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Distinct Glycolysis Inhibitors Determine Retinal Cell Sensitivity to Glutamate-Mediated Injury

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In this study, we analyzed how distinct glycolysis inhibitors influenced the redox status of retinal cells, used as a neuronal model. Three different approaches were used to inhibit glycolysis: the cells were submitted to iodoacetic acid (IAA), an inhibitor of glyceraldehyde 3-phosphate dehydrogenase, to 2-deoxy-glucose (DG) in glucose-free medium, which was used as a substitute of glucose, or in the absence of glucose. The redox status of the cells was evaluated by determining the reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). By the analysis of dose-response curves of MTT reduction, IAA showed values of $IC_{50} = 7.02 \times 10^{-5}$ M, whereas DG showed values of $IC_{50} = 7.42 \times 10^{-4}$ M. Upon 30 min-incubation, glucose deprivation, per se, did not significantly affect MTT reduction. We also evaluated the reduction of MTT as an indicator of cell injury by exposing the cells to 100 μM glutamate during the decrement of glycolysis function. In the presence of glutamate, for 2 h, there was a decrease in MTT reduction, which was potentiated in the presence of DG (10-20% decrease), in the presence of IAA (about 30% decrease) or in glucose-free medium (about 30% decrease). Major changes observed by the MTT assay, upon exposure to glutamate, indicative of changes in the redox status of retinal cells, were concomitant with variations in intracellular ATP. Under glucose deprivation, endogenous ATP decreased significantly from 38.9 ± 4.4 to 13.3 ± 0.7 nmol/mg protein after exposure to 100 μM glutamate. The results support a different vulnerability of retinal cells after being exposed to distinct forms of glycolysis inhibition.

KEY WORDS: ATP production; glutamate; glycolysis; MTT reduction; retinal cell injury.

INTRODUCTION

Retinal cells, similarly to neuronal cells, are highly dependent on the flux of oxygen and glucose (1) and they were described to contain large sources of endogenous glucose and glycogen (2). Several studies have demonstrated the importance of glycolysis in neuronal function: K^+ uptake, occurring through the Na^+/K^+ pump, was dependent on energy generated by glycolysis (3), stimulation of glycolysis was shown to occur during the initial phase of mitochondrial dysfunction (4) and glycolysis was suggested to prevent non-synaptic release of excitatory amino acids during hypoxia (5). In the retina, histological lesions induced by hypoglycemia-like conditions were shown to be related to glutamate-induced excitotoxicity (6). Previously, we found that hypoglycemia-like conditions, induced in the presence of IAA (iodoacetic acid) or DG (2-deoxy-glucose), significantly decreased the levels of ATP in retina co-cultures, used

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Abbreviations: BME, basal medium of Eagle; DG, 2-deoxy-glucose; IAA, iodoacetic acid; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

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as neuronal models, although the ATP levels after chemically-induced hypoxia did not change significantly (7). A decrement in the energy levels of the cells, even if small, may account for the disturbance of intracellular calcium concentration (8), which may evoke the release of neurotransmitters. An increase in the release of excitatory amino acids has been considered responsible for the excitability of post-synaptic neurons, the accumulation of free fatty acids and the production of free radicals, contributing to neuronal degeneration. Glutamate excitotoxicity was shown to be involved in the pathogenesis of neuronal loss associated with neurological diseases and during ischemia situations (9).

In this study, we analyzed the influence of inhibiting glycolysis on the redox status of retinal cells, used as neuronal models. During glycolysis inhibition, the cells were submitted to IAA, DG or glucose deprivation. The vulnerability of retinal cells, submitted to glutamate exposure under situations that reduced glycolysis function and then returned to glucose medium, was also evaluated. The redox status of the cells was evaluated by the measurement of two parameters, the reduction of MTT (10-14) and the production of ATP. The data obtained may help to determine the threshold of glycolysis inhibition below which cell damage inevitably occurs. Metabolic inhibition was shown to decrease the efficacy of retinal cells to reduce MTT, rendering the cells vulnerable during glycolysis dysfunction, which mediates the potentiating effects of glutamate excitotoxicity. These data may contribute to clarify how cells respond to different energy requirements and the extent of neuronal damage resulting from different levels of hypoglycemia, in vivo.

