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Mechanisms of sodium azide-induced changes in intracellular calcium concentration in rat primary cortical neurons

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Abstract

An intracellular calcium ($[Ca^{2+}]_i$) increase is involved in sodium azide (NaN_3)-induced neurotoxicity, an *in vitro* model of brain ischemia. In this study the questions of possible additional sources of calcium influx, besides glutamate receptor activation, and of the time-course of NaN_3 effects have been addressed by measuring $[Ca^{2+}]_i$ in rat primary cortical cultures with the FURA-2 method. Basal $[Ca^{2+}]_i$ of neuronal populations was concentration-dependently increased 30 min, but not 24 h, after a 10-min NaN_3 (3–30 mM) treatment; conversely, the net increase induced by electrical stimulation (10 Hz, 10 s) was consistently reduced. All the above effects depended on glutamate release and consequent NMDA receptor activation, since the NMDA antagonist MK-801 (1 μM) prevented them, and the spontaneous efflux of $[^3H]$-D-aspartate from superfused neurons was concentration-dependently increased by NaN_3. In single neuronal cells, NaN_3 application progressively and concentration-dependently increased $[Ca^{2+}]_i$ (to 177 ± 5% and 249 ± 7% of the controls, 4 and 12 min after a 10 mM-treatment, respectively). EGTA (5 mM) pretreatment reduced the effect of 10 mM NaN_3 (to 118 ± 5% at 4 min, and to 148 ± 10% at 12 min, respectively), while 1 μM cyclosporin A did not. Both MK-801 and CNQX (a non-NMDA glutamate antagonist, 10 μM) prevented NaN_3 effect at 4 min (to 147 ± 8% and 153 ± 5%, respectively), but not at 12 min after NaN_3 treatment. Conversely, 10 μM verapamil and 0.1 μM ω-conotoxin (L- and N-type calcium channel blockers, respectively) significantly attenuated NaN_3 effects at 12 min (to 198 ± 8% and 164 ± 5%, respectively), but not at 4 min; the P/Q-type calcium channel blocker, agatoxin, 0.3 μM, was ineffective. These findings show that the predominant source of calcium increase induced by NaN_3 is extracellular, involving glutamate receptor activation in a first step and calcium channel (mainly of the N-type) opening in a second step.
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Keywords: Sodium azide; Neuronal cultures; Intracellular calcium; Glutamate efflux; Glutamate antagonists; Calcium channel blockers

1. Introduction

Sodium azide (NaN_3) has many laboratory and industrial applications and is widely used as a preservative in aqueous laboratory reagents, as a pesticide, as a necessary component in the manufacturing of rubber as well as a gas-generating agent in automobile air bags. Its relevance to human health has become increasingly evident as the industrial production and consequent occupational exposure has grown (Chang and Lamm, 2003). Human toxicity of NaN_3 mainly resides in its potentially life-threatening, hypotensive effect, due to its direct vasodilator properties induced by activation of inward rectifier K^+ channels and Na-K-ATPase (Qamirani et al., 2006). Among the mechanisms of action reported for NaN_3 (Ando, 1990; Bennett et al., 1996; Tatarko and Bumpus, 1997; Shahidullah et al., 2002), the most relevant is cytochrome *c* oxidase-respiratory chain complex IV-inhibition (Leary et al., 2002). The latter mechanism makes NaN_3 an interesting tool in neurotoxicity studies. In fact, a treatment with NaN_3, either alone or in combination with the glycolysis blocker 2-deoxyglucose, can mimic the energy depletion which occurs during brain ischemia; such a protocol has been used to induce "chemical ischemia" in neuronal cultures (Varming et al., 1996; Jørgensen et al., 1999; Grammatopoulos et al., 2002, 2004), in brain slices (Djali and Dawson, 2001; Cavallini et al., 2005; Selvatici et al., 2006), as well as in *in vivo* experiments (Bennett et al., 1996; Vecsei et al., 2001). Our previous work, carried out in brain slices, assessed the effects of chemical ischemia a) at the presynaptic level, by measuring neurotransmitter release (Cavallini et al., 2005), b) at the transductional level, by analyzing the changes induced in the different isoforms of protein kinase C (Selvatici et al., 2006), and on the mitogen-activated protein kinase cascade (Siniscalchi

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et al., 2006). The roles played by glutamate (Glu) and nitric oxide, and the calcium-dependence of the observed effects were underlined and discussed in the above papers.

The increase in intracellular calcium ($[Ca^{2+}]_i$) due to excessive release of Glu and consequent over-stimulation of its receptors, mainly of the N-methyl-D-aspartate (NMDA) type (Choi, 1995; Phillis and O'Regan, 2003), is considered the cellular event that triggers the delayed neuronal death induced by ischemia (''excitotoxicity'': Rothman and Olney, 1987; Choi and Rothman, 1990; Choi, 1995; Kristian and Siesjo, 1998). Accordingly, the NaN_3-induced energy failure leads to a derangement of the cellular steady-state and to a reversible increase in $[Ca^{2+}]_i$, involving Glu receptors (Jørgensen et al., 1999). However, whether any additional/alternative mechanism(s), besides Glu receptor activation, underlies such an action is not fully known. We felt the latter question especially intriguing, since it has been pointed out that the diversity of Ca^{2+} entry sources may have different functions in various cellular phenomena (Furuyashiki et al., 2002; Berridge et al., 2003) and that the source of Ca^{2+} entry, not the Ca^{2+} load, is the main cause of the neurotoxic potential of Ca^{2+} ions (Sattler et al., 1998).

