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Increased mitochondrial respiration maintains the mitochondrial membrane potential and promotes survival of cerebellar neurons in an endogenous model of glutamate receptor activation

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Abstract

It is thought that the combination of extracellular glutamate accumulation and mitochondrial damage is involved in neuronal death associated with brain ischemia and hypoglycemia, and some neurodegenerative diseases such as Huntington's disease. However, the mechanism whereby those two factors interact together to trigger neurodegeneration in this and other neurodegenerative disorders is still elusive. Here, we have addressed this issue using a model of mild and sustained accumulation of extracellular glutamate in cerebellar cultured neurons, which are mostly glutamatergic and commonly used to study glutamate neurotoxicity. The resulting stimulation of glutamate receptors triggered a $\sim$ 50% persistent increase in mitochondrial respiration that was associated with free radicals formation, and which was found to be necessary to prevent the collapse of the mitochondial membrane potential ($\Delta\psi_m$) and apoptotic cell death. In fact, hampering the glutamate-mediated increase in mitochondrial respiration with an inhibitor of the mitochondrial respiratory chain stopped neurons from producing free radicals, but led them to undergo rapid and profound $\Delta\psi_m$ collapse and apoptotic cell death. Thus, we suggest that the formation of reactive oxygen species by glutamate receptor activation is the unavoidable consequence of an increase in the mitochondrial respiration aimed to prevent $\Delta\psi_m$ collapse and neurodegeneration. These results may be relevant to understand the pathophysiology of those neurodegenerative diseases associated with both mitochondrial respiratory chain and glutamate transporter defects.

Keywords: free radicals, glutamate, glutathione, mitochondria, neurodegeneration, neuroprotection.

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Glutamate receptor over-stimulation following extracellular accumulation of the amino acid has been involved in the pathophysiology associated with brain ischemia, hypoglycemia, and certain neurodegenerative disorders, such as Huntington's disease (Bittigau and Ikonomidou 1997). It is well known that glutamate neurotoxicity is exacerbated by mitochondrial failure, and that even low concentrations not sufficient to induce neuronal death become neurotoxic in the presence of mitochondrial toxins. During the ischemic episode there is a prompt decline in energy levels and a large increase in the extracellular concentration of glutamate and aspartate (Benveniste *et al*. 1984). In addition, prolonged decreases in mitochondrial activity after brain ischemia have been described (Anderson and Sims 1999). Diminished activity of the different components of the mitochondrial electron transport chain has also been linked to Alzheimer's, Parkinson's and Huntington's diseases, suggesting that mitochondrial damage is involved in the pathogenesis of these disorders. The regulation of the extracellular concentrations of glutamate depends on its removal by transporter proteins. As glutamate transporters are energy and sodium

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Abbreviations used: 7-AAD, 7-amino-actinomycin D; DCF, dichlorofluorescein; $\Delta\psi_m$, mitochondrial membrane potential; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; L-NAME, L-N^{ω}-nitroargininemethyl ester; 3-NP, 3-nitropropionic acid; PDC, L-*trans*-pirrolydine 2,4-dicarboxylate; ROS, reactive oxygen species.

dependent carriers that can operate in the reverse direction during neuronal energy failure, mitochondrial damage would potentiate the excitotoxic neuronal death during extracellular glutamate accumulation (Benveniste *et al*. 1984; Rossi *et al*. 2000). In fact, *in vivo* administration of L-*trans*-pirrolydine 2,4-dicarboxylate (PDC) – an inhibitor of glutamate uptake seen to increase extracellular concentration of glutamate in the striatum and the hippocampus – does not cause neuronal damage in rats (Massieu *et al*. 1995). Similar results have been found in cultured cerebellar granule neurons (Cebers *et al*. 1999; García and Massieu 2001). However, the administration of PDC to rats previously treated with subtoxic concentrations of the mitochondrial toxin 3-nitro-propionic acid (3-NP) or the glycolysis inhibitor, iodoacetate induces large lesions into those brain areas (Sánchez-Carbente and Massieu 1999; Massieu *et al*. 2000), in good agreement with previous *in vivo* and *in vitro* studies showing that metabolic inhibition potentiates glutamate neurotoxicity (Novelli *et al*. 1988; Greene and Greenamyre 1995). These results suggest that, whereas glutamate, at the concentration reached in the extracellular space during these conditions, or mitochondrial impairment *per se* are insufficient factors to cause neurodegeneration, the combination of both trigger neuronal death. However, the underlying mechanism responsible for such synergistic effect is yet unknown.

