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Characterization of a Chemical Anoxia Model in Cerebellar Granule Neurons Using Sodium Azide: Protection by Nifedipine and MK-801

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Induction of chemical anoxia, using sodium azide in cerebellar granule cells maintained in primary culture, was evaluated as an in vitro assay for screening of potential neuroprotective compounds. The purpose of this study was to evaluate sodium azide as an alternative to cyanide salts, compounds which, despite their unfavorable characteristics, are often used in assays for chemical anoxia. The viability of neuronal cultures after treatment with azide, with or without preincubation with calcium channel blockers, tetrodotoxin (TTX), or glutamate receptor antagonists, was monitored by subsequent incubation with the tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), followed by isopropanol extraction and spectrophotometric quantification of cellularly reduced MTT. The azide-induced degeneration of neurons was shown to be dependent on the concentration as well as on the duration of incubation with submaximal concentrations of azide. Incubation of the neurons with nifedipine, a blocker of L-type voltage-sensitive calcium channels (L-VSCC), or with the noncompetitive N-methyl-D-aspartate (NMDA) subtype glutamate receptor antagonist MK-801, prior to addition of submaximal concentrations of azide, significantly attenuated azide-induced neuronal death. Blockers of N-type and Q-type VSCC (ω-conotoxin MVIIA and MVIIC, respectively) and the P-type VSCC blocker ω-agatoxin IVA had no effect in this assay. The sodium channel blocker TTX was without effect when added to neurons under depolarizing conditions, but potently and effectively protected cells when experiments were performed in a nondepolarizing buffer. The results show that chemical anoxia induced by incubation of cultured neurons with azide leads to detrimental effects, which may be quantitatively monitored by the capability of the cells to reduce MTT. This procedure is a suitable method for screening of compounds for possible protective effects against neuronal death induced by energy depletion. In addition, the results suggest involvement of L-type VSCC as well as of glutamate receptors in the pathways leading to neuronal degradation induced by energy depletion in cerebellar granule neurons. This would further support the notion that these pathways might be important in neurodegeneration induced by cerebral ischemia or anoxia.

Key words: cerebellar granule neurons, anoxia, hypoxia, calcium, calcium channels, VSCC, sodium azide

INTRODUCTION

Normal functioning of neurons is dependent on a constant supply of oxygen and glucose. If this supply is abolished for just a few minutes, a cascade of events is started, ultimately leading to degeneration of the cells (Goldberg and Choi, 1993). A key element in this cascade seems to be overstimulation of glutamate receptors, which is known to lead to neurodegeneration (Rothman, 1984; Meldrum, 1985). The neurotoxicity induced by glutamate as well as by anoxic/hypoglycemic conditions is dependent on extracellular calcium concentration (Choi, 1985, 1987; Frandsen and Schousboe, 1992; Goldberg and Choi, 1993), and a correlation has been established between intracellular calcium accumulation and glutamate- or anoxia-induced cell death (Hartley et al., 1993; Goldberg and Choi, 1993). This is consistent with the "calcium hypothesis" proposed by Schanne et al. (1979), which states that calcium overload might be the common final pathway of cell death. However, the mechanisms of calcium toxicity have not been entirely elucidated. For example, it has been shown that elevation of intracellular calcium concentration ($[Ca^{2+}]_i$) to a defined level caused by Ca^{2+}-influx through different channels (gated by either glutamate or voltage) did not cause the same level of neurodegeneration (Tymianski et

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al., 1993; Witt et al., 1994). Nevertheless, glutamate- and calcium-induced neurotoxicity are closely interrelated, since a major route for calcium influx is through glutamate receptor-gated channels (Choi, 1987; Frandsen and Schousboe, 1992), and since synaptic release of glutamate is a process triggered by influx of calcium through voltage-sensitive calcium channels (VSCC) (Katz and Miledi, 1967; Stanley, 1993).