Thus, this study was designed to clarify such issue and to disclose possible differences in the mechanisms underlying early vs. delayed effects of NaN_3.

With the experiments reported here, performed in primary cultures of rat cortical neurons: (i) we examined the early (30 min after the treatment) and delayed (24 h after the treatment) NaN_3 effects on $[Ca^{2+}]_i$; (ii) we verified the Glu-releasing action of NaN_3 by determining the efflux of radiolabelled neurotransmitter; (iii) we investigated, in single neuronal cells, the origin of the observed increase in $[Ca^{2+}]_i$ using suitable pharmacological tools. Moreover, we discussed the effects observed with NaN_3 (''chemical ischemia'') also in comparison to our previous data obtained with another protocol widely used to mimic ischemic damage, namely Glu exposure in cortical neurons (Siniscalchi et al., 2005).

2. Materials and methods

2.1. Cell cultures and treatments

Sprague–Dawley rats were used within 24 h after birth. All the procedures were carried out in accordance with European Community and national laws and policies, and were approved by the Ethics Committee of the University of Ferrara.

Cell cultures were prepared as previously described (Beani et al., 1994). Plastic Nunc dishes (Ø 35 mm) coated with poly-L-lysine 20 μg/ml were used to plate the cells (1×10^6 per dish). For the $[Ca^{2+}]_i$ determination experiments (see below) rectangular (28 mm × 10 mm) or round (Ø 24 mm) glass coverslips were laid on the bottom of the dishes. Cells were cultured in a modified Neurobasal medium supplemented with Gibco B-27 serum-free supplement 2%, gentamycin sulphate 50 μg/ml and Glutamax 500 μM. Cytosine arabinoside 5 μM was added 48 h after plating to prevent glial cell proliferation. Correction of the volume (2 ml/dish) was made by adding 0.3–0.4 ml of fresh medium on the fourth day of incubation. The cultures were kept at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. Determination of intracellular calcium concentrations ($[Ca^{2+}]_i$)

At the 7th–11th day *in vitro*, one half of the medium was withdrawn and set aside and NaN_3 3–30 mM was added for 10 min; then the cells were rinsed with fresh medium and returned to the incubation medium that had been set aside. The other drugs were added during NaN_3 treatment and maintained throughout the entire experiment.

The average $[Ca^{2+}]_i$ changes were measured in neuronal populations consisting of about 10,000 cells (see Beani et al., 1994). Either immediately or 24 h after drug treatment, the culture medium was changed to a Krebs-Ringer buffer (KRB: 118 mM NaCl, 4.8 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM KH_2PO_4, 10 mM Hepes, 10 mM glucose, pH 7.4), the cells were loaded with 1 μM FURA-2/AM for 30 min at 37 °C, then the coverslip was gently removed from the bottom of the dish and inserted, at a 30° angle versus the excitation beam, into a fluorimeter cuvette containing 2 ml of KRB. Fluorescence recording and subsequent $[Ca^{2+}]_i$ calculation were performed using a LS-50 Perkin Elmer luminescence spectrometer. After subtracting background fluorescence, caused by the coverslip plus the poly-L-lysine coat and the monolayer of cells, $[Ca^{2+}]_i$ was calculated by measuring the fluorescence intensity (502 nm emission) with excitation at 340 and 380 nm, according to the following equation (Grynkievicz et al., 1985):

$$[Ca^{2+}]_i = \frac{K_d(R - R_{min})}{(R_{max} - R)}\left(\frac{F_0}{F_s}\right)$$

where K_d is 225 nM, R the ratio 340:380 of indicator fluorescence, R_{min} the ratio 340:380 of FURA 2 in the Ca^{2+}-free solution, R_{max} the ratio of FURA 2 in the presence of saturating Ca^{2+}concentration (1 mM $CaCl_2$) and F_0/F_s is the ratio of 380 nm excitation fluorescence at zero and saturating Ca^{2+} levels.

The electrically evoked increase in $[Ca^{2+}]_i$ was measured as previously described (Beani et al., 1994). Field electrical stimulation was performed by applying rectangular pulses of alternate polarity (duration 1 ms, intensity 30 mA, frequency 10 Hz) delivered from a constant-current generator. The electrically evoked increase in $[Ca^{2+}]_i$ in 10 s was estimated by subtracting the basal (b) nM $[Ca^{2+}]_i$ from the $[Ca^{2+}]_i$ peak value (St); this difference (St − b) was expressed in nM.

In other experiments, changes restricted to cell body $[Ca^{2+}]_i$ of single neurons were determined according to Tognetto et al. (2001). Cells grown onto round glass coverslips (Ø 24 mm) were placed in KRB solution and were loaded with FURA-2 AM 2 μM, for 30 min at 37 °C. The glass coverslip served as the bottom of a teflon chamber, placed on the stage of a Nikon Eclipse TE 300 microscope. Changes in 340: 380 ratio were recorded with a dynamic image analysis system (Laboratory Automation 2.0; RCS, Florence, Italy). Neurons were allowed

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