EXPERIMENTAL PROCEDURE

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). All other chemicals used were purchased from Sigma (USA).

Preparation of Retinal Cell Cultures. Primary cultures of retinal cells were obtained from 8-day-old chick (White Leghorn) embryos, as described previously (8, 15). Briefly, the retinas were dissected free from other ocular tissues and dissociated with trypsin in a Ca^{2+}/Mg^{2} - free Hank's balanced salt solution for 15 min, at 37 °C. The digested tissue was centrifuged and the pellet was resuspended in BME containing Earle's salts and L-glutamine, 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). After appropriate dilution, the cells were plated on Costar 12-multiwell plates pre-coated with poly-L-lysine (0.1 mg/ml) at a density of 0.4×10^6 cell/cm^2. The cells were cultured at 37 °C in an atmosphere of 95% air and 5% CO_2. Similar cultures of retinal cells were previously described to be highly enriched in amacrine neuron-like cells (16, 17). These cultures also contain neurons resembling bipolar cells and few glial cells (5).

Induction of Glycolysis Inhibition and Analysis of MTT Reduction. Five days after plating, the retinal cells were incubated with inhibitors of glycolysis to induce conditions of chemical hypoglycemia. The culture medium was removed and the cells were pre-incubated, for 10 min, in a sodium 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. The inhibition of glycolysis function was induced with IAA, which inhibits the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase, with DG in glucose-free medium, which substitutes glucose in the incubation medium and is not metabolized by the cells, or simply in the absence of glucose. Incubations were carried out at 37 °C in the presence (5.6 mM) or in the absence of glucose, for the periods of time indicated in the Figure legends. Control cells were incubated in the absence of chemical inducers, in the presence of glucose. When indicated, the cells were exposed to glutamate (100 µM), in the presence or in the absence of glucose, for 2 h. Retinal cells were also submitted to glutamate after 30 min-incubation in the presence of specific glycolytic inhibitors.

The efficacy of retinal cells to reduce MTT into formazan, an insoluble product, was performed by following the method described by Mosmann (10). Briefly, after incubation of retinal cells, the medium was removed and MTT (final concentration 0.5 mg/ml in sodium saline solution containing glucose, prepared just before using and maintained in the dark) was added to the cells. Incubation with MTT was performed in sodium saline solution. After incubation for 3 h, at 37 °C, to obtain a maximal formation of formazan cristals, an equal volume of acid-isopropanol (0.04 M HCl in isopropanol) was added and mixed thoroughly at room temperature until all formazan crystals were dissolved. The absorbance of the samples was measured at 570 nm, against a blank containing the solution of MTT and acid-isopropanol. The efficacy of retinal cells to reduce the MTT was expressed as a percentage of OD of control cells, in the absence of glycolytic inhibitors or glutamate, because the number of cells was not significantly changed after each treatment.

Intracellular ATP Measurements. Retinal cells were extracted with 0.3 M perchloric acid after the incubation for 2 h in the absence or in the presence of glutamate (100 µM) in cells pre-incubated (30 min) with glucose medium in the absence or in the presence of IAA (25 µM) or without glucose. The acidic extract was centrifuged at 15800 xg, for 5 min, at 0–4 °C. The pellets were analyzed for total protein (18), whereas the supernatants were neutralized with 10 M KOH in 5 M Tris. After neutralization the samples were centrifuged at 15800 xg, for 10 min, at 0–4 °C, and stored at −80 °C until HPLC injection. The supernatants were assayed for ATP, by separation in a reversed phase HPLC (19), eluted at a flow rate of 1.2 ml/min with 100 mM KH_2PO_4, pH 7.4, and 1% methanol. The nucleotides were detected at 254 nm, for 6 min.

Data Analysis. Throughout the text, data are expressed as means ± SEM of the indicated number of experiments. The IC_{50} values for IAA and DG were determined by using the sigmoidal equation (variable slope) of Graph Pad Prism. Statistical significance was determined by using the unpaired two-tailed Student's t-test and by using the one-way ANOVA Tukey post-test for multiple comparisons. A P value < 0.05 was considered significant.