Primary cultures of mouse cerebellar granule cells are widely used as a model of glutamatergic neurons. It is well-known that these cultures are highly enriched in granule cells (80–90%), with little glial contamination (Messer, 1977; Schousboe et al., 1985). It has been shown that anoxia induces increased release of glutamate from cerebellar granule cells (Drejer et al., 1985). The depolarization-coupled release of glutamate from these cells can be inhibited by verapamil (Belhage et al., 1992), dihydropyridines (Huston et al., 1990), and ω-conotoxin (Elster et al., 1994), and is therefore dependent on Ca^{2+}-influx through L- and N-type VSCC.

A number of in vitro models have been proposed to mimic certain aspects of ischemic/anoxic brain injury. An obvious experimental approach to hypoxia is to replace the oxygen surrounding cultured neurons with nitrogen. This method can be troublesome, since prenatal or early postnatal rodent brain tissue is quite resistant to oxygen depletion, and complete removal of oxygen is difficult. Thus, 24 hr of anoxia is often necessary in order to reach significant injury (Rothman, 1984; Marcoux et al., 1989; Goldberg and Choi, 1993; Dessi et al., 1993; Lin et al., 1993), although simultaneous exclusion of glucose (in vitro "ischemia") will accelerate the process (Goldberg and Choi, 1993). Incubation of neuronal cultures with cyanide has been extensively used as an assay for "chemical anoxia." Using this method, Patel et al. (1993) showed that NMDA receptors mediate anoxic injury in hippocampal neurons. Still, even though the intracellular calcium concentration is rapidly elevated by cyanide (Patel et al., 1993), several hours of incubation are needed before cell death is seen (Dubinsky and Rothman, 1991; Lin et al., 1993; Patel et al., 1993). Moreover, at physiological pH, cyanide is almost exclusively present as free acid, which will evaporate rapidly, and therefore cyanide concentration cannot be controlled. In the present assay, sodium azide (NaN_3), another inhibitor of oxidative phosphorylation (Vigers and Ziegler, 1968; Vasilyeva et al., 1982; Noumi et al., 1987), was evaluated as an alternative to cyanide to generate "chemical anoxia."

MATERIALS AND METHODS

Materials

Pregnant (8-day) NMRI (The Naval Medical Research Institute, USA) mice were obtained from Bomholtgaard Breeding and Research Center, Ltd., Ry, Denmark. ω-conotoxin MVIIA was purchased from Peninsula Laboratories Ltd, Merseyside, UK; ω-agatoxin IVA and ω-conotoxin MVIIC were from Alomone Labs, Jerusalem, Israel, and MK-801 was from Merck, Sharp & Dohme, Essex, UK. Horse serum and culture media were purchased from Life Technologies (GIBCO), Roskilde, Denmark. All other compounds were obtained of highest commercial quality from Sigma Chemical Co. (St. Louis, MO).

Cell Cultures

Mouse cerebellar granule neurons were cultured essentially as described by Messer (1977). Briefly, 7-day-old mice were decapitated and the cerebella removed under sterile conditions. After manual disintegration, the tissue was triturated with trypsin (0.025% w/v) and DNAse (0.005% w/v) for 15 min at 37°C. The cells were suspended at a concentration of 3×10^6 cells/ml in a slightly modified Dulbecco's Minimum Essential Medium (DMEM) which contained horse serum (10% (v/v)), penicillin (333 U/ml), paraaminobenzoic acid (1 μg/ml), L-glutamine (0.5 mM), insulin (0.08 U/ml), and potassium chloride (23.8 mM). The cell suspension was subsequently inoculated into poly-L-lysine-coated 24-well multidishes, 0.5 ml suspension/well. After 48 hr in culture, cytosine arabinoside (5 μM, final concentration) was added in order to prevent glial proliferation.

The cells were kept in culture for 9–11 days at 37°C (5% CO_2/95% O_2) before experiments were carried out. The culture medium was not changed during the culture period.