In order to address this issue, we have used a well-established model of glutamate receptor activation based on the inhibition of glutamate uptake, which leads to a mild and sustained increase in the extracellular concentration of glutamate (García and Massieu 2001). We have found evidence consistent with the notion that glutamate receptor activation triggers an enhancement in mitochondrial oxygen consumption focused to prevent the loss of mitochondrial membrane potential ($\Delta\psi_m$), and the apoptotic neuronal death. We believe that these results may be of relevance to understand the mechanism of neurotoxicity in certain pathophysiological conditions, such as ischemia, hypoglycemia, or Huntington's disease, in which, in addition to mutated Huntingtin, decreased activity of complexes II, III and IV of the electron transport chain (Gu *et al*. 1996; Browne *et al*. 1997; Panov *et al*. 2002) and decreased glutamate transporter (Arzberger *et al*. 1997) has been described.

Experimental procedures

Culture of cerebellar neurons

Cerebellar granule neurons in primary culture were obtained from 7–8-day-old Wistar rats as described (García and Massieu 2001). Cells were seeded at 1.5×10^6 cells/well in 6-wells Nunc (Roskilde, Denmark) plastic Petri dishes previously coated with poly D-lysine (15 μg/mL) and incubated for 9 days *in vitro* in Basal Eagle's Medium, supplemented with 10% fetal bovine serum, L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 μg/mL), amphotericin B (0.25 μg/mL), and KCl (25 mM). Glucose (5 mM) and cytosine arabinoside (10 μM) were added 24 h after plating to inhibit the proliferation of non-neuronal cells.

Cell treatments

After 9 days *in vitro*, neurons were incubated in the presence of the glutamate uptake inhibitor PDC (500 μM) or the succinate dehydrogenase inhibitor 3-NP (500 μM), either alone or in combination, as previously described (García and Massieu 2003), for different time periods (5 min to 4 h). In some experiments, cells were incubated in the presence of MK-801 (10 μM), superoxide dismutase (100 U/mL), catalase (100 U/mL) and N^{ω}-nitro-L-arginine monomethyl ester (L-NAME, 1 mM), which were added 5 min prior to PDC addition.

Flow-cytometric analyses of mitochondrial membrane potential and apoptotic death

$\Delta\psi_m$ and apoptotic death were determined in the same cellular suspension by flow cytometry, essentially as previously described (Almeida *et al*. 2001; García-Nogales *et al*. 2003). Briefly, cells were first smoothly scraped off the plates in order to minimize cellular disruption, and then they were incubated with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, 3 μM), followed by incubation with APC-conjugated annexin-V and 7-amino-actinomycin D (7-AAD) (Becton-Dickinson Biosciences, Franklin Lakes, NJ, USA). Data acquisition was performed using a FACScalibur flow cytometer (Becton Dickinson Biosciences), equipped with a 15 mW argon ion laser tuned at 488 nm, using the CellQuest software (Becton-Dickinson Biosciences). Photomultiplier settings were adjusted to detect JC-1 monomer fluorescence signals on the FL1 detector (green fluorescence, centered around 525 nm), and JC-1 aggregate fluorescence signals on the FL2 detector (red fluorescence, centered around 590 nm). Data analysis was performed using the Paint-A-Gate PRO software (Becton-Dickinson Biosciences). Mean fluorescence intensity values for FL1 and FL2, expressed as relative linear fluorescence channels (arbitrary units scaled from channel 0–10 000) were obtained for all experiments. The relative aggregate/monomer (red/green) fluorescence intensity values were used for $\Delta\psi_m$ data presentation. Preliminary experiments focused to validate this protocol were performed using the mitochondrial permeability pore inhibitor, cyclosporin A (10 μM for 15 min), which showed no effect on FL2/FL1 ratio (~ 1.3 a.u., not shown) when compared to untreated cells. Furthermore, the FL2/FL1 ratio values (~ 0.2 a.u., not shown) obtained after adding FCCP (5 μM) to the cell suspensions were similar to those obtained with antimycin A (10 μg/mL). Annexin V-APC and 7-AAD signals were acquired on the FL4 and FL3 channels, respectively, to quantitatively determine the percentage of apoptotic cells. In all cases, the analyzer threshold was adjusted on the FSC channel to exclude most of the subcellular debris in order to reduce the background noise due to the cellular disruption during scrapping. To exclusively quantify cells showing the early apoptotic feature phosphatidylserine translocation, we determined those annexin V positive cells that were 7-AAD negative. As 7-AAD only binds DNA to those cells having a non-intact plasma membrane (due to both disruption during scrapping

