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NMDA receptors contribute to the survival promoting effect of high potassium in cultured cerebellar granule cells

María Armanino, María C. Gravielle, M. Natalia Marangoni, Mónica L. Fiszman *

Instituto de Investigaciones Farmacológicas, CONICET, Buenos Aires, Argentina

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Abstract

The present report further analyzes the survival promoting effect of high potassium, a condition that mimics neural activity in cultured cerebellar granule cells, an excellent model to study trophic mechanisms induced by depolarization and trophic factors. We found that the survival promoting effect measured at 7 days in vitro (DIV 7) of depolarizing potassium concentrations (25 mM KCl), added at DIV2, is partially prevented by adding at DIV 2 the non-competitive NMDA blocker MK801 (10 μM). The concentration of MK801 used blocks completely the survival promoting effect of a supramaximal effective concentration of NMDA (100 μM). The addition at DIV 2 of anti-brain derived neurotrophic factor (anti-BDNF) antibody, failed to modify the effect of high potassium. The present report provides evidences that in cultured cerebellar granule cells, high potassium-induced survival promoting effect is due in part by the activation of NMDA receptors. The effect does not require the presence of BDNF.

Keywords: Depolarization; Brain-derived neurotrophic factor; Cerebellar granule cells; NMDA; Development

Neural activity exerts a trophic role during development and maturation of the central nervous system; the development of the cerebellum is modulated by neural activity (Burgoyne et al., 1993; Fiszman and Schousboe, 2004; Gallo et al., 1987). One of the in vitro conditions that mimics neural activity is the exposure to depolarizing potassium concentrations. The effects of high potassium on the development of cerebellar granule cells are well documented and vary according with the stage of cell maturation. They include, increase in proliferation when added within the first 24 h in vitro (Borodinsky and Fiszman, 1998), differentiation of early postmitotic cerebellar granule cells (day 2 in vitro; DIV 2) and a long-term survival increase of the mature phenotype (Borodinsky et al., 2002). In all cases a calcium influx triggered by voltage-gated calcium channels activation is the main mechanism of action (Borodinsky et al., 2002; Dolmetsch et al., 2001; Gallo et al., 1987). Furthermore, NMDA receptors activation promotes long-term survival of cerebellar granule cells (Balázs et al., 1989), a process that involves calcium-dependent cascades (Hack et al., 1993).

Stimulation of NMDA receptors in granule cells facilitates the availability of BDNF by increasing BDNF-mRNA and the amount of protein in the culture media (Bessho et al., 1993; Bhave et al., 1999; Marini et al., 1998) a neurotrophin that plays a crucial role in the development of many regions of the brain (Kaplan and Miller, 2000), including the cerebellum (Segal et al., 1995), and supports survival of cerebellar granule cells (Gao et al., 1995).

To further understand neuronal survival mechanisms, we explored the contribution of the endogenous released glutamate in high potassium-induced increase in young cerebellar granule cells survival grown under serum-free conditions for up to 7 days. We found that part of the trophic effect induced by high potassium is through NMDA receptor activation. We also found that in contrast to what was

Abbreviations: BDNF, brain derived neurotrophic factor; NMDA, *N*-methyl-D-aspartic acid; DIV, days in vitro; MTT, 3-[4,5-dimethylthia-zol-2yl]-2,5-diphenyl tetrazolium bromide; BSA, bovine serum albumin

* Corresponding author at: Department of Physiology and Biophysics, BSB221, Georgetown University, School of Medicine, 3900 Reservoir Road, Washington, DC 20007, USA. Tel.: +1 202 687 8096; fax: +1 202 687 1447.

E-mail address: mlf29@georgetown.edu (M.L. Fiszman).

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described with NMDA, released glutamate failed to increase the concentration of BDNF.

We postulate that the survival promoting effect of high potassium is due in part to endogenously released glutamate that activates NMDA receptors.

