Paradoxical Potentiation by Low Extracellular Ca^{2+} of Acute Chemical Anoxic Neuronal Injury in Cerebellar Granule Cell Culture

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Received February 19, 1991; Accepted June 21, 1991

ABSTRACT

Acute chemical anoxic injury was produced in primary cerebellar granule cell cultures incubated with iodoacetate (IAA) alone or IAA combined with potassium cyanide (KCN). Cytotoxicity was assessed using Trypan blue exclusion or LDH release. Four millimolars of KCN induced approx 30% neuron death at 3 h, whereas >50% cell death was produced by 0.2 mM IAA. No potentiation of cytotoxicity was observed by IAA + KCN. A total of 0.2 mM IAA produced an early major reduction of intracellular ATP prior to the onset of neuron injury or reduction in intracellular glutathione (GSH). Medium Na^+ replacement by choline, K^+ , or methylglucamine protected against IAA-induced neuronal injury, reduced the rate of decline of intracellular ATP but had no effect on intracellular GSH. Some 80% neuronal survival was obtained when Na^+ was deleted from the medium even after the intracellular ATP had been reduced to <10% of control. Removal of Ca^{2+} from the medium had no effect on control culture, Trypan blue exclusion, GSH, or ATP, but potentiated the onset and magnitude of IAA-induced cytotoxicity, ATP and GSH decline. Loading of granule cells with the Ca^{2+} chelator Fura-2 did not influence

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IAA-induced cytotoxicity in control or low Ca^{2+} media. Addition of 50 μM glutamate had a minimal cytotoxic effect over 3 h and the combined addition of 0.2 mM IAA plus 50 μM glutamate did not potentiate IAA-induced injury. The glutamate receptor antagonists, D-2-amino-5-phosphonovaleric acid (APV) or kynurenate did not block IAA-induced injury in control medium but inhibited the potentiation of toxicity seen in the low Ca^{2+} medium.

This study suggests the use of IAA as a chemical anoxic agent in cerebellar granule cell culture. The early, dose-dependent decline in ATP may be dissociated from GSH change. Acute IAA-induced injury is Na^+/Cl^- dependent but paradoxically potentiated in low Ca^{2+} medium. The low Ca^{2+} potentiated component was sensitive to glutamate/NMDA receptor antagonists and associated with reduction of intracellular GSH.

Index Entries: Cerebellar granule neuron culture; chemical anoxia; iodoacetate; reduced glutathione; ATP; cytotoxicity; low medium Ca^{2+} ; glutamate receptor.

INTRODUCTION

Observations using dissociated neurons in tissue culture have provided new insights into the pathophysiology of neuronal injury produced by hypoxia, unaccompanied by other complicating variables such as might exist in vivo, slice or brain derived organelle preparations. Rothman (1983,1984) revealed that hippocampal neuron cultures deteriorated in the absence of oxygen but such cultures tolerated prolonged anoxia when synaptic activity was blocked by elevated Mg^{2+} , suggesting a role for the synaptic release of excitatory neurotransmitters, most likely glutamate and/or aspartate, in the mediation of anoxic neuronal injury. Surprisingly, the excitatory amino acids were neurotoxic in medium that contained no added Ca^{2+} , in agreement with observations of Price et al. (1985), casting doubt on the hypothesis that Ca^{2+} entry was responsible for the neuronal death (Berdichevsky et al., 1983; Coyle, 1983). The maintenance of cytosolic Ca^{2+} at a physiological level depends on energy-dependent transport systems and it is expected that hypoxic, anoxic, or ischemic conditions known to deplete intracellular ATP would likely cause a disruption of Ca^{2+} homeostasis (Brattin et al., 1984; Jewell et al., 1982; Johnson et al., 1987; Nicotera et al., 1989). Others have also questioned the involvement of Ca^{2+} in mediating anoxic cell injury (Allen and Orchard, 1983; Cobbold et al., 1985; Pascoe and Reed, 1989) and the precise mechanism and contribution of the role of Ca^{2+} is unclear.

