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Role of glutathione metabolism in the glutamate-induced programmed cell death of neuronal-like PC12 cells

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Abstract

In addition to its well-known interaction with ionotropic and metabotropic receptors, glutamate may, at high concentrations, interfere with a cystine-glutamate antiport designated as X_c^- and lead to a significant decrease in cystine uptake and intracellular glutathione level. These effects, in turn, may induce death in various cellular bodies including astrocytes, rat glioma cells and cortical neurons in culture. In the present paper we demonstrate that the toxicity evoked by glutamate in a neuronal-like model is indeed related to the metabolism of glutathione since glutamate toxicity is preceded by a significant depletion of intracellular glutathione and is abolished in the presence of precursors of glutathione synthesis such as cystine and *N*-acetylcysteine. It also appears that prolonged incubation in cystine-free medium leads to cell detachment and death, a phenomenon which is progressively abolished in the presence of increasing concentrations of cystine. In addition, buthionine sulfoximine, a known inhibitor of glutathione synthesis, also induces cell lysis with a time-course very similar to that of glutamate. However, depletion of glutathione is probably not sufficient to trigger the death signal since cycloheximide, which inhibits the toxic effect of both glutamate and buthionine sulfoximine, does not block the decrease in cellular glutathione content induced by these drugs. Our results therefore confirm that oxidative stress and intracellular glutathione depletion are able to trigger programmed cell death in neuronal-like cells, although the exact nature of the death mechanisms remains largely unknown.

Keywords: Glutamate; Oxidative stress; Glutathione depletion; Programmed cell death

1. Introduction

Exposure of primary cultures of fetal neurons to glutamate and glutamate analogs (10–100 μM) leads to a rapid cytotoxic action which involves the binding of the excitotoxin to both NMDA and non-NMDA receptors (Beal, 1992; Choï et al., 1987; Garthwaite and Garthwaite, 1991; Goldberg et al., 1987). The complete mechanisms leading to cell degeneration are not fully elucidated but appear to imply the opening of receptor-coupled channels and a transient accumulation of cytosolic free calcium (MacDermott et al., 1986; Schurr and Rigor, 1993; Siesjö, 1990). In addition to these well-characterized receptor-mediated effects, glutamate appears to exert a distinct cytotoxic action at very high concentrations (5–10 mM). This glutamate-induced cytotoxicity is not related to classical glutamate receptors but is likely mediated through an inhibition of cystine uptake leading to a depletion of cellular glutathione synthesis and level (Murphy et al., 1990; Schubert et al., 1992). Several authors have described the existence of a Na^+-independent cystine/glutamate antiport designated as X_c^- (Bannaï, 1986; Kato et al., 1993; Lerner, 1987). It has been suggested that a high concentration of extracellular glutamate may inhibit this carrier and therefore block the uptake of cystine which is necessary for glutathione biosynthesis (Murphy et al., 1989). Given the importance played by glutathione in the defence against oxidative stress (Di Monte et al., 1992; Makar et al., 1994; Pellmar et al., 1992; Yu, 1994), such a prolonged depletion of intracellular glutathione may, under certain circumstances, lead to cell degeneration (Coyle and Puttfarcken, 1993; Ratan et al., 1994a). This mode of glutamate-induced toxicity has been previously described in several neuronal but also non-neuronal cell types (Cho and Bannaï, 1990; Davis and Maher, 1994; Piani and Fontana, 1994). More recently, evidence has been provided to indicate that this glutamate-induced toxicity is blocked by inhibitors of

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macromolecular synthesis (Ratan et al., 1994a; Serghini et al., 1994) and may thus represent a model of apoptosis or programmed cell death. This assumption has been questioned recently by Ratan et al. (1994b) who described in cultures of embryonic rat cortical neurons that cycloheximide, by blocking protein synthesis, could spare the intracellular pool of cysteine and thus allow a sufficient synthesis of glutathione to prevent oxidative stress. We have previously described, in a clonal pheochromocytoma line (PC12), a mode of cell death induced by very high concentrations of glutamate and quisqualate, and inhibitable by actinomycin D and cycloheximide (Froissard and Duval, 1994; Serghini et al., 1994). In the present work we provide additional evidence to suggest that this toxic effect of glutamate is indeed a programmed event linked to the intracellular metabolism of glutathione.