1. Experimental procedure

1.1. Cerebellar granule cells cultures

Animal care and use was in agreement with institutional guidelines and the GCULA (1996). Cell suspensions were obtained from cerebella of 6–8-day-old Sprague–Dawley rats and placed in Krebs–Ringer solution supplemented with 33 mM glucose. The tissue was cut into 1 mm pieces and incubated in saline containing 0.025% trypsin (Sigma, St. Louis, MO), 1.2 mM $MgSO_4$ and 3 mg/ml bovine serum albumin (BSA, Sigma) for 15 min at 37 °C, with continuous agitation. Enzymatic digestion was stopped with 1 mg/ml ovomucoid (trypsin inhibitor, Sigma) and the tissue was mechanically dissociated with Pasteur pipettes of two different diameters (25 strokes), in saline containing ovomucoid and 0.01% DNAse (Roche, Indianapolis, IN). The cell suspension obtained was sedimented at 150 g for 10 min and the pellet resuspended in Neurobasal media containing 5.4 mM KCl, supplemented with B27 (Neurobasal, Gibco, Grand Island, NY). Cells were seeded onto 96 multiwells (300,000 cells/well) or 24 multiwells (300,000 cells/well), precoated with poly-D-lysine (MW 300,000, Sigma).

1.2. Cell counting

Cerebellar granule cells were seeded on 24 multiwells and allowed to grow for 7 days. Then, cells were counted in three fields of quadruplicate samples (1 mm^2) using an ocular with a grid, under phase-contrast optics at 400× magnification. Vital neurons were identified as phase-bright small rounded cells (6–7 μm diameter) with two or more processes which, in preliminary experiments, revealed positive to the neuronal marker Tetanus Toxin Fragment C (Borodinsky and Fiszman, 1998).

1.3. MTT assay

Cerebellar granule cells were seeded on 96 multiwells and, at DIV 7, 10 μl of 5 mg/ml 3-[4,5-dimethylthia-zol-2yl]-2,5-diphenyl tetrazolium bromide (MTT, Sigma) were added to each well and the cells were incubated for 4 h in a humidified atmosphere (37 °C, 5% CO_2). One hundred microliters of solubilization solution (10% SDS in 10 mM HCl) were added to each well and the plates allowed to stand overnight in the incubator (37 °C, 5% CO_2). The absorbance was measured using a scanning multiwell spectrophotometer (ELISA reader) at a wavelength of 570 nm.

1.4. Drug treatments

Agonists: NMDA (10–30–100 μM), 25 mM KCl, BDNF (20–100 ng/ml) were added at DIV 2. Antagonists: Anti BDNF antibody (10 μg/ml) and MK801 (10 μM) were added 30 min before the addition of agonists. All drugs were purchased from Sigma (St. Louis, MO) except for BDNF and anti-BDNF that were purchased from Promega (Madison, WI, USA).

1.5. Data and statistics

Data are expressed as the mean ± S.E.M. Statistical significance was examined by a one-way ANOVA with a Bonferroni post-test where $P < 0.05$ was considered significant.

2. Results

Cerebellar granule cells were obtained from 6- to 8-day-old rats and were treated at DIV 2 with 100 μM NMDA or high potassium. There was a significant increase in cells number with both treatments, as determined by cell counting (Fig. 1A, $P < 0.01$, ANOVA) and MTT absorbance at 570 nm. (Fig. 1B, $P < 0.006$, ANOVA).