and necrosis by the treatments), we considered to be apoptotic those cells that were annexin V^+-7-AAD^-.

GSH, NADPH and NADH determinations

GSH concentrations were measured by the enzymatic method described by Tietze (1996) with the modifications described by Dringen and Hamprecht (1996) (see also García-Nogales *et al.* 2003). NADH and NADPH concentrations were measured in the cells exactly as described by García-Nogales *et al.* (2003).

Determination of oxygen consumption

Oxygen consumption was determined with a Clark-type electrode (Rank Brothers, Cambridge, UK) in the intact, resuspended cells at 37°C in buffered Hanks' solution. The rates of oxygen consumption were calculated from the slopes (monitored for at least 15 min per trace), and expressed as nmol of oxygen consumed per minute per 0.5×10^6 cells.

Measurement of reactive oxygen species

The formation of intracellular reactive oxygen species (ROS) was assessed using the dye carboxy-H_2DCFDA (20 μM) (Hempel *et al.* 1999). Its desterification product, carboxy-dichlorofluorescein (carboxy-H_2DCF), is cleaved by esterases and remains entrapped within the cells, hence being prone to ROS-mediated oxidation to fluorogenic carboxy-DCF (Hempel *et al.* 1999). To evaluate the formation of superoxide anion, we used hydroethidine, which is specifically oxidized by superoxide as previously reported (Bindokas *et al.* 1996). Thus, after the incubation periods, cells were washed and further incubated in the presence of hydroethidine (3.2 μM) for 20 min at 37°C. After fixing and mounting the cells in coverslips, the number of fluorescent neurons was counted in 10 different fields per coverslip from three independent experiments with the aid an image analyzer (NIH Image 1.6 for Macintosh) and expressed as percentages.

Determination of complex I activity

For the determination of enzyme activity, cells were washed with ice-cold phosphate-buffered saline and collected, centrifuged and resuspended in 300 μL of 0.1 M potassium phosphate buffer (pH 7.0). Cell suspensions (containing approximately 3–4 mg of protein/mL) were frozen and thawed three times to ensure cell lysis. Complex I (NADH–ubiquinone oxidoreductase) activity was measured by determining the rotenone-sensitive NADH oxidation using ubiquinone-5 (coenzyme Q1, Sigma, St Louis, MO, USA) as the electron acceptor (Ragan *et al.* 1987) in a Hitachi U2000 spectrophotometer (Hitachi Ltd, Tokio, Japan). Enzyme activity is expressed as nanomoles per minute per milligram of protein.

Protein determinations

Protein concentrations were determined in cells solubilized with 0.1 M NaOH as Lowry *et al.* (1951), using bovine serum albumin as standard.

Statistical analysis

Data are expressed as means ± SEM values. Statistical analysis was carried out by one-way analysis of variance (ANOVA), followed by a Fisher's least significant difference multiple comparison test.