RESULTS

Time-Course of MTT Reduction Upon Recovering from Glycolysis Inhibition. Fig.1 shows the time-depen-

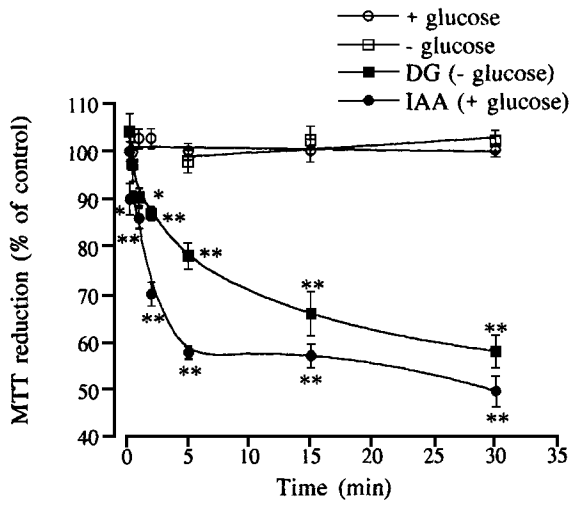

Fig. 1. Time-dependent changes in MTT reduction induced by inhibitors of glycolysis function.
Retinal cells were exposed to 0.5 mM IAA in glucose medium, to 2 mM DG in the absence of glucose, to glucose-free medium, or to medium containing glucose (5.6 mM), for 0.25, 0.5, 1, 2, 5, 15 or 30 min, at 37 °C. The MTT colorimetric assay was performed as described under Experimental procedure. Data are the means ± SEM of two to four experiments, each run in triplicates. Statistical significance: *P < 0.01 or **P < 0.001 as compared to controls in the presence of glucose, but in the absence of glycolytic inhibitors.

dent changes of MTT reduction in retinal cells exposed to inducers of glycolysis inhibition, for 15 sec and up to 30 min. In the absence of glucose, there were not significant changes in MTT reduction as compared to control conditions, in the presence of glucose. Nevertheless, in the presence of IAA (0.5 mM) or DG (2 mM) there was a significant decrease in MTT reduction after 15 sec ($P < 0.05$) or after 1 min ($P < 0.01$), respectively. MTT reduction in the presence of IAA or DG was similar after 15 or 30 min (Fig.1). We also analyzed the influence of glycolytic inhibitors on the release of lactate dehydrogenase (LDH), after incubation for 5, 15 or 30 min, although we could not find significant differences between each cell treatment and the controls, in cells incubated in the presence of glucose (not shown).

Dose-Response Curves of MTT Reduction Induced by Iodoacetic Acid or Deoxyglucose. We next studied the dose-response curves of IAA (Fig.2-A), or DG (Fig.2-B), incubated for 30 min, in the efficacy of retinal cells to reduce MTT. The concentrations of IAA ranged from 25×10^{-6} to 2×10^{-3} M, whereas the concentrations of DG ranged from 100×10^{-6} to 10×10^{-3} M. The efficiency to reduce MTT was evaluated by determining the IC_{50} values, the concentration of IAA or DG providing 50% loss of MTT reducing ability by retinal cells in culture. In the presence of IAA, the IC_{50} was