During studies on the ionic modulation of iodoacetate (IAA)-induced chemical anoxia in cerebellar granule cells in culture, we observed a paradoxical potentiation of neural cell death in low $(\text{Ca}^{2+})_e$. This report examines the role of $(\text{Ca}^{2+})_e$ in mediating IAA-induced anoxic cell death and the contribution of excitotoxic amino acids or receptors in the mediation of neuronal death.

MATERIALS AND METHODS

EDTA, EGTA, bovine pancreatic deoxyribonuclease I, insulin, γ -aminobutyric acid, fluorodeoxyuridine, transferrin, disodium ATP, FLE-55 firefly lantern extract, reduced glutathione, iodoacetate (sodium salt), tetrodotoxin, kynurenic acid (4-hydroxyquinoline-2-carboxylic acid), and APV (DL-2-amino-5-phosphonovaleric acid) were obtained from Sigma Chemical Company (St. Louis, MO). Trypan blue (0.4% in 0.85% saline) was obtained from Flow Laboratories, Inc. Fura-2 AM was obtained from Molecular Probes.

Cerebellar Granule Cell Culture

Cerebellar granule neuron cultures were prepared from 4- to 6-d-old Sprague-Dawley rats (Sarafian and Verity, 1986). Following dissociation and washing, cells were plated at a density of 5×10^5 mL in 12 well plastic culture plates precoated with poly-L-lysine.

Assay for Neurocytotoxicity

The release of lactate dehydrogenase (LDH) or trypan blue exclusion were used to quantitate neuronal toxicity. For LDH release, the incubation medium was removed at appropriate time periods and stored at 4°C until assay. The residual LDH activity remaining was assayed following lysis by addition of 25 μ L 5% Triton X-100 to 500 μ L medium. The absolute (total culture) or percent LDH release was determined by following the decrease in absorbance ($A_{340\text{nm}}$) of NADH in medium containing 30 mM potassium phosphate buffer, pH 7.4, 0.4 mM sodium pyruvate, and 50 μ M NADH. In the Trypan blue exclusion assay, 0.5 mL of freshly diluted (1:10) stock (0.4%) Trypan blue solution was added to the cultures following careful removal of medium. Four mid-circumferential fields were evaluated in which Trypan blue positive cells and total cells were estimated and summated. A measure of percent Trypan blue positive cells was taken as an index of cellular toxicity. The estimate is likely to give a lower rate of toxicity owing to loss of dead cells during the wash. This criticism was partially obviated by obtaining total number of cells before and after treatment. Under all experimental conditions, the difference was not greater than 8%.

Biochemical Assays

The ATP content of the culture was determined in 0.5 mL 0.3M perchloric acid extracts neutralized with 50 μ L 2M potassium carbonate, centrifuged and assayed within 3 h (Seidman and Verity, 1987).

Content of reduced glutathione (GSH) in the culture was assayed following two extractions with 150 μ L 0.1M formic acid using the fluorometric method of Hissin and Hilf (1976). The reaction system consisted of 2 mL 0.1M sodium phosphate, 5 mM EDTA, pH 8.0 to which was

added 100 μ L culture extract and 0.1 mL freshly prepared o-phthalaldehyde (1 mg/mL methyl alcohol). After incubation with appropriate standards for 15 min at room temperature, readings were obtained at excitation 350 nm and emission 420 nm in a Farrand spectrofluorimeter.

Culture protein was determined on the residual culture or native culture following perchloric acid extraction for ATP determination. Following addition of 300 μ L 0.5M NaOH, cells were scraped, incubated for 2 h at 37°C and protein concentration determined on 10–50 μ L aliquots by the method of Lowry et al. (1951) using bovine serum albumin as protein standard.

Composition of Defined Chemical Media

Media composition was modified slightly (Verity et al., 1990) from that of Choi (1987). The control medium (CSS) contained 120 NaCl; 5.4 mM KCl; 0.8 mM MgCl_2 ; 1.8 mM CaCl_2 ; 25 mM Tris-HCl, pH 7.4; 3 mM NaHCO_3 ; and 15 mM glucose. Na-free medium contained equimolar choline chloride for NaCl and omission of NaHCO_3 . The low Ca^{2+} medium omitted CaCl_2 but contained 0.3 mM EGTA.