2. Materials and methods

PC12 cells were grown routinely in Dulbecco's minimal essential medium supplemented with 10% heat-inactivated fetal calf serum, 5% inactivated horse serum (Biological Industries, Israel), 1% of a solution containing 10 000 U/ml penicillin G, 10 mg/ml streptomycin and 25 μg/ml amphotericin B in 0.9% NaCl (Sigma) and 2 mM L-glutamine (Sigma). The cells were subcultured twice a week by gentle scraping at a concentration of 8–10 000 cells/cm^2 in 6-well plates (2 ml/well) or in 25-cm^2 culture flasks (5 ml/flask). The effects of the various drugs tested were generally assessed 48 h after seeding.

2.1. Determination of lactate dehydrogenase activity

Glutamate toxicity was determined using the trypan blue exclusion procedure or by measuring the release of lactate dehydrogenase (LDH) in the culture medium.

LDH activity was determined spectrophotometrically at 340 nm according to the method described by Wroblewski and Ladue (1955). Values are expressed as percentage of dead cells, dividing the activity recovered in the medium by the sum of the released activity and that remaining in the cells, measured after 0.1% Triton X-100 lysis.

2.2. Determination of cellular glutathione content

The amount of intracellular glutathione was measured using the method of Tietze (1969), modified slightly as described below. At the end of the incubation in the presence of glutamate or of the test compound, the culture medium was carefully removed for LDH determination. Each well then received 400 μl of ice-cold 10% trichloroacetic acid. After 10 min of vigorous shaking, the acid-soluble fractions were collected and centrifuged at 21 000 $\times g$ for 10 min at 4°C.

The supernatants were then brought to neutral pH using 3 M NaOH. 50 μl aliquots of the supernatants were then mixed with 250 μl of 5,5′-dithionitrobenzoic acid (0.96 mg/ml in 0.1 M phosphate buffer pH 7.4, 5 containing 5 mM EDTA). After 20 min incubation at room temperature, the concentration of glutathione was determined by following the change in absorbance at 412 nm induced by the addition of NADPH (250 μl, 0.59 mg/ml) and glutathione reductase (450 μl, 5 U/ml). Values were expressed as ng of glutathione/mg cellular protein.

After digestion of the cell pellets by 0.1 M NaOH, protein concentrations were determined according to Lowry et al. (1951) using bovine serum albumin as a standard.

2.3. Morphological studies

After 16 h treatment by glutamate (10 mM) or buthionine sulfoximine (BSO, 30 μM) the cells were detached by gentle scraping and centrifuged at 800 rpm during 5 min in a cytofuge. The cells attached to the glass slide were then fixed with a mixture of chloroform-methanol-acetic acid and stained for 30 min at room temperature with the DNA-specific dye 4,6-diamino 2-phenylindone (final concentration 1 μg/ml).

All the compounds used in these experiments, actinomycin D, cycloheximide, glutamate, buthionine sulfoximine, EDTA, NADPH, dithionitrobenzoic acid, glutathione reductase, bovine serum albumin, 4,6-diamino 2-phenylindone (DAPI) were purchased from Sigma (St. Louis, MO, USA) and were of analytical grade. Cystine-free medium was obtained from Life Technologies (Pontoise, France). Statistical analyses were carried out using the Student's *t*-test.

3. Results

As described previously (Froissard and Duval, 1994), treatment of PC12 cells by 10 mM glutamate leads to a pronounced cell death (≅ 70%) which is apparent within 10–12 h of glutamate treatment and maximum at 24 h. When measuring in parallel the intracellular concentration of glutathione, we observed that glutamate induces within 5–6 h of treatment (i.e., before any significant increase in LDH release or detachment of the cells from the bottom of the culture flasks) a striking decrease in intracellular glutathione (from 2.4 ± 0.50 ng/mg protein in the absence of glutamate ($n = 10$), to 0.39 ± 0.09 ng/mg protein 6 h after treatment by 10 mM glutamate ($n = 7$, $P < 0.01$ vs. control)).