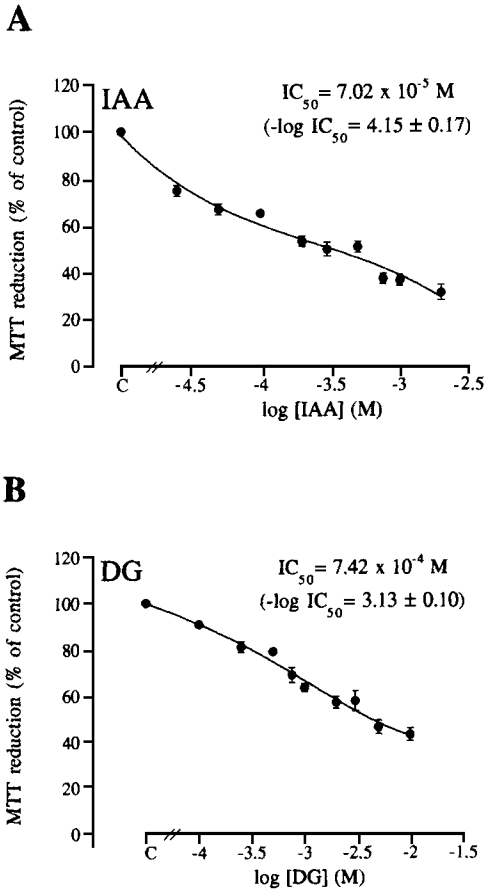

Fig. 2. Dose-response curves of IAA and DG on the reduction of MTT in retinal cells.
The cells were exposed to IAA (25×10^{-6} M to 2×10^{-3} M) in the presence of glucose (A) or to DG (100×10^{-6} M to 10×10^{-3} M) in the absence of glucose (B), for 30 min, at 37 °C. MTT reduction was determined as described under Experimental procedure. Data are the means ± SEM of two to four experiments, each run in triplicates. The graph curves were plotted following the equations:
$y = -360.14 - 303.32x - 78.51x^2 - 7.24x^3$ ($r = 0.99$) for the dose-response curve of IAA (A), or $y = 173.53 + 198.51x + 98.45x^2 + 18.40x^3 + 1.23x^4$ ($r = 0.99$) for the dose-response curve of DG (B). The IC_{50} values were calculated by using the sigmoidal equation (variable slope) of the Graph Pad Prism. Data obtained in the presence of IAA or DG showed statistical significance ($P < 0.001$) as compared to the controls (C), in the presence of glucose.

7.02×10^{-5} M (-log IC_{50} = 4.15 ± 0.17), whereas in the presence of DG, the IC_{50} was 7.42×10^{-4} M (-log IC_{50} = 3.13 ± 0.10). These data indicated that specific inhibition of glycolysis in retinal cells in culture, in the presence of IAA or DG, highly decreased the retinal cells efficiency to reduce MTT.

Susceptibility of Retinal Cells to Glutamate Upon Glycolysis Inhibition. In order to investigate the importance of distinct glycolytic inhibitors in the susceptibility of retinal cells to glutamate, we analyzed their efficacy