Results

L-*trans*-Pirrolydine 2,4-dicarboxylate and 3-nitropropionic acid depolarize mitochondrial membrane potential but do not induce neuronal death

In our previous study, we have shown that incubation of cerebellar neurons with PDC (500 μM) increases extracellular glutamate concentrations to values ranging from 18 to 35 μM (García and Massieu 2001) without inducing neuronal death at least up to 4 h of incubation (García and Massieu 2003). However, in the presence of subtoxic concentrations of the mitochondrial toxin 3-NP, PDC triggers cell death (García and Massieu 2001, 2003). Now, we have investigated the mechanisms involved, and we first focused on mitochondrial membrane potential. Here, we show that inhibition of glutamate uptake with PDC induced a rapid, but moderate (by ~ 30%) decrease of $\Delta\psi_m$ that was sustained for, at least, the following 4 h of incubation (Fig. 1a). Such a PDC-dependent loss of $\Delta\psi_m$ was NMDA receptor-mediated, as it was fully prevented by MK-801 (JC-1 fluorescence values, 1.21 ± 0.11, 0.80 ± 0.09, 1.11 ± 0.10, arbitrary units, for

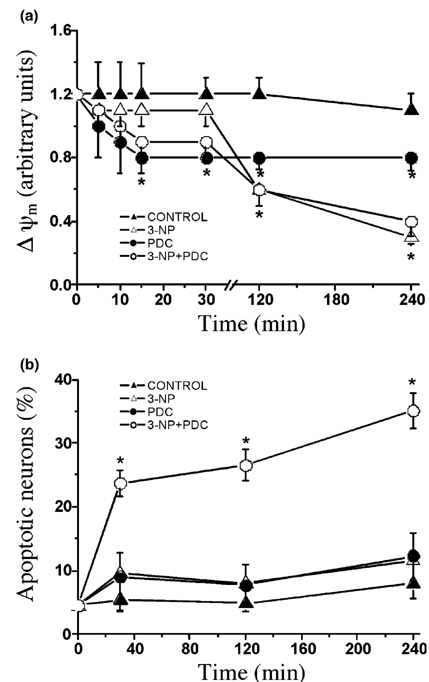

Fig. 1 Time course changes in (a) mitochondrial membrane potential ($\Delta\psi_m$) and (b) apoptotic death as measured in cerebellar neurons after inhibition of glutamate uptake (PDC, 500 μM), inhibition of succinate dehydrogenase (3-NP, 500 μM) or a combination of both. $\Delta\psi_m$ and apoptosis (annexin V^+/7-AAD^- cells) were measured by flow cytometry as described in Experimental Procedures. Results are mean ± SEM values from four to six independent experiments. *$p < 0.05$ vs. control. 3-NP, 3-nitropropionic acid; PDC, L-*trans*-pirrolydine 2,4-dicarboxylate.

control, PDC and PDC + MK-801, respectively). On the other hand, the inhibition of succinate dehydrogenase activity with 3-NP (500 μM) did not alter $\Delta\psi_m$ during the first 30 min of exposure, but induced a large depolarization after 2 h that declined further at 4 h (Fig. 1a). Co-incubation of PDC with 3-NP did not result in additional decay of $\Delta\psi_m$ as compared with 3-NP alone (Fig. 1a), but enhanced apoptotic cell death from as soon as 30 min (Fig. 1b). In contrast, PDC or 3-NP treatments, alone, did not increase the percentage of apoptotic cells, at least up to 4 h of incubation (Fig. 1b).

Mitochondrial membrane depolarization induced by 3-nitropropionic acid is dependent on NADH concentrations.

As show in Fig. 1(a), treatment with 3-NP led neuronal $\Delta\psi_m$ to collapse after 2 h. In view that 3-NP inhibits both succinate dehydrogenase and complex II of the mitochondrial respiratory chain, we next investigated whether 3-NP-induced mitochondrial depolarization was associated with the loss of reducing equivalents (NADH and NADPH), which are involved in the maintenance of $\Delta\psi_m$. As shown in Fig. 2(a), NADH concentrations diminished significantly 2 and 4 h after 3-NP exposure, well correlating with the observed depolarization of the mitochondrial membrane (Fig. 1a). In contrast, exposure of neurons to PDC did not alter NADH concentrations at any of the time periods studied (Fig. 2a), and the coincubation of 3-NP with PDC had no additive effect on NADH concentrations when compared to those induced by 3-NP alone. NADPH concentrations were not altered by any of these treatments (Fig. 2b).