The effect of glutamate on cellular glutathione content is very rapid since it is obvious within 1–2 h of treatment (not shown). Quisqualate, which is highly cytotoxic at concentrations above 1–3 mM (Froissard and Duval, 1994), similarly induces a marked decrease in cellular glutathione whereas in contrast homocysteate (1 mM), which has been

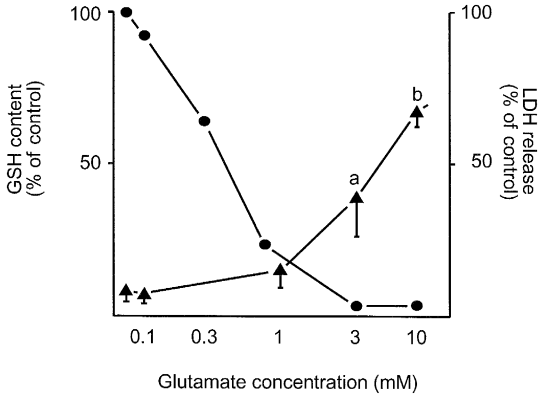

Fig. 1. Effect of increasing concentrations of glutamate on cellular glutathione content (●, measured 6 h after glutamate addition) and cell viability (▲, measured 24 h after glutamate treatment). Each value represents the mean (±S.D.) of duplicate determinations in 7–10 different experiments. [a] $P < 0.05$ vs. control; [b] $P < 0.01$ vs. control. Results of a typical experiment. Similar dose–response curves were obtained in three different experiments.

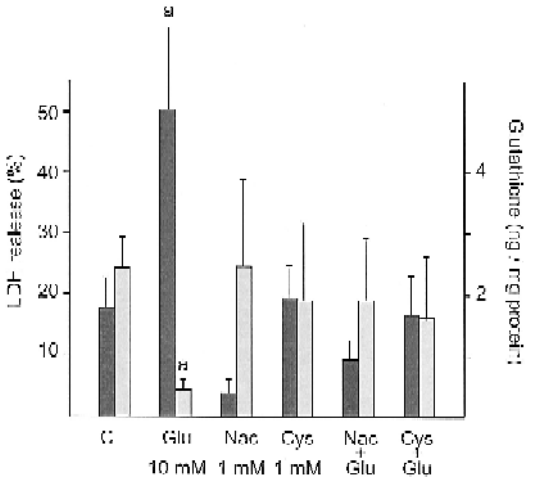

Fig. 2. Effect of precursors of glutathione biosynthesis on cell viability and cellular glutathione content in control and glutamate-treated cells. Glutathione (□) was measured 6 h following glutamate addition and cell viability was assessed by measuring LDH release (■) in parallel samples, 24 h after glutamate treatment. Each value represents the mean (±S.D.) of 4–10 values obtained in different experiments. [a] $P < 0.05$ as compared to the corresponding control. NaC: *N*-acetylcysteine, Cys: cystine, Glu: glutamate.

described to block the X_c^- antiport and to deplete cellular glutathione, fails in our model to induce glutathione decrease or toxicity (data not shown).

We have measured the effect of increasing concentrations of glutamate (0.1–10 mM) on both cellular glutathione (determined 6 h after glutamate addition) and cell viability (measured 24 h after treatment). As shown in Fig. 1, increasing concentrations of glutamate lead to a progressive decrease in cellular glutathione content followed by a significant lysis. It should be noted that the time-course of this drop in cellular glutathione content varies from one experiment to another with the lowest concentration being reached after 5–8 h of glutamate treatment.

In order to test the putative relationship between glutamate-induced cell death and decrease in cellular glutathione content, we have determined in parallel the effect of known precursors of glutathione metabolism on both parameters. As shown in Fig. 2, *N*-acetylcysteine (1 mM) and L-cystine (1 mM) do not exert any toxic effect (in the case of *N*-acetylcysteine, we even observed a decrease in the percentage of spontaneous cell death), and do not alter significantly the level of intracellular glutathione. However, both compounds were able to completely block the glutamate-induced decrease in cellular glutathione content and the glutamate toxicity.

We have previously described that the minimal period of treatment by glutamate required to set in motion the lytic mechanism is 6 h. Indeed, washing of the glutamate after 2 or 4 h of treatment fails to trigger any significant mortality above control, when measured 24 h later; whereas medium renewal after 6 h of glutamate treatment induces nevertheless a significant loss of viability, indicating that this lag phase is critical (Froissard and Duval, 1994).

We have thus determined the effect of cystine addition (1 mM) on glutamate-induced toxicity, when cystine was added 3 or 6 h following glutamate treatment. As shown in Fig. 3, even when added 6 h after glutamate treatment cystine completely blocks its toxic effect.