Statistical Analysis

Data are expressed as mean $\pm$ SEM and statistical evaluation performed by Student's *t*-test for small populations. Values represent individual microwell cultures from numerous cultures plated over a 9-month period.

RESULTS

Effect of Iodoacetate (IAA) With and Without KCN on Chemical Anoxic Neuronal Injury

To mimic a state of cell anoxia, iodoacetate (IAA) was used in combination with KCN to prevent the production of ATP from anerobic metabolism (Nicotera et al., 1989). Four millimolar iodoacetate alone or in combination with 2 mM KCN produced a progressive significant release of lactate dehydrogenase (LDH) during 3 h (Fig. 1). KCN alone revealed minimal cytotoxicity (LDH release) even at 3 h. Of note was the apparent protection by KCN of the cytotoxicity induced by IAA expressed as the percentage LDH release at all time periods examined (Fig. 1). However, extrapolation to 4–5 h revealed similar percentage LDH release (data not shown). Two millimolar KCN in the presence or absence of IAA had no effect on the LDH activity assay (data not presented).

When cytotoxicity was examined in terms of Trypan blue exclusion (Fig. 2), parallel, asymptotic curves were produced by IAA in the presence or absence of KCN. KCN alone produced approx 30% cell death at 3 h in keeping with the LDH release data. The two methods for the

Fig. 1. Effect of iodoacetate (IAA, 4 mM) alone or combined with potassium cyanide (KCN, 2 mM) on lactic dehydrogenase (LDH) release from cerebellar granule cell cultures. Cultures were washed and incubated in 0.5 mL basal medium (CSS), 37°C. Values represent mean $\pm$ SEM (n = 7–11 cultures).

cytotoxicity assay using IAA are not strictly complimentary during early evolution of cell injury: The dichotomy likely reflects the greater sensitivity of the Trypan blue assay in identifying early cell injury.

Dose-Dependent Effect of IAA on Cytotoxicity, Cellular ATP, and Reduced Glutathione (GSH)

The lack of potentiation of IAA-induced cytotoxicity by KCN suggested that IAA alone was sufficient to induce a chemical anoxic perturbation of intracellular ATP and/or glutathione. A plot of IAA concentration against cytotoxicity at 2 h revealed significant cell death at IAA concentrations <0.1 mM (Fig. 3), 40-fold less than used in the preliminary cytotoxicity experiments. Although it is recognized that IAA inhibits enzymes in the glycolytic pathway by modification of sulfhydryl groups, other thiols including glutathione also react with IAA. Intracellular ATP and glutathione concentration at 1 h in the presence of 0.2 mM IAA revealed a major decline in ATP without significant change in the specific activity of reduced glutathione (Table 1). At this time there was no measurable cytotoxicity. Hence, at this concentration of IAA (used in subsequent experiments), no early effect on intracellular thiol concentration was observed.

Fig. 2. Effect of 4 mM IAA alone or in combination with 2 mM KCN on cytotoxicity in cerebellar granule cell culture determined by the Trypan blue exclusion assay. In this experiment, cell death was expressed as number of Trypan blue positive cells assessed after enumerating stained and unstained cells in four circumferential representative fields. 850 ± 90 cells are equivalent to 100% of staining. Values represent mean $\pm$ SEM ($n = 7-16$ cultures).

Fig. 3. Plot of IAA vs cytotoxicity as assessed by Trypan blue stained cells. Cultures were washed and incubated in 0.5 mL CSS medium for 2 h, 37°C . Values represent mean $\pm$ SEM ($n = 3-5$ cultures).

Table 1
Effect of Iodoacetate, 0.2 mM on Cerebellar Granule
Cell ATP and Reduced Glutathione (GSH)

	nmol/mg protein ⁻¹	
	ATP	GSH
Control	39.1 ± 9.3	22.9 ± 0.5
Iodoacetate, 0.2 mM	7.0 ± 1.1	21.4 ± 1.6

Determinations represent mean ± SEM ($n = 5-6$) of specific activities following 1 h incubation with 0.2 mM iodoacetate, 37°C in control (CSS) medium.