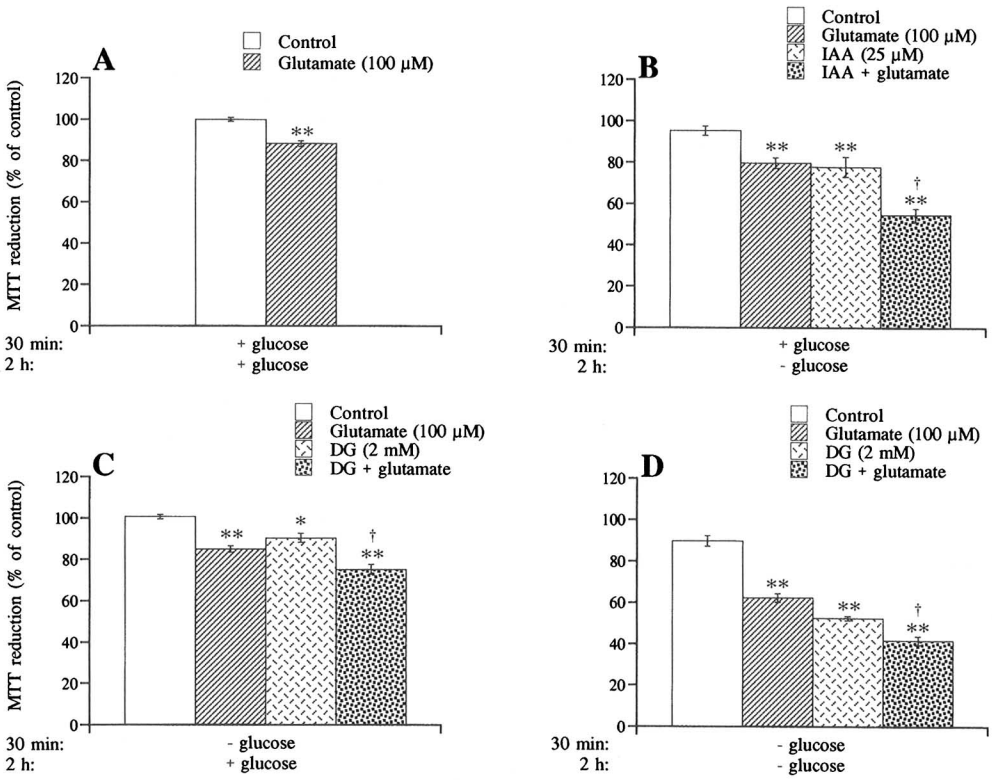

Fig. 3. Influence of inhibition of glycolysis and glutamate on the reduction of MTT by retinal cells. Retinal cells were pre-incubated for 30 min in the presence (A, B) or in the absence of glucose (C, D). Inhibition of glycolysis was also induced in the presence of IAA (25 μM), as shown in B, or with DG (2 mM), as shown in C or D, prior to glutamate (Glu) incubation. The cells were further exposed to 100 μM glutamate, in the presence (A, C) or in the absence of glucose (B, D), but in the absence of IAA or DG, for 2 h. Then, they were incubated with MTT (0.5 mg/ml) in Na^+ medium containing glucose, for 3 h, as described under Experimental procedure. Data, expressed as a percentage of MTT reduction in control retinal cells incubated in the presence of glucose for 30 min plus 2 h (A), are the means ± SEM of duplicates or triplicates from two to eight experiments. Statistical significance: *$P < 0.01$ or **$P < 0.001$ as compared to the respective control; †$P < 0.01$ as compared to similar conditions, in the absence of glutamate.

to reduce MTT after being exposed to IAA, DG or glucose-free medium, for 30 min. This period of time was used as a pre-incubation period sufficient to induce almost maximal effects (Fig.1), which was followed by 2 h exposure to 100 μM glutamate, in the presence or in the absence of glucose. In these experiments, IAA was used at a concentration closer to the IC_{50} value (about 70 μM) determined in Fig. 2, so that glutamate toxicity could be detected. The inhibition of MTT reduction observed in the presence of 0.5 mM IAA, after 30 min-incubation, was of about 60%, partly masking further changes observed upon incubation with glutamate (not shown). Fig. 3-A shows that glutamate alone in the presence of glucose decreases slightly, although significantly, the reduction of MTT (88.3 ± 1.4%, $P < 0.001$), as compared to the control, in the absence of glutamate. In the presence of glutamate, in cells submitted to glucose deprivation for 2 h, there was a decrease in MTT reduction to 79.9 ± 2.6% of control (Fig.3-B). MTT data obtained in the presence of IAA (25 μM) alone, followed by incubation in the absence of glucose, with no IAA added, was of 78.1 ± 4.8% of control. Nevertheless, we observed a further decrement in MTT reduction when the last two treatments were applied. Treatment of retinal cells with IAA, followed by incubation with glutamate in the absence of glucose, presented values of MTT reduction of 54.7 ± 3.3% ($P < 0.01$, as compared to incubation with IAA in the absence of glutamate or glutamate exposure without IAA pretreatment), indicating potentiating effects induced by glutamate.

In Fig. 3-C we observed a significant, but smaller, decrement in MTT reduction upon treatment with DG (2 mM) in the absence of glucose, for 30 min, followed