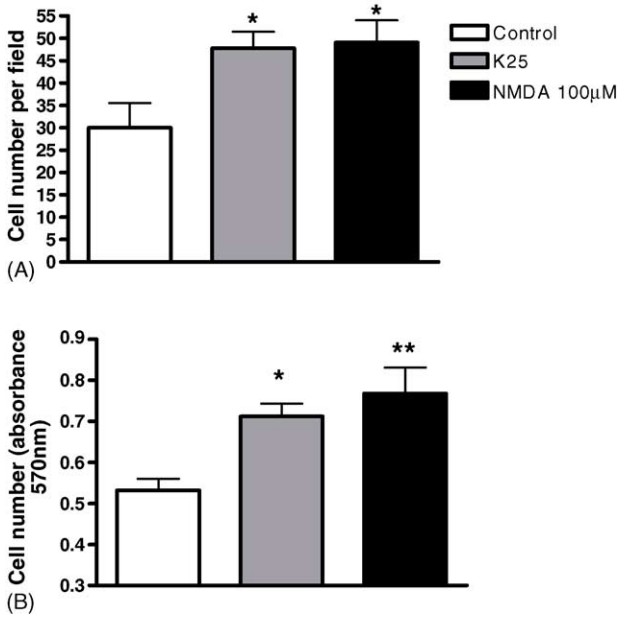

Fig. 1. Effect of 100 μM NMDA and 25 mM KCl (25 K) on (A) neuronal number measured by cell counting ($P < 0.01$, ANOVA) and (B) assessed by MTT assay ($p < 0.006$, ANOVA). (A) Cells were counted in three fields of quadruplicate samples (1 mm^2) using an ocular with a grid, under phase-contrast optics at 400× magnification. (B) Cultures were incubated with MTT for 4 h, and then exposed to solubilization solution overnight, absorbance at 570 nm was measured. In all assays NMDA, 25 mM KCl or saline were added 2 days after seeding. Data are represented in A as cells per field and in B as absorbance units at 570 nm, and represent mean ± S.E.M. of 4–10 experiments. Statistical comparisons: $^{*}P < 0.05$; $^{**}P < 0.001$ control vs. treatment.

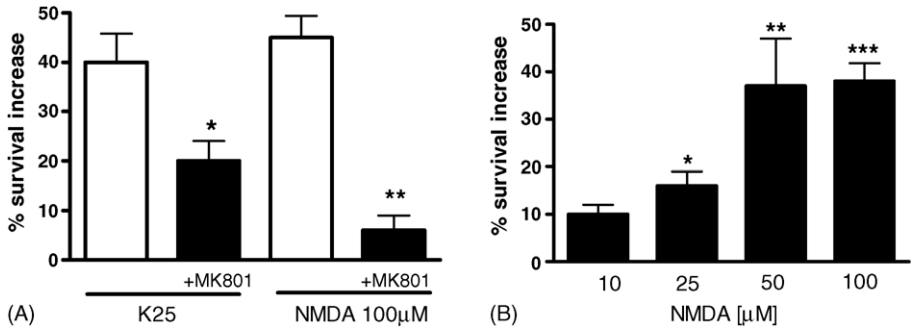

Fig. 2. Contribution of NMDA receptors to 25 mM KCl survival promoting effect. (A) The increase in survival induced by high potassium was partially blocked by 10 μM MK801. MK801 completely blocked the effect induced by 100 μM NMDA ($P < 0.001$ ANOVA). (B) The survival promoting effect of NMDA is concentration-dependent ($P < 0.0005$, ANOVA) being 100 μM the maximal effective concentration. Data are expressed as percentage increase in absorbance at 570 nm over saline (control) treated cultures. Mean ± S.E.M. of 3–10 experiments. Statistical comparisons: $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$ control vs. treatment. Ordinates: data are expressed as percentage increase in the survival over untreated (plated with 5.4 mM KCl) samples.

To test the contribution of NMDA receptors to the survival promoting effect of high potassium, we measured the effect of 25 mM KCl in the presence of 10 μM MK801, a selective NMDA blocker. We found that MK801 blocked by about 50% the effect of high potassium. On the other hand, MK801 blocked completely the survival promoting effect of 100 μM NMDA (Fig. 2A), a maximal effective concentration of NMDA (Fig. 2B). Interestingly, we found that the threshold concentration of NMDA is lower than that obtained in cells plated with serum-containing media (Fiszman, unpublished observations). To evaluate the contribution of BDNF to the effect observed with 25 mM KCl we test the effect of 25 mM KCl in the presence of a blocking BDNF antibody. Anti-BDNF failed to decrease the effect observed with 25 mM KCl while it blocked partially the effect of NMDA (Fig. 3A). As expected the antibody effectively blocked the effect of exogenously applied BDNF (Fig. 3B).

3. Discussion

Cerebellar cultures obtained from 6- to 8-day-old rodent pups express a wide variety of excitatory amino acids receptors and almost the majority of the cells in the culture are granule cells that synthesize glutamate (Carlson et al., 1998). The present report provides evidences that endogenously released glutamate may contribute to the survival promoting effect induced by depolarizing potassium concentrations by activating NMDA receptors in this cell preparation.