Murphy and coworkers have described in a neuronal line as well as in immature cortical neurons (Murphy et al., 1989, 1990) that incubation of the cells in a cystine-free medium is sufficient to trigger cell death whereas in contrast Schubert et al. (1992) showed in PC12 cells that cystine depletion does not induce cell lysis and even inhibits glutamate toxicity. We have thus grown our cells for 24 h in a cystine-free medium supplemented with increasing concentrations of cystine. Fig. 4 shows that 24 h

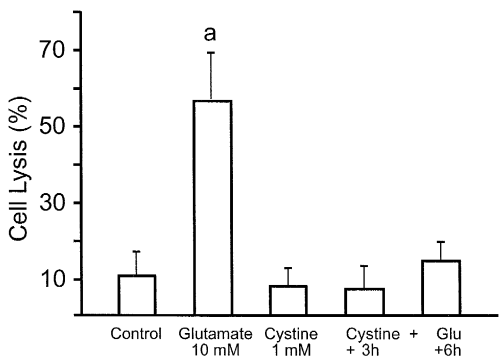

Fig. 3. Effect of delayed cystine addition on the glutamate-induced cell toxicity. At the beginning of the experiment the cells were treated with 10 mM glutamate. In addition some samples received cystine 1 mM either 3 h or 6 h following glutamate treatment. The percentage of dead cells (as estimated by trypan blue dye exclusion) was determined 24 h later and compared to that of control, untreated, samples or of samples treated with cystine alone. Each value represents the mean (±S.D.) of three different experiments, [a] $P < 0.05$ vs. control.

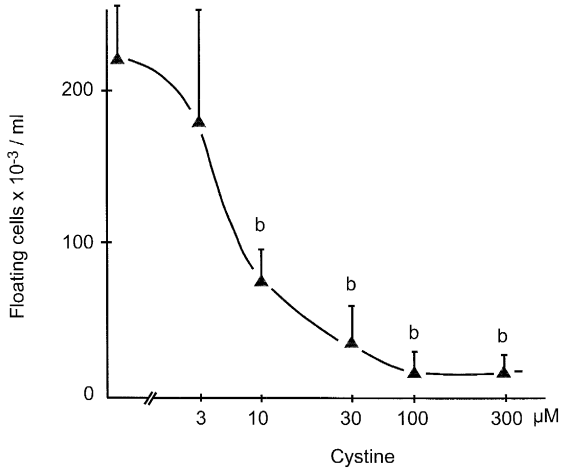

Fig. 4. Effect of cystine deprivation on cell viability. At the beginning of the experiments the culture medium was renewed by cystine-free medium supplemented with increasing concentrations of cystine (3–300 μM). After an additional 24 h incubation we measured the number of floating (dead) cells in the culture supernatant. Each value represents the mean (±S.D.) of three different experiments. [b] $P < 0.01$ vs. control (no cystine).

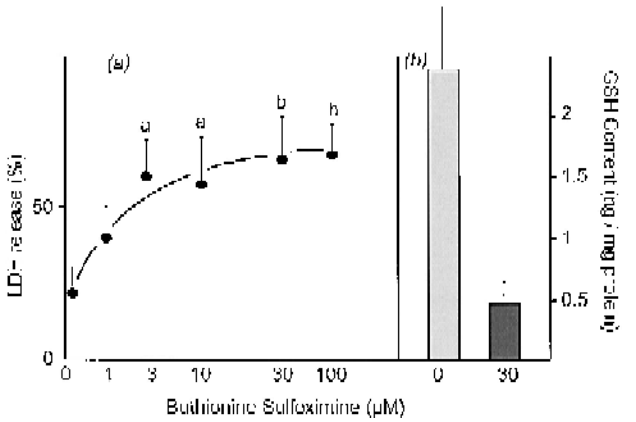

Fig. 5. (a) Effect of increasing concentrations of BSO on cell viability. Cell death was monitored by the determination of LDH release at the end of a 24 h treatment period. Each value represents the mean (±S.D.) of individual determination in 4–12 experiments. [a] $P < 0.05$ vs. control; [b] $P < 0.01$ vs. control. (b) Effect of 6 h treatment with 30 μM BSO on cellular glutathione content. Each value represents the mean (±S.D.) of four different experiments. [b] $P < 0.01$ vs. control.

incubation in cystine-free medium is associated with a significant detachment of the cells from the bottom of the culture flasks. In addition, we observed using the trypan blue exclusion procedure that most of the cells floating in the culture supernatant are indeed dead cells (88.7 ± 7.9%, $n = 4$). Addition of cystine led to a striking reduction of cell detachment and death, with almost no floating cells in the presence of 100–300 μM cystine.