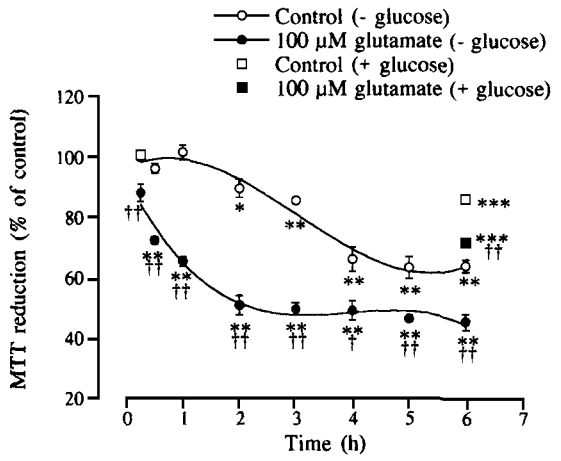

Fig. 4. Time-dependent changes in MTT reduction induced by glutamate under glucose deprivation.
Retinal cells were pre-incubated for 30 min in the absence of glucose, similarly as performed in Fig. 3-D. Then, the cells were incubated in the absence (white circles) or in the presence (black circles) of 100 μM glutamate in glucose-free medium, for 15 min and up to 6 h. The cells were also incubated with glucose for 30 min plus 15 min or 6 h, in the absence (white squares) or in the presence (black squares) of 100 μM glutamate. The results, calculated as a percentage of data observed in the presence of glucose for 30 plus 15 min (controls, not significantly different from data observed in the absence of glucose), are the means ± SEM of triplicates from three to four experiments. Statistical significance: †$P < 0.01$, ‡$P < 0.001$ as compared to data in the absence of glutamate; *$P < 0.05$, **$P < 0.01$ or ***$P < 0.001$ as compared to data obtained after 15 min, under similar experimental conditions.

by incubation in medium containing glucose (90.5 ± 2.2%, $P < 0.01$). Retinal cells incubated in the presence of DG and exposed to glutamate in the presence of glucose also showed a higher decrement in MTT reduction (75.3 ± 2.4%, $P < 0.01$ as compared to incubation with DG, in the absence of glutamate). The effect of glutamate in decreasing the reduction of MTT in the presence of glycolytic inhibitors was of about 30% in the presence of IAA and about 10-20% in the presence of DG, a large effect as compared to the effect mediated by these inhibitors when incubated separately.

We also tested the effect of incubating the retinal cells in the absence of glucose on MTT reduction (Fig. 3-D). The incubation with glutamate (100 μM) in the absence of glucose (2 h), in cells pre-exposed to glucose-free medium, highly decreased the reduction of MTT to 62.4 ± 2.1% ($P < 0.001$ as compared to the respective control). This inhibition was greater than that observed when glutamate was incubated in the absence of glucose, in cells pre-incubated in the presence of glucose. The effect of DG was also examined by incubating the cells in glucose-free conditions for 2 h. Under these conditions, MTT reduction was also greatly decreased (52.6 ± 1.1%) and 100 μM glutamate potentiated that decrease (41.9 ± 2.0%), as shown in Fig. 3-D. Comparison of data shown in Fig. 3-C and D, in cells incubated with DG, indicated that MTT reduction is favored by exposure of retinal cells to glucose-containing medium.

We next examined the time-dependent changes in MTT reduction elicited by 100 μM glutamate (Fig.4), in cells deprived of glucose during all the experimental procedure and exposed to glutamate, because a large decrease in MTT reduction was observed under these conditions (Fig. 3-D, second bar). Figure 4 shows that glutamate potentiated the increase in cell injury, as determined by the reduction of MTT after incubation with glutamate (100 μM) for 15 min (88.1 ± 2.9%, $P < 0.001$ as compared to the respective control.) Maximal effects were observed after exposure of the cells to glutamate for 2 h (50.7 ± 3.3% versus 89.5 ± 3.0% observed in the absence of glutamate), At 4 h and up to 6 h, glutamate-mediated cell injury, evaluated by the difference between treatment in the presence and in the absence of glutamate, was decreased, because cells submitted to glucose deprivation showed already a high susceptibility, with values reaching 63.8 ± 2.0% after 6 h in the absence of glutamate (Fig.4). After 6 h in the presence of glucose, glutamate potentiated cell injury at a similar extent, when compared to conditions in the absence of glucose (Fig.4).