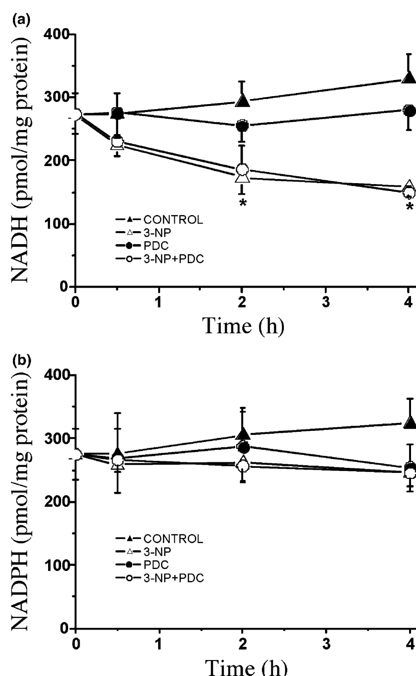

Fig. 2 Time course changes in (a) NADH and (b) NADPH, as measured in cerebellar neurons after inhibition of glutamate uptake (PDC, 500 μM), inhibition of succinate dehydrogenase (3-NP, 500 μM) or a combination of both. NADH and NADPH concentrations were measured by chemiluminiscence, as described in Experimental Procedures. Results are mean ± SEM values from four to six independent experiments. $*p < 0.05$ vs. control. 3-NP, 3-nitropropionic acid; PDC, L-*trans*-pirrolydine 2,4-dicarboxylate.

Inhibition of glutamate uptake by L-*trans*-pirrolydine 2,4-dicarboxylate increases oxygen consumption in cerebellar granule neurons

As shown in Fig. 1(a), PDC produced a brief and rapid $\Delta\psi_m$ decrease that was sustained for the following 4 h. In order to elucidate whether any further $\Delta\psi_m$ collapse could have been prevented through the stimulation of the respiratory chain, the rate of oxygen consumption was monitored after 2 h of PDC exposure. Control neurons consumed oxygen at a rate of 0.31 nmol O_2 per min per 0.5×10^6 cells (Table 1). However, incubation of cells with PDC triggered an increase in the rate of oxygen consumption by 1.4-fold, whereas 3-NP had no effect (Table 1). To elucidate whether PDC-induced increase in oxygen consumption was associated to the activation of NMDA receptors, a group of cells were also incubated in the presence of the NMDA receptor antagonist, MK-801. As shown in Table 1, MK-801 prevented the increase in oxygen consumption induced by PDC. To ascertain whether such an increase in oxygen consumption was due to mitochondrial function, 3-NP was added to PDC-treated respiring cells, and we observed a 95% blockade of oxygen consumption (not shown). In addition, we have previously reported that another mitochondrial toxin, sodium azide, potentiates PDC-mediated cellular death in a similar experimental paradigm (García and Massieu 2001). In view that an increase in oxidative stress up-regulates complex I activity, at least in glial cells (Vásquez *et al.* 2001), we sought to determine whether such a putative increase would

Table 1 Inhibition of glutamate uptake with L-*trans*-pirrolydine 2,4-dicarboxylate (PDC) increases the rate of oxygen consumption in cerebellar neurons

	Rate of oxygen consumption (nmol O_2/min/0.5×10^6 cells)
Control	0.31 ± 0.01
3-NP	0.29 ± 0.02
PDC	0.44 ± 0.04*
PDC + MK-801	0.21 ± 0.07†

Oxygen consumption was determined after 2 h exposure of neurons to PDC (500 μM) or 3-nitropropionic acid (3-NP: 500 μM). MK-801 (10 μM) was incubated with PDC. Values are mean ± SEM of three independent experiments. $*p < 0.05$ vs. control values; $†p < 0.05$ vs. PDC.

account for the observed increased respiration rate. However, treatment of cells with PDC (500 μM, 2 h) resulted in no change in the activity of complex I (control, 4.4 ± 0.1; PDC, 4.2 ± 0.7 nmol/min/mg of protein; $n = 3$).