Chemical Anoxia and Cell Viability

In order to establish an anoxia-like condition, azide was added to the cell cultures as dilutions in Hepes-buffered saline (HBS) (120 mM NaCl, 5 mM KCl, 0.62 mM $MgSO_4$, 1.8 mM $CaCl_2$, 10 mM Hepes, and 6.1 mM glucose, pH 7.4). The volume added was 25 μl; in control wells, 25 μl HBS were added. After the desired incubation time (1 hr, except for the time-dependence experiments for azide), each well was washed once with HBS (500 μl), 250 μl MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 0.2 μg/μl in HBS) were added, and the incubation was continued for 15 min at 37°C. MTT is reduced only in viable cells with NADH (nicotinamide adenine dinucleotide, reduced form) as a cosubstrate (Mosmann, 1983; Schiff et al., 1985; Holt et al., 1987; Dorman et al., 1993), and this reaction is not directly inhibited by azide (Berridge and Tan, 1993).

The blue formazan product formed was extracted from the cultures by addition of 315 μl isopropanol/40 mM HCl to each well, followed by gentle shaking for 10

min on a Vortex Genie 2 (Scientific Industries Inc., Bohemia, NY).

Four 100-μl aliquots from each well were transferred to a 96-well microtiter plate, and the optical density at 540 nm was determined (Dynatech MR5000, Dynatech Labs, Inc., Chantilly, VA).

In order to test various calcium or sodium channel blockers and glutamate receptor antagonists for neuroprotective effects in this assay, granule cells were preincubated with the compound in question (25 μl in HBS) for 10 min prior to addition of azide (25 μl in HBS, final concentration 4.5 mM), and the cells were incubated for another 60 min (37°C, 5% CO_2/95% O_2). The viability of neurons was determined as described above.

The influence of the relatively high concentration of potassium (~24 mM) in the culture medium was investigated by replacement of the medium by HBS or by HBS with 25 mM potassium (HBS-25K, equimolar substitution of sodium by potassium). After preincubation at 37°C for 15 min with or without TTX, azide was added to the cultures. Since cultures incubated in HBS were less sensitive to azide, the concentration of azide was chosen at 10 mM for HBS and 4.5 mM for HBS-25K. After incubation for 60 min at 37°C, viability of the neurons was determined as described above.

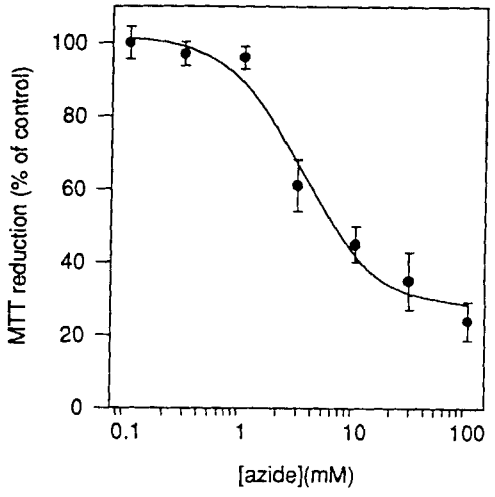

Fig. 1. Effect of exposure to azide on reduction of MTT by cerebellar granule cells. Primary cultures of mouse cerebellar granule cells (see Materials and Methods) were incubated for 60 min at different concentrations of sodium azide (see Materials and Methods). Cells were washed and incubated with MTT for an additional 15 min. The cellularly reduced formazan formed from MTT was extracted with isopropanol/HCl and quantitated spectrophotometrically at 570 nm. The results are means of three experiments ± SD, indicated by vertical bars. The curve was drawn by computer fitting (SigmaPlot, Jandel Scientific GmbH, Erkrath, Germany).

RESULTS

In the present study, incubation of primary cultures of mouse cerebellar granule cells with sodium azide was used as a model for anoxia. Subsequent incubation of the neurons with the tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and spectrophotometrical quantification of cellularly reduced MTT were used as a viability assay. The ability of the neurons to reduce MTT was found to be dose-dependently inhibited by azide (Fig. 1). The incubation time in this experiment was 60 min, and under these conditions half-maximal inhibition of MTT reduction was observed at ~4.5 mM azide. Using this azide concentration, dependency on the duration of the azide treatment on the viability of the cells was studied (Fig. 2). Under these conditions, 50–70% of the neurons died after 60 min of incubation (see Table I and Fig. 2).