Changes in Intracellular ATP Levels Induced by Glycolytic Inhibitors and Glutamate. The analysis of the susceptibility of retinal cells to glycolytic inhibitors and glutamate was also performed by measuring the levels of intracellular ATP, in order to evaluate a relationship between loss of MTT reduction and altered metabolic function. Incubation of retinal cells with glutamate (100 μM) in the presence of glucose did not affect significantly the levels of ATP, as shown in Table I. Nevertheless, incubation with glutamate in the absence of glucose, in cells pre-incubated in glucose medium (Table I-A), significantly decreased ATP production, from 55.5 ± 3.5 nmol/mg protein in the absence to 33.0 ± 2.4 nmol/mg protein ($P < 0.001$) in the presence of glutamate. Pre-incubation with IAA in glucose medium (Table I-B) did not significantly influence the ATP levels in the absence of glutamate exposure. Glutamate exposure in glucose-free medium, after a pre-incubation with IAA, significantly decreased the ATP levels to 26.9 ± 3.1 nmol/mg protein ($P < 0.001$). Moreover, the levels of intracellular ATP in retinal cells incubated in the absence of glucose and glutamate (Table I-C) were significantly changed ($P < 0.05$) as compared to pre-incubation with glucose. Upon 100 μM glutamate

Table I. Effect of Reduced Glucose Metabolism on the Intracellular Levels of ATP in Retinal Cells Exposed to Glutamate

	ATP (nmol/mg protein)			
	Post-incubation			
	+ glucose		− glucose	
Pre-incubation	− glutamate	+ glutamate	− glutamate	+ glutamate
A) + glucose	52.6 ± 4.7	57.9 ± 3.6	55.5 ± 3.5	33.0 ± 2.4[a]
B) + IAA	n.d.	n.d.	57.2 ± 1.6	26.9 ± 3.1[a]
C) − glucose	n.d.	n.d.	38.9 ± 4.4[b]	13.3 ± 0.7[a]

Retinal cells were pre-incubated for 30 min in the presence (A) or in the absence of glucose (C), or in the presence of 25 μM IAA in glucose medium (B). Then, the cells were exposed to 100 μM glutamate (+glutamate) for 2 h (post-incubation) in the absence or in the presence of glucose. Intracellular ATP (in nmol/mg protein) was analyzed by reversed phase HPLC, after perchloric acid extraction and neutralization with KOH/Tris. Data are the means ± SEM of five to seven separate experiments. Statistical significance (ANOVA test for multiple comparisons): [a]$P < 0.001$ as compared to similar experimental conditions in the absence of glutamate (− glutamate); [b]$P < 0.05$ as compared to pre-incubation with glucose (A), in the absence of glutamate; n.d., not determined.

exposure under glucose deprivation, ATP decreased significantly to 13.31 ± 0.7 nmol/mg protein ($P < 0.001$, Table I-C). These results indicated that glutamate potentiated the decrement of ATP caused by glucose deprivation. The decrease in ATP levels induced by glutamate in cells completely deprived of glucose was also observed in the presence of 10 μM glutamate (not shown).

DISCUSSION

In the present study, we showed that retinal cells in culture are differently vulnerable to glycolysis inhibition. IAA or DG caused a dose-dependent decrease in MTT reduction, showing $IC_{50} = 7.02 \times 10^{-5}$ M or $IC_{50} = 7.42 \times 10^{-4}$, respectively. These data indicated that, a specific inhibition of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase by IAA, or the substitution of DG for glucose inhibited glycolysis and highly decreased the efficiency of retinal cells to reduce MTT (Fig. 1 and 2). Exposure to glutamate (100 μM) during glycolysis inhibition decreased the cellular redox status, as observed by a decrement in MTT reduction and by a decrease in endogenous ATP.