Inhibition of glutamate uptake with L-*trans*-pirrolydine 2,4-dicarboxylate induces reactive oxygen species production and decreases GSH concentrations

As an increase in the rate of oxygen consumption would enhance oxidative metabolism, we sought to investigate whether PDC treatment led to an increase in ROS production. Approximately 22% of control neurons showed carboxy-DCF fluorescence, indicating basal ROS production (Fig. 3a). However, the presence of PDC significantly increased the number of fluorescent cells as from 30 min of treatment, and remained elevated after 2 and 4 h (Fig. 3a). This effect was inhibited by catalase (100 U/mL) and by MK-801 (10 μM) (Fig. 3b). In contrast, 3-NP alone did not increase the number of fluorescent cells at any of the times investigated (Fig. 3a). Furthermore, the exposure of neurons to PDC plus 3-NP did not increase the number of fluorescent cells when compared with PDC alone (Fig. 3a). In order to ascertain whether superoxide anion was a major ROS component under our conditions, we next used hydroethidine, i.e. a fluorescent probe previously shown to specifically detect superoxide anion in cultured neurons (Bindokas *et al.* 1996). As shown in Table 2, 2 h after treatment, neither PDC nor 3-NP alone were sufficient to generate detectable superoxide, whereas the combination of PDC with 3-NP significantly increased the number of fluorescent cells. To investigate whether ROS production was associated with impairment of GSH status, GSH concentrations were determined in the same experimental conditions. As shown in Fig. 4(a), PDC treatment progressively decreased GSH concentrations, reaching the statistical significance after 2 and 4 h of exposure. In contrast, 3-NP treatment, alone, did not alter GSH concentrations (Fig. 4a). When 3-NP was combined with PDC, GSH was not further decreased when compared with PDC treatment alone (Fig. 4a). The decrease in GSH concentrations observed 2 h after PDC treatment was not prevented by superoxide dismutase (100 U/mL) or L-NAME (1 mM) (results not shown). However, it was partially prevented by catalase (100 U/mL) and MK-801 (10 μM) (Fig. 4b).

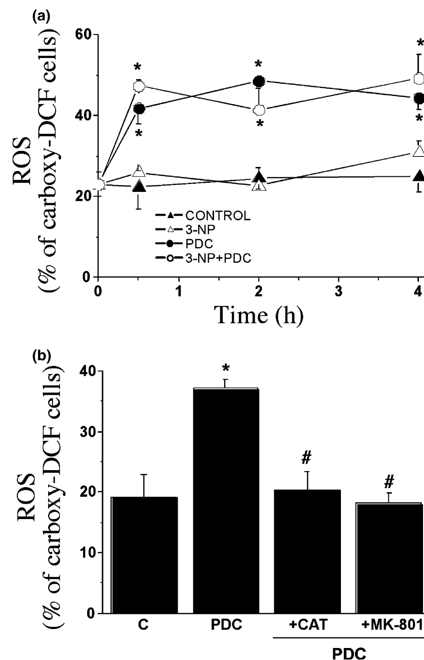

Fig. 3 Inhibition of glutamate uptake by PDC induces ROS production in cultured cerebellar granule cells. (a) ROS production were determined after exposure of cells to PDC (500 μM) or 3-NP (500 μM), either alone or in combination. (b) ROS production were also measured at 2 h after PDC (500 μM) treatment, either in the absence or in the presence of catalase (100 U/mL) or MK-801 (10 μM). ROS production was assessed by determination of oxidation products of carboxy-DCF. Data are mean ± SEM values from three (carboxy-DCF) independent experiments. **p* < 0.05 vs. control; #*p* < 0.05 vs. PDC. CAT, catalase; DCF, dichlorofluoroscein; 3-NP, 3-nitropropionic acid; PDC, L-*trans*-pirrolydine 2,4-dicarboxylate; ROS, reactive oxygen species.