Relationship of Cell Viability, ATP, and GSH Levels During IAA-Induced Chemical Anoxia

A composite plot of the change in intracellular ATP, reduced glutathione (GSH), and cytotoxicity in the presence of 0.2 mM IAA is presented in Fig. 4. During the first hour, no cytotoxicity was evident in the face of a considerable reduction in intracellular ATP but no change in intracellular GSH. Over the subsequent 3 h, ATP levels dropped to zero and Trypan blue stained cells approached 70%. Only modest reduction of GSH was evident, likely secondary to passive leakage from damaged cells.

Ionic Dependence of IAA-Induced Neurotoxicity

Hypoxic and glutamate neurotoxicity can be separated into two components on the basis of time course and ion dependence (Choi, 1987, 1988; Goldberg et al., 1986; Rothman, 1983). An early component marked by neuronal swelling was dependent on extracellular Na^+ and Cl^- , whereas the later second component was dependent on extracellular Ca^{2+} . Other investigators (Rothman, 1985; Shier and Dubourdieu, 1985) have also demonstrated a Na^+ and/or Cl^- mediated step in cell killing. Replacement of Na^+ by choline in the 0.2 mM IAA culture significantly protected against neuronal injury as measured by Trypan blue exclusion or LDH release, had minimal effect on intracellular GSH, and reduced the rate of decline of intracellular ATP (Fig. 4 and Table 2). Greater than 80% neuron survival was evident at 3 h in Na^+ deficient medium even after intracellular ATP was reduced to <10% of control.

A comparison of Na^+ replacement by K^+ , methylglucamine, or choline (Table 2) revealed the considerable cytoprotection given by all the Na^+ substitutes even in the presence of ATP reduction. In all cases, no difference in intracellular glutathione was evident as compared with the control (CSS) medium. Tetrodotoxin, 2 μM did not protect cells during IAA-mediated injury.

Fig. 4. Effect of 0.2 mM IAA on the evolution of cytotoxicity as assessed by ATP (A), GSH (B) content, and Trypan blue exclusion (C) in cerebellar granule cell cultures incubated in CSS medium, 37°C. Control profiles in CSS were contrasted with those obtained by equimolar replacement of NaCl by choline chloride. Values represent means $\pm$ SEM ($n = 4-6$ cultures).

Table 2
Comparison of Na⁺ Substitution on Iodoacetate-Induced Neural Injury in Cerebellar Granule Cell Culture

	ATP (nmol/ culture)	GSH (nmol/ culture)	Trypan blue stained (%)
CSS	0.03	1.86	62
KCl	0.04	1.86	20
Methylglucamine	0.07	1.38	7
Choline-Cl	0.47	1.56	6

NaCl in CSS medium was replaced by 130 mM N-methyl-D-glucamine; 115 mM choline chloride or 115 mM KCl. Incubation for 2 h, 37°C with 0.2 mM iodoacetate prior to measurement of LDH release into supernatant, Trypan blue exclusion, GSH, and ATP assays. Values are mean of duplicate experiments.

Effect of $[Ca^{2+}]_e$ on Iodoacetate-Induced Neurotoxicity

Previous studies demonstrated a dependence on extracellular $[Ca^{2+}]_e$ for the delayed phase of glutamate- or hypoxia-induced neuronal injury. Paradoxically, removal of Ca^{2+} from the external medium was found to potentiate 0.2 mM IAA-induced cytotoxicity in cerebellar granule cell cultures. Accelerated cytotoxicity (Fig. 5) was also accompanied by a further decline in ATP and intracellular GSH even at 30 min, a time at which no significant cytotoxicity was observed.

Effect of Intracellular Ca^{2+} Chelation on Iodoacetate-Induced Cytotoxicity

If a sustained increase in cytosolic Ca^{2+} is implicated in the genesis of IAA-anoxic cell injury, then pretreatment and loading of granule cells with the Ca^{2+} chelator Fura-2 should delay the onset of neurotoxicity while having no influence on the IAA-induced loss of ATP or reduced glutathione (Nicotera et al., 1989). Granule cells were loaded with 15 μ M Fura-2 AM for 45 min in CSS medium. Cultures were washed free of extracellular Fura-2 AM and returned to the control CSS or the low Ca^{2+} medium containing 0.2 mM IAA. Assessment of cytotoxicity at 3 h revealed no protection by the Ca^{2+} chelator whether examined in the low $[Ca^{2+}]_e$ or control medium (Table 3) suggesting that an increase in $[Ca^{2+}]_i$ may not be a principal mechanism in the mediation of acute phase IAA-induced injury.