Using an azide concentration of 4.5 mM and 60 min of incubation time, the effect of potentially protective compounds was tested. The L-type calcium channel blocker nifedipine potently attenuated azide-induced neurodegeneration, with maximal protection at 300 nM (Fig. 3). The blockers of N- and Q-type VSCC, ω-conotoxins MVIIA and MVIIC, respectively, the blocker of P-type VSCC, ω-agatoxin IVA (Table I), and the sodium channel blocker TTX (data not shown) were all without effect under these conditions. However, preincubation

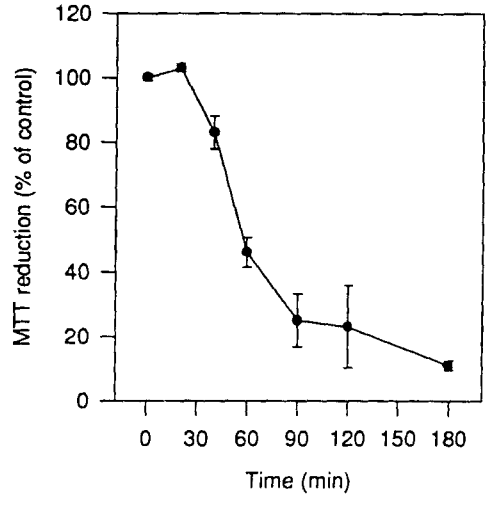

Fig. 2. Time course of azide-induced cell damage. Parallel cultures of cerebellar granule cells (see Materials and Methods) were incubated with azide (final concentration 4.5 mM) for different periods of time. The data point at 0 min indicates MTT reduction by control cultures (added HBS only). After incubation, cells were washed, MTT was added, and the incubation was continued for another 15 min. Cellular reduction of MTT was determined as described in Materials and Methods. The results are means of three experiments ± SD, indicated by vertical bars.

TABLE I. Effect of Calcium Channel Blockers on Azide-Induced Neurotoxicity*

Compound	MTT reduction (% of control)	
	Azide only	Azide + compound
ω-conotoxin MVIIA (0.1 μM)	32 ± 12	34 ± 7.1
ω-agatoxin IVA (0.1 μM)	39 ± 4.2	31 ± 4.7
ω-conotoxin MVIIC (0.1 μM)	38 ± 10	40 ± 0.6

*Parallel cultures of cerebellar granule neurons were incubated in culture medium with or without ω-conotoxin MVIIA, ω-conotoxin MVIIC, or ω-agatoxin IVA for 10 min prior to addition of azide (4.5 mM, final concentration). After 60 min of incubation, cells were washed, MTT was added, and cellular reduction of MTT was monitored as described in Materials and Methods. Results are means of three experiments ± SD.

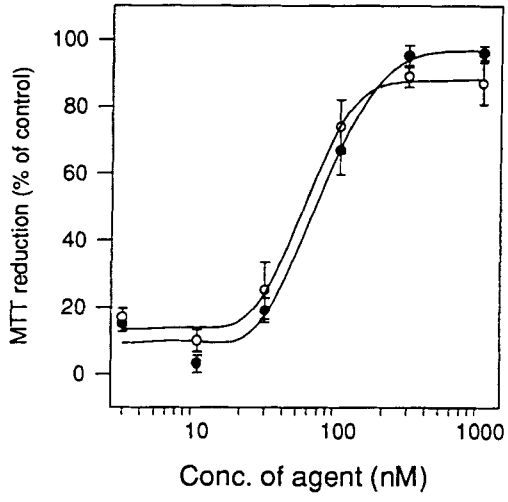

Fig. 3. Effect of MK-801 and nifedipine on neurotoxicity induced by azide. Parallel cultures of cerebellar granule cells (see Materials and Methods) were incubated with nifedipin (solid circles) or MK-801 (open circles) for 10 min prior to azide addition (final concentration, 4.5 mM). Controls were incubated in HBS only. After 60 min of incubation, cultures were washed and MTT was added as described in Materials and Methods. Cell viability was determined by following the ability to metabolize MTT (see Materials and Methods). Results are averages of three experiments, with SD shown as vertical bars. The curves were drawn by computer fitting (SigmaPlot).

with the noncompetitive NMDA antagonist MK-801 resulted in a significant increase in MTT reduction after azide exposure (Fig. 3), i.e., protection of the cells.