Buthionine sulfoximine, a relatively specific inhibitor of glutathione biosynthesis (Griffith, 1982) has been frequently used to manipulate the level of glutathione both in vitro and in vivo (Mizui et al., 1992). We have thus tested the effect of increasing concentrations of buthionine sulfoximine (1–100 μM) on cell viability and glutathione content.

As shown in Fig. 5, treatment by doses of buthionine sulfoximine as low as 3 μM leads, 24 h later, to a significant decrease in cell viability. This loss of viability is also preceded by a marked reduction of cellular glutathione, which represents 15–25% of that in control after 6 h of treatment by 30 μM buthionine sulfoximine, i.e., well before any sign of cell detachment and death. Thus, as previously described in primary neuronal cultures and in various cell lines (Davis and Maher, 1994; Murphy et al., 1989, 1990; Schubert et al., 1992), there appears to be an inverse relationship between cell viability and glutathione content. However, the nature of this link remains a matter of debate. The effect of glutamate on cell viability has been described to be blocked by inhibitors of macromolecular synthesis such as actinomycin D and cycloheximide, thus suggesting that glutamate-induced cell death corresponds to a programmed event, requiring genetic expression (Ratan et al., 1994a; Serghini et al., 1994). However, Ratan et al. (1994b) recently argued that cycloheximide, by blocking protein synthesis, may prevent cysteine from being incorporated into proteins and may thus indirectly favour glutathione synthesis, avoiding the oxidative stress and death associated with glutathione depletion. We have thus measured in parallel the effect of cycloheximide treatment on both the decrease in glutathione content and cell viability triggered by glutamate.

As shown in Fig. 6, cycloheximide (0.1 μg/ml), which is only moderately cytotoxic by itself, is able to block the toxic action of glutamate but does not inhibit the glutamate-induced decrease in cellular glutathione.

As in the case of cystine depletion, cell death induced by glutamate but also by buthionine sulfoximine is preceded by cell rounding and detachment of the cells from the bottom of the culture flasks. Fig. 7 shows that treat-

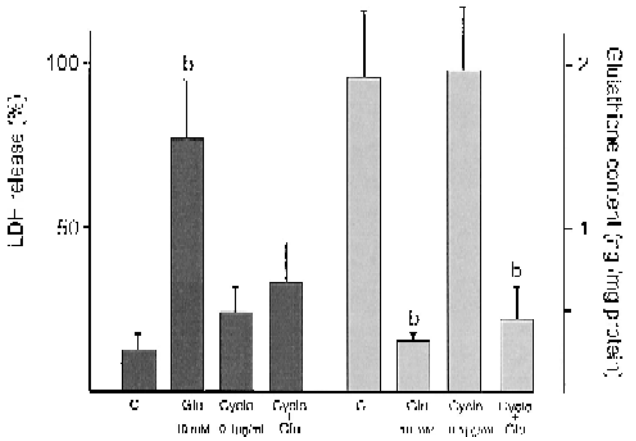

Fig. 6. Effect of cycloheximide and/or glutamate on cell viability (■) and glutathione content (□). Each value represents the mean (±S.D.) of individual values in 5–8 experiments. [b] $P < 0.01$ vs. the appropriate control (C).

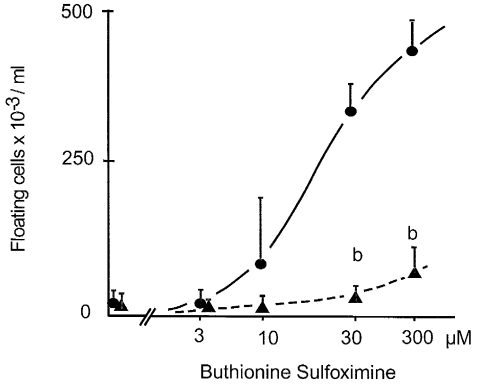

Fig. 7. Effect of cycloheximide on the toxicity induced by buthionine sulfoximine. The cells were incubated in the presence of increasing concentrations of buthionine sulfoximine (3–100 μM) in the absence (●) or presence of cycloheximide (▲, 0.1 μg/ml). After 24 h incubation, the number of floating cells was determined under a light microscope. Each value represents the mean (±S.D.) of individual determinations in four different experiments. [b] $P < 0.01$ vs. control (no cycloheximide).

ment with increasing concentrations of cell buthionine sulfoximine indeed induces within 24 h a striking detachment and death of the cells. This effect is almost completely abolished in the presence of cycloheximide, which nevertheless does not block the depletion of glutathione level due to buthionine sulfoximine treatment (not shown).