Fig. 5. Effect of "low Ca^{2+} " medium (omission of CaCl_2 , 0.3 mM EGTA) alone or in presence of 0.2 mM IAA-supplemented cerebellar granule cell culture, 37°C , as compared to 0.2 mM IAA in CSS. Cytotoxicity was assessed by ATP (A) and reduced glutathione (GSH) (B), both expressed as nmol/culture and percentage LDH release (C). Values represent mean $\pm$ SEM ($n = 5-7$ cultures).

Table 3
Effect of Fura-2 AM Loading on IAA-Induced
Cytotoxicity

	Trypan blue stained cells (%)	
	– Fura	+ Fura
Low Ca^{2+}	60 $\pm$ 11	72 $\pm$ 9
Control CSS	29 $\pm$ 4	36 $\pm$ 7

Granule cells were loaded in CSS containing 15 μM Fura-2 AM, 37°C for 45 min. Following washing, 0.2 mM IAA-induced cytotoxicity was determined at 3 h by Trypan blue exclusion in control CSS medium (1.8 mM Ca^{2+}) or low Ca^{2+} medium. Values are mean $\pm$ SEM of three experiments.

Role of Glutamate Receptor in Mediation of IAA-Induced Anoxic Neuronal Injury

Rothman (1984) provided convincing evidence for the synaptic release of excitatory transmitter in rat hippocampal neuron culture associated with the mechanism of irreversible anoxic injury. Glutamate is a prominent excitatory amino acid in cerebellar granule cell culture (Gallo et al., 1982; Nicoletti et al., 1987) and may be released following prolonged anoxic depolarization. We examined indirectly the role of presumed endogenously released glutamate and/or aspartate into the culture medium during IAA-induced anoxia. Addition of 0.5 mM D-2-amino-5-phosphonovaleric acid (APV), the selective NMDA receptor antagonist, or 0.5 mM kynurenate did not block IAA-induced injury in CSS medium (Table 4). However, both receptor antagonists significantly reduced the potentiation of toxicity seen in the low $[\text{Ca}^{2+}]_e$ medium, suggesting that the added component of neural injury observed in low $[\text{Ca}^{2+}]_e$ may be mediated via the NMDA receptor site.

Novelli et al. (1988) demonstrated that glutamate became neurotoxic via the N-methyl-D-aspartate (NMDA) receptor when intracellular energy levels were reduced. They showed that glutamate was toxic for cerebellar granule cells in the absence of glucose, oxygen, or added KCN. We postulated, therefore, that in the presence of IAA, associated with limited cellular energy, the cytotoxic action of glutamate would be revealed and would demonstrate synergism with IAA. However, glutamate (50 μM) alone revealed only minimal effect over 3 h, not accompanied by significant Trypan blue staining (Fig. 6). Paradoxically, incubation of cultures in IAA plus glutamate revealed less cytotoxicity as compared to IAA alone at 2 and 3 h of incubation (Fig. 6). Although the explanation for the apparent glutamate protection of IAA neurotoxicity is unclear, these experiments did not reveal expected synergism or potentiation of IAA injury by glutamate at 1 h as was previously demonstrated by Novelli et al. (1988). We found no dose-dependent effect for the apparent

Table 4
Effect of Glutamate Receptor Antagonists on IAA-Induced Chemical Anoxic Injury in Cerebellar Granule Cell Culture

Addition	Trypan blue stained (%)	
	CSS	Low Ca^{2+}
Control,		
0.2 mM IAA	40.3 ± 6.7	67.1 ± 1.7
APV, 500 μM	35.4 ± 6.8	$28.8 \pm 3.5^*$
Kynureate, 500 μM	38.3 ± 4.6	$33.9 \pm 3.4^*$

Neurons were cultured, washed, and placed in CSS or low Ca^{2+} medium for 2 h, 37°C with 0.2 mM iodoacetate and 500 μM APV or kynureate. Viability was assessed by Trypan blue exclusion. Values are means $\pm$ SEM, $n = 4-8$ microwell cultures.