The above-mentioned experiments were carried out by adding test compounds directly to the culture media containing ~24 mM potassium. In order to evaluate significance of the depolarizing condition, selected experiments were repeated using the same protocol, except that the media were changed to HBS or HBS-25K. Since cultures incubated in HBS were less sensitive to azide, 10 mM azide used in these experiments. This azide concentration resulted in neuronal degeneration corresponding to that observed when 4.5 mM NaN_3 was added to cultures in HBS-25K or in culture medium (see Table I). Experiments carried out in HBS-25K were in all cases quantitatively similar to the ones using culture medium (see Table I and Fig. 4). However, preincubation with TTX potently prevented toxicity induced by 10 mM azide, when the experiment was performed in HBS (Fig. 4), i.e., under nondepolarizing conditions.

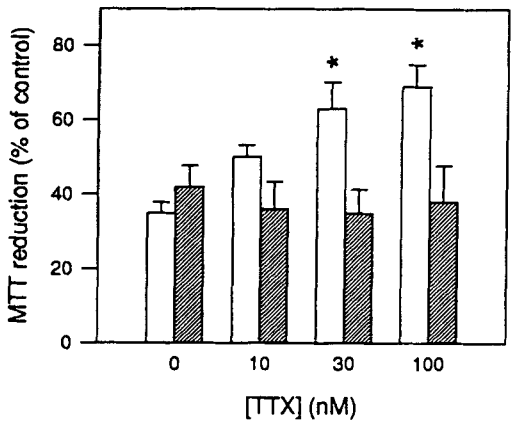

Fig. 4. Effect of TTX on azide neurotoxicity under depolarizing or nondepolarizing conditions. Culture media of parallel cerebellar granule cell cultures (see Materials and Methods) were exchanged to Hepes-buffered saline (HBS, open bars) or HBS containing 25 mM potassium (HBS-25K, hatched bars) and kept at 37°C. Ten min after addition of TTX or control HBS ("0 nM"), azide was added and incubation was continued for another 60 min. In order to reach comparable levels of degeneration, 4.5 mM azide was used with HBS-25K, whereas neurons incubated in HBS were exposed to 10 mM azide (final concentration). Subsequent incubation with MTT and determination of cellularly reduced MTT were performed as described in Materials and Methods. Results are averages of three experiments ± SD, indicated by vertical bars. Asterisks indicate a statistically significant difference ($P < 0.05$, Student's t-test) from control.

The results quantified using the MTT assay were confirmed by examination of the morphological changes that followed azide treatment. Some of these observations are illustrated in Figure 5. Control cultures exhibited phase-bright cells with fasciculated processes, indicating that the major part of these neurons is intact (Fig. 5A). Incubation of a parallel culture with 4.5 mM azide for 60 min drastically changed the morphology of the neurons. Cells were shrunken with vacuoles and had degenerated neurites (Fig. 5B), indicating that the majority of neurons were dead or significantly damaged, in keeping with the results of the MTT viability assay (Figs. 1, 2). It appears from Fig. 5C,D that incubation of parallel cultures with nifedipine (Fig. 5C) or MK-801 (Fig. 5D) protected the neurons from the anoxic damage. How-