We have in parallel experiments used DAPI staining to monitor the nuclear morphology of cells treated by either glutamate or BSO. Fig. 8a indicates that nuclei from

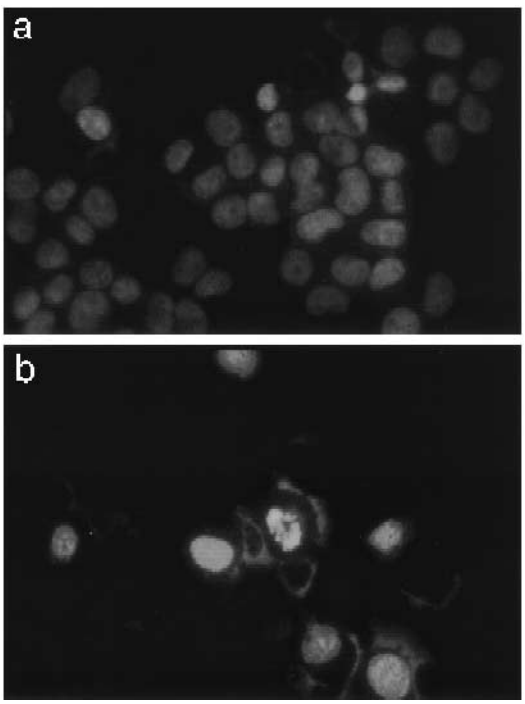

Fig. 8. BSO treatment induces morphological changes typical of apoptosis. (a) DAPI staining of control cells. (b) DAPI staining of BSO-treated cells (30 μM, 16 h of treatment) – note the spherical fragments of nuclei characteristic of apoptosis. Magnification 40×10.

Table 1
Effect of glutamate and BSO on cellular glutathione levels under serum-free conditions

	Glutathione (ng/mg protein)
Control	1.53±0.56
Glutamate 10 mM	0.52±0.36 [a]
BSO 30 μM	0.41±0.34 [a]

48 h after seeding, the culture medium was renewed with serum-free DMEM and the cells were treated for 6 h by glutamate (10 mM) or BSO (30 μM). The cellular glutathione content was then determined according to the colorimetric method of Tietze (1969) and compared to that of control samples. Each value represents the mean (±S.D.) of individual determinations in seven distinct experiments. [a] $P < 0.05$ vs. control.

control cells present an almost homogeneous staining with no signs of chromatin condensation or fragmentation. In contrast, we observed in BSO-treated cells several figures showing chromatin fragmentation as well as cell debris with spreaded and faint staining, indeed indicative of an apoptotic process. Similar observations were made in glutamate-treated cells (not shown).

We have previously described that the PC12 subclone used in these experiments is able to survive for 24–48 h in serum-free medium and that, in addition, glutamate fails to induce cell death under these conditions (Froissard and Duval, 1994). We have thus measured the effect of glutamate and of buthionine sulfoximine on cellular glutathione levels under serum-free conditions.

As shown in Table 1, glutamate (10 mM) and buthionine sulfoximine (30 μM), which both fail to induce any significant cell lysis, provoke a very striking reduction of cellular glutathione content within 6 h of treatment. It thus appears that glutathione depletion per se is not sufficient to induce cell death but may likely trigger under certain circumstances a death mechanism involving RNA and protein synthesis.

4. Discussion

The results of the present investigation provide strong evidence to support the existence of a close relationship between the level of intracellular glutathione and cell survival in the PC12 neuronal-like line. Indeed, glutamate-induced cell death is preceded by an early decrease in intracellular glutathione whereas known precursors of glutathione biosynthesis, such as cystine or *N*-acetylcysteine, both block the glutamate-induced decrease in intracellular glutathione and rescue PC12 cells. In addition, incubation in a cystine-free medium leads to cell detachment and death within 24 h of treatment, a process which is abolished in the presence of cystine. We also show that buthionine sulfoximine, a relatively specific inhibitor of glutathione biosynthesis, induces a significant decrease in the level of intracellular glutathione and ap-