*Differences from appropriate control at $p < 0.01$.

Fig. 6. Effect of exogenous L-glutamate on cytotoxicity in cerebellar granule cell cultures alone or in presence of 0.2 mM IAA. Values represent mean $\pm$ SEM ($n = 6$ cultures).

glutamate protection at 50–500 μM assayed at 3 h, 37°C by Trypan blue exclusion or LDD release (data not presented).

DISCUSSION

The present study was designed to examine the feasibility of IAA as a model of chemical anoxic injury in cerebellar granule cell culture analo-

gous to similar studies in hepatocytes (LeMasters et al., 1987) or hepatoma (Nicotera et al., 1989). IAA interacts with cellular thiols including glutathione and enzyme sulfhydryl groups associated with glycolysis. Although assessment of cell viability using Trypan blue exclusion or LDH release revealed no difference in the onset and magnitude of IAA cell injury owing to an apparent inhibition of LDH release that was found with IAA and KCN in combination. Such apparent KCN protection may be partially explained by the more sensitive marker of Trypan blue staining compared to the time-related release of LDH, which indicates more massive membrane permeability. KCN alone was not sufficient to inhibit ATP production via oxidative phosphorylation to cytopathic levels in agreement with an observation of Novelli et al. (1988). IAA, 0.2 mM, induced significant ATP reduction prior to glutathione depletion. It would appear, therefore, that IAA-induced anoxic injury in cerebellar granule cell cultures is not a result of a primary depletion of intracellular GSH as previously suggested in hepatoma at higher concentrations of IAA (Nicotera et al., 1989) but is initiated following early ATP depletion mediated by inhibition of glycolysis.

These observations reveal that in normal ionically balanced medium, IAA-induced neurotoxicity is mediated through Na^+ , Cl^- , and H_2O linked acute neuronal swelling, is blocked by Na^+ replacement, but not blocked by NMDA receptor antagonists or tetrodotoxin suggesting that the Na^+ influx is not through NMDA receptor or voltage-dependent Na^+ channels. Moreover, the cytoprotection in low Na^+ medium is even evident when intracellular ATP has been reduced to <10% of control (Fig. 4). The rate of ATP decline is slowed in low Na^+ medium indicating that the protective effect of low Na^+ is also coupled to ATP sparing owing to lack of need to extrude Na^+ ions back across the plasma membrane.

Although discussion of the exact cause of neuronal death caused by anoxia (Kass and Lipton, 1982; Novelli et al., 1988; Rothman, 1984) and relation to excitatory amino acids is beyond the scope of the present report, glutamate neurotoxicity requires the influx of Ca^{2+} (Choi, 1987; Garthwaite and Garthwaite, 1986; Manev et al., 1989; Rothman, 1985), although a divergent opinion has been advocated by Rothman (1984, 1985). These observations may be partially reconciled by the separation of glutamate neurotoxicity into an acute Na^+ -dependent phase followed by a delayed Ca^{2+} component thought to mediate the mechanism of delayed glutamate neurotoxicity. The present studies with IAA support the Na^+ -dependent hypothesis as they were performed during the analogous acute phase. Paradoxically, IAA-induced neuron death was potentiated in the absence of extracellular Ca^{2+} and it is surprising that the potentiated component in low $[\text{Ca}^{2+}]_e$ would be mediated via a glutamate and/or NMDA receptor mechanism as suggested from the antagonist studies with APV or kynurenate (Table 4). It must be restated that the IAA neurotoxicity produced in normal $[\text{Ca}^{2+}]_e$ is not blocked by

NMDA receptor antagonists and therefore not initially coupled to a glutamate-activated receptor pathway (Choi, 1988). Also, the data contribute little to a further understanding of the Ca^{2+} -dependent delayed mechanism of cell death.