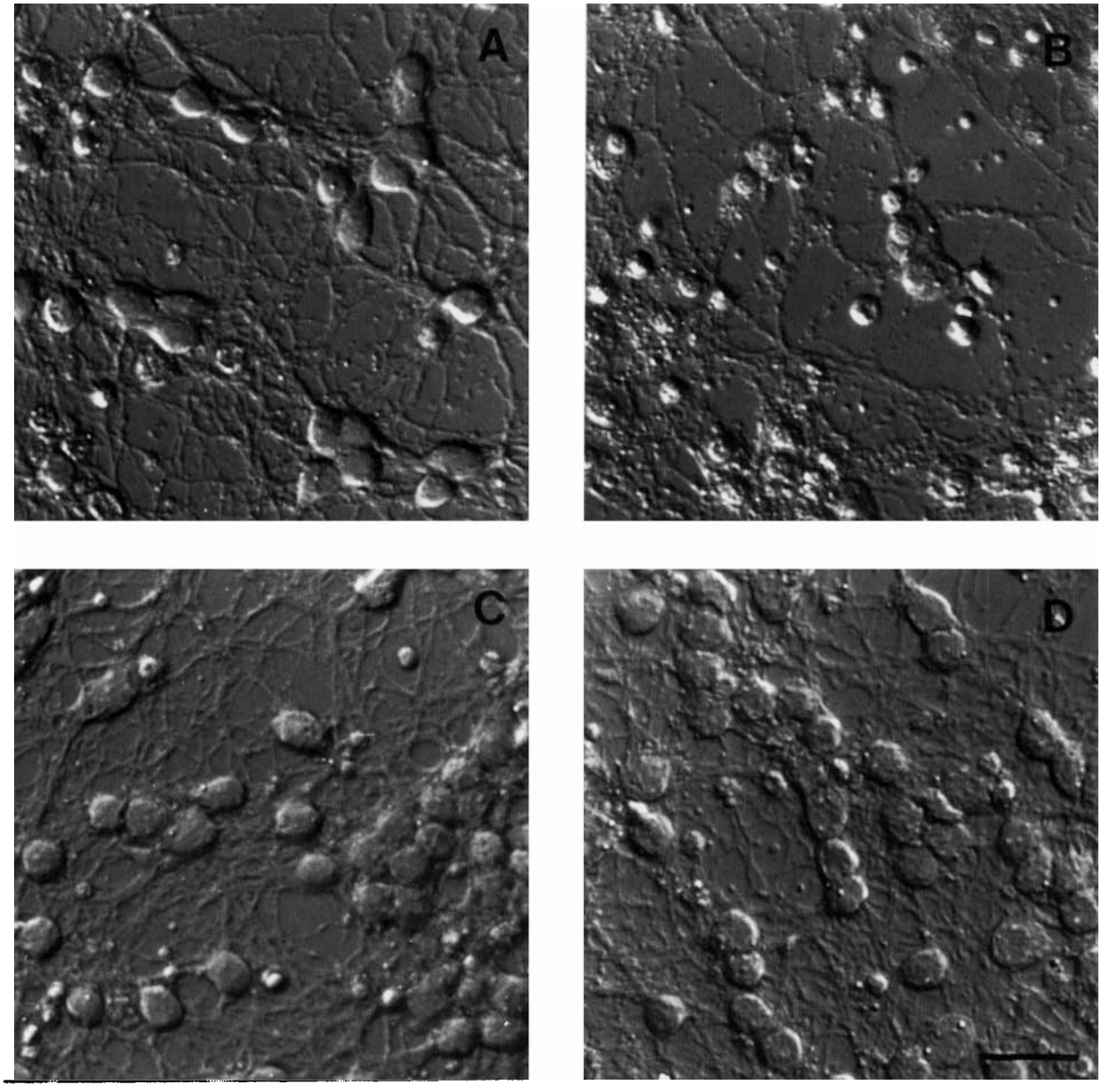

Fig. 5. Phase-contrast photographs of control and azide-treated cerebellar granule cells. Granule cell cultures were prepared as detailed in Materials and Methods. Before photographs were taken, the cells were exposed for 1 hr to the following incubation media: Hepes-buffered saline (HBS) (**A**), or HBS containing: 4.5 mM azide (**B**), 4.5 mM azide + 1 μM nifedipine (**C**), or 4.5 mM azide + 1 μM MK-801 (**D**). Photomicrographs are representative of morphological appearance of cells. Bar, 25 μm.

ever, the cells treated with nifedipine or in particular with MK-801 still appeared swollen compared to controls (Fig. 5A vs. Fig. 5C,D).