pears cytotoxic at concentrations above 1–3 μM. We observed striking similarities between the kinetics of glutamate and BSO-induced decrease in glutathione concentration, which were almost complete within 6 h of treatment, but also between the kinetics of cell lysis. The cytotoxicity due to BSO treatment has a relatively low onset and is only complete after 16–24 h of incubation, a time-course very similar to that reported earlier for glutamate-induced lysis (Froissard and Duval, 1994). It is therefore likely that glutamate kills PC12 cells by triggering the 'oxidative pathway' already described by several groups in both neuronal and non-neuronal cell lines (Cho and Bannaï, 1990; Kato et al., 1993; Murphy et al., 1989, 1990; Piani and Fontana, 1994; Schubert et al., 1992). However, Schubert et al. (1992) described that incubation in cystine-free medium fails to induce cell lysis and simultaneously blocks the toxic action of glutamate. Nevertheless it appears that the pharmacological characteristics of the 'oxidative pathway' may vary from one model to another since we were unable to show any toxic effect of homocysteate on PC12 glutathione content and viability whereas homocysteate has been shown to be cytotoxic in the hybrid cell line N-18-RE-105 (Murphy et al., 1989).

The question now is that of the link between glutathione depletion and cell death. we have shown before (Froissard and Duval, 1994) that the cells are commited to die after 6 h of treatment with glutamate; now we demonstrate that glutamate-induced glutathione depletion reaches its maximum after 5–8 h of treatment. Nevertheless, addition of cystine 6 h after glutamate treatment rescues the cells from the glutamate-induced toxicity, thus suggesting that glutathione depletion is not the end point in the death pathway but only an intermediate, eventually reversible event, in the chain leading to cell lysis.

This conclusion has also been reached recently by Yonezawa et al. (1996) who described that cystine deprivation induces both glutathione depletion and death in cultures of oligodendroglial cells, but that rescue by free radical scavengers and a diffusible glial factor was not associated with a restoration of normal glutathione content. These authors thus suggest that the protecting agents act distal to glutathione.

In the present work, we also observe that under serum-free conditions glutamate and BSO (30 μM) induce a significant decrease in cellular glutathione content but failed to induce cell death.

The fact that actinomycin D and cycloheximide are able to block this glutamate-induced oxidative pathway has been interpreted as evidence in favour of a programmed death event (Ratan et al., 1994a; Serghini et al., 1994). This interpretation has however been recently challenged by Ratan et al. (1994b). They suggested that cycloheximide by blocking protein synthesis and thus the incorporation of cysteine into macromolecules will spare the amino acid pool and allow sufficient glutathione synthesis to avoid oxidative stress. Several of our results are in complete disagreement with their suggestion. We indeed show that cycloheximide while blocking the glutamate-induced lysis fails to block the glutamate-induced decrease in cellular glutathione. We similarly observe that cycloheximide also reduces BSO-induced toxicity but does not affect the depletion of cellular glutathione due to BSO treatment.

In addition, the chromatin fragmentation observed during BSO and glutamate-induced cell death also point toward the conclusion that 'the oxidative pathway' triggered by cystine depletion and BSO treatment indeed represents a programmed event, and is likely mediated through the regulation of gene expression. Yan et al. (1995) recently described that *N*-acetylcysteine promotes the survival of PC12 cells after NGF withdrawal, and that this protecting action of *N*-acetylcysteine is blocked by actinomycin D. The mechanisms by which glutathione may control gene expression remain unknown but several explanations can be proposed.

The role of glutathione may be indirect. Indeed glutathione is known to be one of the major factors controlling the redox status of cells and several proteins including thioredoxins have been described to respond to variations of this status, acting in part through a regulation of gene expression (Chae et al., 1994; Holmgren, 1989). On the other hand, glutathione may exert a direct effect on gene expression since amino acid starvation as well as glutathione depletion have been described to control the expression of several transcription factors such as a Jun/Fos heterodimeric complex (AP-1) and the nuclear factor κB (NF-κB) (Anderson et al., 1994; Mihm et al., 1995; Schenk et al., 1994). Tong and Perez-Polo (1996) have recently described a modulation of AP-1 binding activity during apoptosis due to serum deprivation or during H_2O_2 treatment. Additional experiments are now in progress to evaluate the effect of glutathione depletion on these transcription factors in PC12 cells, but this line may represent a useful model to investigate the mechanisms of the cytotoxic 'oxidative pathway'.

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