Table 2 Effect of L-*trans*-pirrolydine 2,4-dicarboxylate (PDC) and 3-nitropropionic acid (3-NP) on superoxide anion production by cerebellar neurons, as assessed by hydroethidine fluorescence

	Hydroethidine fluorescence (% of positive cells per field)
Control	2.3 ± 0.9
PDC	5.2 ± 1.2
3-NP	5.6 ± 0.3
PDC + 3-NP	10.3 ± 1.5*

Cells were treated with PDC (500 μM), 3-NP (500 μM) or a combination of both for 2 h, washed and further incubated in the presence of hydroethidine. After washing and fixing, cells were mounted and analyzed by fluorescence microscopy. Each value represent the mean ± SEM values from four to six different cell culture preparations. **p* < 0.05 vs. control values.

Discussion

Glutamate uptake inhibition with PDC induced a rapid and modest $\Delta\psi_m$ fall that was sustained further, whereas the inhibition of mitochondrial respiratory chain with 3-NP largely depolarized mitochondria in a delayed (2 h) manner. In contrast to 3-NP, PDC-mediated loss of $\Delta\psi_m$ was not related to the loss of reducing equivalents and therefore to the impairment of mitochondrial electron flow, but rather to calcium influx into the mitochondria after activation of NMDA receptors. This result is in good agreement with the well-known role of calcium influx to the mitochondria in the $\Delta\psi_m$ loss and excitotoxicity (Schinder *et al.* 1996; White and Reynolds 1996; Peng and Greenamyre 1998; Fiskum *et al.*

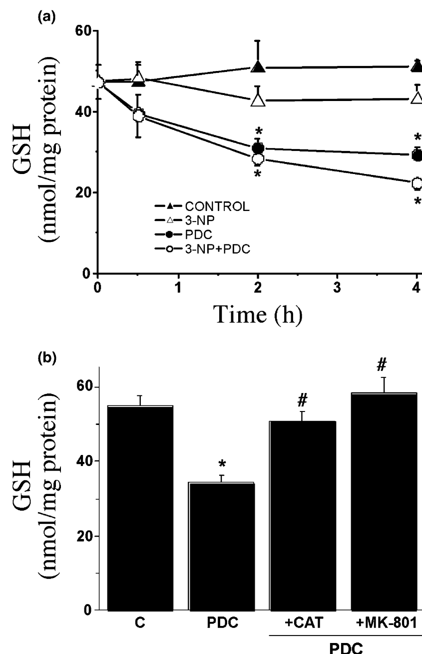

Fig. 4 Inhibition of glutamate uptake by PDC depletes GSH in cultured cerebellar granule cells. (a) GSH concentrations were determined after exposure of cells to PDC (500 μM) or 3-NP (500 μM), either alone or in combination. (b) GSH concentrations were also measured at 2 h after PDC (500 μM) treatment, either in the absence or in the presence of catalase (100 U/mL) or MK-801 (10 μM). GSH concentrations were determined enzymatically as described in Material and Methods. Data are mean ± SEM values from six (GSH) independent experiments. *$p < 0.05$ vs. control; #$p < 0.05$ vs. PDC. CAT, catalase; 3-NP, 3-nitropropionic acid; PDC, L-*trans*-pirrolydine 2,4-dicarboxylate.

1999; Duchen 2000; Nicholls *et al.* 2003). However, none of these treatments affected cell survival, at least during the 4 h investigated. Interestingly, apoptotic cell death was triggered only when both PDC and 3-NP were simultaneously present, despite no further decline in $\Delta\psi_m$ was observed when compared to 3-NP treatment alone. Such a $\Delta\psi_m$ loss would hence be associated with the widely held notion that excitotoxicity occurs through mitochondrial dysfunction leading to ATP depletion (Ankarcrona *et al.* 1995; Pang and Geddes 1997; Almeida and Bolaños 2001; García and Massieu 2003).