It is known that voltage-gated calcium channel activation mediates the trophic effect induced by high potassium in granule cells (Borodinsky et al., 2002; Dolmetsch et al., 2001; Gallo et al., 1987). The effect induced by voltage-gated calcium channel activation during the first days in vitro is direct and it is not due to glutamate release. The lack of effect of glutamate in these cultures is explained by an incapability of these immature cells to synthesize glutamate (Gallo et al., 1987). Furthermore, two effects mediated by 25 mM KCl during the first days in vitro, an increase in immature cerebellar cell proliferation and young cerebellar granule cells differentiation are independent of endogenous glutamate. Interestingly, both effects are mediated by the ras/MAPK pathway (Borodinsky and Fiszman, 1998; Borodinsky et al., 2002), a signal transduction cascade involved in trophic events (Kaplan and Miller, 2000).

We hypothesize that the survival promoting effect of high potassium is accomplished in two steps. First, the

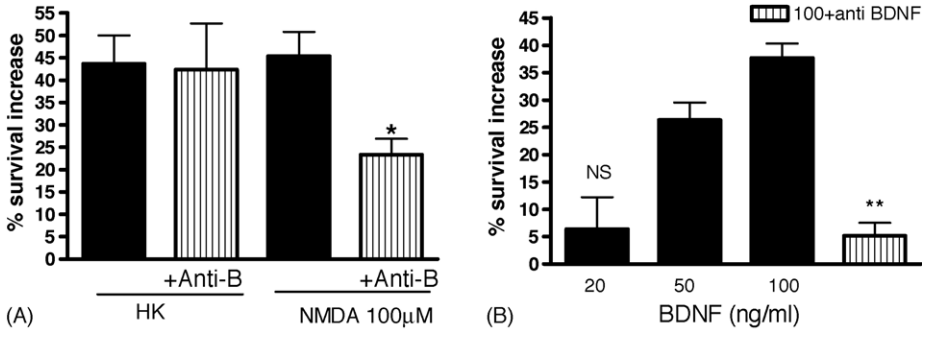

Fig. 3. (A) Effects of treatment with anti-BDNF (Anti-B) antibodies (10 μg/ml) on high potassium (25 mM KCl, HK) and NMDA (100 μM)-induced increase in survival. (B) Dose-response curve to BDNF in the absence and presence of 10 μg/ml of anti-BDNF antibody. Mean ± S.E.M. of three experiments. Statistical comparisons: $^{*}P < 0.05$; $^{**}P < 0.01$ control vs. treatment. Ordinates: data are expressed as percentage increase in the survival over untreated (plated 5.4 mM KCl) samples.

calcium influx triggered by voltage-gated calcium channels activation (at DIV 2, when potassium is added) promotes the maturation of cerebellar granule neurons. Secondly, once they become more differentiated (DIV 3–4, see Borodinsky et al., 2002) and capable to synthesize glutamate, the depolarization induced by 25 mM KCl triggers the release of glutamate and activates NMDA receptors with the concomitant survival increase detected at DIV 7.

NMDA receptors contribute only partially to the effect of high potassium since the non-competitive antagonist MK 801, at a dose that completely blocked the survival-promoting effect of supramaximal effective concentrations of NMDA, induced only a 50% blockade of high potassium survival promoting effect.

NMDA protects young cerebellar granule cells against low K-induced cells loss (Balázs et al., 1989, and our results) and the effect is partially mediated by BDNF released from these cells (Bhave et al., 1999; Marini et al., 1998 our results). However, we found that the effect of high potassium is independent of BDNF since exposure to the anti-BDNF antibody failed to decrease the effect of high *K* in conditions that blocked the effect of exogenous BDNF and the survival promoting activity of NMDA (Fig. 3). It is possible that endogenously released glutamate is not as effective as NMDA to increase the availability of BDNF in the culture. In this regard, it has been reported in this model that glutamate was not effective in increasing BDNF-mRNA while NMDA and other ionotropic agonists did (Bessho et al., 1993). The interplay between different glutamate receptor subtypes may take place, antagonizing the effect of NMDA receptor activation, as was described in the striatum with group II metabotropic receptor agonists (Hanania and Johnson, 1999). Further studies will elucidate this matter. We conclude that the survival promoting effect of high potassium on cerebellar granule cells is mediated in part through NMDA receptor activation. The survival promoting effect of high potassium does not require BDNF.

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