Although MTT reduction has been argued to be carried out by active mitochondria (10-14, 20) we show that reduction of MTT requires the maintenance of glycolysis function, because glycolytic inhibitors, IAA or DG, induced a substantial time-dependent and dose-dependent decrease in MTT reduction (Fig. 1 and 2). A prolonged exposure to glucose deprivation, for 4 h or more (Fig. 4), also decreased the reduction of MTT. These results suggest the need of reducing agents, such as NADH, generated through glycolysis at the level of glyceraldehyde 3-phosphate dehydrogenase, to obtain a maximal reduction of MTT. We also show that distinct forms of glycolysis inhibition affected MTT reduction or the cellular redox status, even when the cells are re-incubated in medium containing glucose. NADH- or NADPH-dependent redox systems were shown to be the principal reductants for MTT, reflecting the redox status of the cells (21, 22). Data in this study suggested that MTT reduction may also represent an early indicator of cell damage, because the decrease in metabolic function, in the presence of glycolytic inhibitors, resulted in a significant decrease in the redox efficacy of cultured retinal cells. Exposure to 100 μM glutamate, for 2 h, under glucose deprivation, increased significantly ($P < 0.05$) the release of LDH (about 13% of total) after incubation in medium containing glucose, as compared to cells incubated in the absence of glutamate. Patel at al. (23) demonstrated that the MTT test fails to give adequate indication of astroglial cell damage induced by the amyloid β-protein as compared with LDH release and trypan blue exclusion methods. The indication of early metabolic failure by the MTT test, rather than overall cell damage, seems to be a good starting point to be examined before irreversible cell damage occurs. Although Liu et al (24) reported that MTT was not permeable to lipid membranes, and MTT reduction was primarily confined to endosomes/liposomes in intact B12 cells, we can not exclude the possibility that different cell types offer distinct membrane permeabilities to MTT and that at least some of the MTT is reduced at the level of the mitochondria. Although the extra-mitochondrial reduction of MTT is highly increased in rat hepatocytes, mitochondrial dehydrogenase activity (20, 22) or cellular generation of superoxide anions may constitute other (minor) sources of MTT reduction (21, 22, 25) Liu et al (24) also showed that in isolated mitochondria, malate plus glutamate or succinate supported MTT reduction. Early (10, 11) and more recent (2-14, 24) studies indicated that MTT is (partly) reduced by active mitochondria, although it can not be dissociated from reduction by non-mitochondrial enzymes (25, 26).

By exposing the retinal cells with glutamate, we were able to determine the influence of metabolic failure of cells in the presence of glycolytic inhibitors, in the extent of cell injury, as evaluated by the cellular redox status and energy levels. Glutamate (100 μM) was shown to potentiate cell injury in the presence of glycolytic inhibitors, as evaluated by a significant loss in

MTT reduction observed in the presence IAA, DG or under glucose deprivation (Fig.3). Major increments in cell injury induced by 100 μM glutamate were observed after pre-incubation with IAA (Fig.3-B) or under glucose-free conditions (Fig. 3-D). A significant decrement in MTT reduction, elicited in the presence of glycolytic inhibitors (Fig. 3), was followed by a significant decrement in intracellular ATP, by 1.6-fold (Table I), suggesting that at least part of the effects observed were due to glycolysis metabolic dysfunction.

Previously, glycolysis inhibition was shown to reduce the ATP levels in rat striatal slices (27) or in retinal co-cultures from chick embryos (7). Cell toxicity associated with hypoglycemia situations has also been refered to be associated with the alteration of Ca^{2+} homeostasis (8, 28, 29), proposed to initiate cell damage. The maintenance of glycolysis has been considered important in partly (8) or totally (5) preventing the non-synaptic release of excitatory amino acids, which are capable of stimulating NMDA and non-NMDA glutamate receptors, causing delayed neurodegeneration in the retina (30). Glycolysis inhibition with IAA was previously shown to produce serious histological lesions in retina from chick embryos. These lesions could be reverted upon application of selective antagonists of glutamate receptors, suggesting the involvement of glutamate receptors on the retina damage caused by metabolic stress. Previously, IAA was demonstrated to cause the inhibition of sodium-dependent glutamate transporter (31). The inhibition of the glutamate transporter was shown to increase the neurotoxicity of glutamate by an enhanced accumulation of extracellular glutamate (31-35). Glucose deprivation was also shown to enhance the release of arachidonic acid (20:4) evoked by glutamate (34, 35) which could contribute to an increased inhibition of the glutamate transporter.

In this work, we showed that retinal cells are differently susceptible to the various inducers of glycolysis inhibition. Furthermore, glutamate potentiated the increase in cell damage through the decrease in intracellular ATP. Because the redox status of retinal cells, recovering from glucose deprivation, was not significantly changed up to 2 h in the absence of glucose (Fig.4) data highly suggest a short "therapeutic window" for complete recover of neuronal cells in the penumbra area after an ischemic insult.

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