DISCUSSION

The azide ion (N_3^-) has multiple cellular effects. In addition to inhibition of cytochrome aa_3 and of ATP synthesis (Vigers and Ziegler, 1968; Vasilyeva et al., 1982; Noumi et al., 1987), azide affects superoxide dismutase (Misra and Fridovich, 1978) and DNA synthesis (Ciesla et al., 1974), effects that might all contribute to the toxicity of this compound. It has also been speculated that the neurotoxicity of azide might be related to enhanced excitatory transmission as a consequence of con-

version of azide into nitric oxide (Smith et al., 1991). Thus, the exact pathways leading to degeneration are not entirely clear, although a likely explanation is that a decrease in the intracellular ATP level leading to a loss of Na^+/K^+ homeostasis and hence depolarization of the cell membrane is responsible for the catastrophic prolonged opening of VSCC. Apart from the potential toxicity induced by increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by this pathway per se, opening of VSCC in the nerve terminals, increased release of glutamate, and hence overstimulation of postsynaptic glutamate receptors, could be responsible for further increase in $[Ca^{2+}]_i$. Additionally, the depolarization will relieve the voltage-dependent Mg^{2+}-block of the NMDA-gated cation channel, leading to further Ca^{2+} influx (Zeevalk and Nicklas, 1992). The importance of these relationships is supported by the demonstration of the significant protective effects of nifedipine and MK-801 against azide-induced cell damage. The lack of effect of ω-conotoxins MVIIA and MVIIC is consistent with recent results showing only marginal inhibition by these toxins of glutamate release from granule cells (Elster et al., 1994; Varming et al., unpublished).

The results obtained in this in vitro chemical anoxia assay correlate well with findings by other investigators using other methods. Exposure of neurons to cyanide has repeatedly been used as a model for anoxia. Patel et al. (1993) found that the competitive NMDA receptor antagonist APV (2-amino-5-phosphonovalerate) protected hippocampal neurons from cyanide-induced injury. Using a more direct method, i.e., replacement of oxygen by nitrogen, Dessi et al. (1992, 1993) showed protection by MK-801 as well as by riluzole, an inhibitor of glutamate release. A protective action of MK-801, but not of dihydropyridines, was also found by others using cerebral cortical neurons (Marcoux et al., 1989; Goldberg and Choi, 1993). The discrepancy with regard to the protective action of dihydropyridines should be viewed in light of the fact that in the present study nifedipine presumably acts indirectly by preventing glutamate release (T. Varming, unpublished observations), while in cortical neurons this is not possible since these cells are GABAergic (Drejer et al., 1987). Finally, several in vivo studies have demonstrated protective action of nifedipine and/or MK-801 in ischemia-induced neuronal damage (cf. Frandsen and Schousboe, 1993).

The finding that TTX protected under resting but not under depolarizing conditions is interesting. When extracellular potassium concentration is raised to 25 mM, the membrane potential will, according to the Nernst equation, set at ~−45 mV, and hence the sodium channels will be largely inactivated. Thus, under these conditions, there will be no involvement of action potentials, which otherwise might contribute to neurodegeneration. It may be speculated that the time course of azide-induced cell death may reflect a drift of the membrane potential towards less negative values, and that a certain threshold potential should be reached in order to activate the VSCC which regulate glutamate release and thus initiate activation of the degeneration cascade. This hypothesis could explain why lower doses of azide were needed for degeneration to occur, when the neurons were already depolarized.

It is concluded that this combined assay is a simple and reliable method for screening of compounds potentially protective against anoxic neuronal death. In the acute version described here, the assay may be used as a crude screening system, which should be effective in identifying potential neuroprotective calcium and sodium channel blockers, inhibitors of glutamate release, and glutamate receptor antagonists. Such compounds could become valuable neuroprotective therapeutics for the treatment of severe neurodegenerative conditions such as those seen following stroke.

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