, & de Silva, 1996).

NMDA receptor-mediated increase in retinal excitotoxicity may result from a reduction in the Mg^{2+} blockade of the NMDA receptor channel due to the depolarization of retinal cells (Rego et al., 1998). Under this perspective, Zeevalk & Nicklas (1992) showed that glycolysis inhibition reduced the Mg^{2+} blockade of NMDA receptors, increasing the activation of NMDA receptors for lower agonist concentrations. Moreover, MK-801 was reported to reduce cortical neuronal injury after an extended period (6–8 h) of glucose deprivation (Monyer, Goldberg, & Choi, 1989).

In retinal cells, glutamate-induced neurotoxicity was also associated with the entry of Ca^{2+} through the receptor-associated channels (Ferreira et al., 1996). A decrease in cell viability and the impairment of energy metabolism in the presence of low glutamate concentrations in the retinal cells may be explained by the uncoupling of oxidative phosphorylation and a decrease in the activity of mitochondrial complexes after NMDA receptor activation (Atlante et al., 1996; Rego, Santos, & Oliveira, 2000). Furthermore, glutamate receptor activation has been also associated with the production of reactive oxygen species, like nitric oxide (Gunasekar et al., 1995) and the superoxide anion (Lafon-Cazal et al., 1993). Although our study was focused on determining the involvement of NMDA receptor-mediated changes, non-NMDA receptor activation may have also contributed to retinal cells toxicity. Activation of AMPA receptors by kainate was previously shown to decrease MTT reduction and enhance Ca^{2+} entry in chick amacrine-like cells (Ferreira, Duarte, & Carvalho, 1998).

4.2. High glutamate concentrations counteract the decrease in cell viability and ATP production upon transient glucose deprivation

In contrast with cells submitted to glucose deprivation only, in retinal cells submitted to the post-incubation period glutamate dose-dependently enhanced MTT reduction and intracellular ATP levels. The maintenance of intracellular ATP levels in the presence of high glutamate may have ensured the maintenance of retinal cells viability.

Mitochondria have been recognised to play a fundamental role in the metabolism of glutamate, either in glial cells or in the neurons. As the external glutamate increases, apart from the conversion to glutamine in glial cells, the metabolism of glutamate via the trichloroacetic acid cycle is enhanced (McKenna, Sonnewald, Huang, Stevenson, & Zielke, 1996b). In the retinal cells in culture, AOAA affected glutamate transamination specially at high glutamate concentrations, suggesting the involvement of active mitochondria. Nevertheless, changes in MTT reduction or intracellular ATP levels were not significantly affected by AOAA, and endogenous pyruvate levels were not significantly altered, implicating that high glutamate might have proceeded through mitochondrial oxidative deamination by glutamate dehydrogenase in order to maintain intracellular ATP production and cell viability. In the neuronal tissue, glutamate was previously shown to be preferentially oxidised rather than transaminated (Kauppinen & Nicholls, 1986). Furthermore, the possibility of mitochondrial glutamate usage as an energy substrate upon glucose deprivation may help to reduce the need for glycolysis and temporarily fulfil the energetic needs of retinal neurons.

Since major changes in the retinal cells were observed after transient glucose deprivation, upon the post-incubation period, the present results may be pertinent to evaluate the fate of neuronal cells during reperfusion after in vivo retinal ischemia, particularly near the transition from the ischemic core to the penumbra area, where cells inevitably die by necrosis and release large amounts of glutamate to the extracellular space. Although prone to injury due to the reduction in oxygen and glucose, and the release of excitatory amino acids, the neurons in the penumbra area remain viable for several hours. Thus, the maintenance of neuronal activity by glucose, together with an excess of glutamate may be crucial for determining the degree and progression of cell death. Although the glial cells have been reported to play an important role in the protection of neuronal cells (Zeevalk & Nicklas, 1997), further studies will be required to determine the metabolic pathways of glutamate in retinal cells submitted to partial inhibition of energy metabolism and to distinguish this process from a possible down regulation of NMDA receptor function. Because, in most studies, the retinal cells have shown to produce similar responses as compared to other neuronal culture models, our results may also be applicable to other neuronal cells.

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