Interestingly, the dual inhibition of succinate dehydrogenase and complex II of the mitochondrial respiratory chain occurring in the cerebellar neurons exposed to 3-NP (García and Massieu 2001) was insufficient to trigger immediate $\Delta\psi_m$ loss. Such $\Delta\psi_m$ loss occurred only after 2 h of 3-NP treatment, and was well correlated with NADH, but not NADPH depletion, strongly suggesting that, during this time, $\Delta\psi_m$ would be maintained at the expense of complex I-mediated NADH oxidation. Only when endogenous NADH content is exhausted, due to the inhibition of succinate dehydrogenase activity, the subsequent complex I dysfunction will fail to properly maintain $\Delta\psi_m$. In any case, $\Delta\psi_m$ loss induced under this condition does not appear to compromise neuronal survival, suggesting that mitochondrial depolarization, *per se*, would be insufficient to cause neuronal death.

Non-toxic glutamate receptor activation during PDC exposure partially decreased but did not disrupt completely $\Delta\psi_m$. To understand the mechanism whereby $\Delta\psi_m$ was not collapsed and cells survived to such mild glutamate receptor activation, we next investigated the rate of mitochondrial oxygen consumption. Cells responded to PDC treatment by increasing the rate of mitochondrial oxygen consumption. Such an increase in oxygen consumption would be associated with $\Delta\psi_m$ maintenance, because $\Delta\psi_m$ in PDC-treated cells was lost only when mitochondrial respiratory chain was inhibited. Furthermore, the stimulation of mitochondrial respiration was accompanied by enhanced ROS production and GSH depletion, in good agreement with previous studies reporting that superoxide anion would be formed during glutamate excitotoxicity (Lafón-Cazal *et al.* 1993; Dugan *et al.* 1995). However, no detectable superoxide was observed after 2 h with PDC alone, whereas it did increase ROS production, suggesting that superoxide may be rapidly transformed into hydrogen peroxide. In fact, catalase, but not superoxide dismutase, prevented PDC-mediated dichlorofluorescein fluorescence and GSH depletion. Moreover, the decrease in GSH might be associated with the recently described impaired GSH biosynthesis – a consequence of the lack of the GSH precursor, glutamate – in a similar model of excitotoxicity (Chen and Swanson 2003; Himi *et al.* 2003; Ré *et al.* 2003). However, this is not the case under our circumstances, because both GSH depletion and ROS production induced by PDC were prevented by MK-801. These results strongly suggest that ROS production and GSH depletion are the consequence of NMDA receptors activation by the glutamate accumulated in the extracellular medium.

In conclusion, our results suggest that the accumulation of endogenous extracellular glutamate after inhibition of its transporters induces a partial $\Delta\psi_m$ loss that is compensated by the stimulation of mitochondrial respiratory chain activity, which leads to ROS production and GSH deficiency in a manner dependent on NMDA receptor activation. Such a compensative mechanism appears to support neuronal survival, unless there is concomitant mitochondrial respiratory chain deficiency hampering such a neuroprotective strategy. Prolonged inhibition of mitochondrial respiratory chain activity has been observed after cerebral ischemia (Anderson and Sims 1999) and in postmortem brain tissue samples obtained from Huntington's disease subjects (Gu *et al.* 1996; Browne *et al.* 1997). Moreover, the excitotoxicity associated with these pathologies has been suggested to be a consequence of decreased glutamate transport (Arzberger *et al.* 1997; Mitani and Tanaka 2003). These observations suggest that in addition to the expansion of the CAG codon in

Huntingtin protein associated with Huntington's disease patients, an excitotoxic mechanism, probably precipitated by a progressive decline in mitochondrial function, might be involved in the pathogenesis of this disease (Zeron *et al*. 2001; Panov *et al*. 2002). Thus, our results may contribute to understanding the mechanisms involved in neuronal death associated with cerebral ischemia and certain neurodegenerative disorders, such as Huntington's disease.

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