Addition of 50 μM glutamate to cultures in the presence of IAA failed to induce accelerated neurotoxicity as would be expected from the data of Novelli et al. (1988). It is possible that IAA interacts ("blocks") at the glutamate receptor preventing glutamate activation of Na^+ and/or Ca^{2+} movement, or that IAA interferes with the voltage-dependent Mg^{2+} block of the NMDA receptor channel. No neuron death was observed in the presence of 50 μM glutamate in CSS medium containing Mg^{2+} and glucose in agreement with Novelli et al. (1988). Glutamate interaction at the kainate receptor was untested but the evolution of kainate-induced neurotoxicity is known to occur gradually over many hours (Favaron et al., 1988), a time scale not examined in this protocol.

What, then, is the mechanism(s) that may account for the potentiated IAA-induced neurotoxicity in low $[\text{Ca}^{2+}]_e$? It is noteworthy that Choi (1987, 1988) also found that the acute phase of neuronal swelling associated with glutamate exposure was potentiated in the absence of medium Ca^{2+} . Evidence presented here suggests that the low Ca^{2+} component of early neurotoxicity is NMDA receptor coupled owing to inhibition by the NMDA antagonists kynurenate and APV. This observation argues against an impaired operation of the membrane $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Nachsen et al., 1986) operating to exchange excess internal Na^+ ions for extracellular Ca^{2+} . A second possible explanation for the low $[\text{Ca}^{2+}]_e$ potentiation of injury stems from the studies of Orrenius and colleagues (1988) and Pascoe and Reed (1987, 1989) who have demonstrated a reduction of intracellular $[\text{Ca}^{2+}]_i$, protein thiols including GSH and loss of alpha-tocopherol following incubation of cells in low $[\text{Ca}^{2+}]_e$. Under conditions of low GSH and ATP there would be accelerated oxidative stress accounting for cytotoxicity. Some support for this hypothesis was observed in the accelerated early loss of GSH in low Ca^{2+} + IAA (Fig. 5). This attractive mechanism does not account for the protection by the NMDA antagonists, which clearly point to a role for the NMDA receptor complex, unless "activation" of the receptor was directly associated with loss of GSH and oxidative injury.

Thirdly, low $[\text{Ca}^{2+}]_e$ may potentiate the release of voltage-dependent Mg^{2+} from the NMDA receptor complex leading to sensitization of the receptor to low concentrations of glutamate/aspartate. Certainly, Mg^{2+} release from the NMDA receptor complex is known to potentiate glutamate activity with subsequent lethal ion flux (Mayer et al., 1984; Nicoletti et al., 1987; Novelli et al., 1988; Nowak et al., 1984). A significant increase in culture medium glutamate concentration will occur upon depolarization in cerebellar granule cell culture (Gallo et al., 1982; Holopainen and Kontro, 1988) thereby providing an appropriate potentiating signal. Kauppinen et al. (1986) demonstrated that Ca^{2+} chelation by EGTA

caused partial depolarization of the synaptosomal membrane and activation of a voltage-dependent, tetrodotoxin-sensitive Na^+ channel. In this scenario, low Ca^{2+}_e activates Na^+ influx through a voltage-sensitive channel, leading to partial depolarization, further loss of Mg^{2+} at the NMDA receptor, activation of Na^+ , Cl^- , and H_2O influx at the NMDA receptor (blocked by NMDA antagonists) and hence potentiation of neuronal injury. These deleterious effects do not occur in the "low Ca^{2+} " medium in the absence of IAA, because the intracellular ATP is maintained allowing for activation of the Na^+ extrusion pump and maintained membrane polarization.

In conclusion, these studies support the use of IAA as a chemical anoxic agent in primary cerebellar granule cell culture. IAA cytotoxicity was modulated by medium Na^+/Cl^- and potentiated in low $[\text{Ca}^{2+}]_e$ medium. The low Ca^{2+} potentiated component was glutamate receptor antagonist sensitive but also associated with an accelerated reduction in intracellular GSH, which may influence the activity of the glutamate/NMDA receptor complex.

ACKNOWLEDGMENTS

This work was partially supported by USPHS ES02573, ES04722, and the UCLA Foundation (World Boxing Council). Marites Torres was a scholar under the auspices of the Associated Western Universities Summer Research Program.

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