Li^+ protects nerve cells against destabilization of Ca^{2+} homeostasis and delayed death caused by removal of external Na^+

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Abstract In experiments with fura-2 loaded cultured rat cerebellar granule cells we have compared the changes in $[Ca^{2+}]_i$ homeostasis produced by replacement of external Na^+ with the organic cation *N*-methyl-D-glucamine (NMDG) or Li^+. The Na^+/NMDG replacement caused an increase in baseline $[Ca^{2+}]_i$ and a considerable delay in $[Ca^{2+}]_i$ recovery following a glutamate (Glu) pulse in almost all the cells. In contrast Na^+/Li^+ replacement usually did not change baseline $[Ca^{2+}]_i$ and produced only a small (if any) delay in the post-glutamate $[Ca^{2+}]_i$ recovery. Previously [Storozhevykh et al. (1998) FEBS Lett. 431, 215–218] we revealed that perturbation of $[Ca^{2+}]_i$ homeostasis caused by Na^+/NMDG replacement cannot be explained by a reversal of the Na^+/Ca^{2+} exchange but is mainly due to Ca^{2+} influx through NMDA channels activated by Na^+ dependent release of endogenous excitatory amino acids ('reversed Glu uptake'). In the present work we confirmed this conclusion and obtained evidence suggesting that in contrast to NMDG Li^+ interferes with the 'reversed Glu uptake' triggered by removal of external Na^+. Thus it has been shown that the addition of Li^+ (20 mM) to a Na^+-free NMDGcontaining solution suppressed both the perturbation of $[Ca^{2+}]_i$ homeostasis and delayed neuronal death caused by Na^+/NMDG replacement. Li^+ is also able to abolish the $[Ca^{2+}]_i$ response induced by PDC which at high concentrations (>200 μM) is shown to stimulate the release of endogenous Glu. In contrast to Na^+/Li^+, Na^+/NMDG replacement greatly enhances $[Ca^{2+}]_i$ increase caused by PDC. Control experiments showed that Na^+/Li^+ replacement does not decrease the $[Ca^{2+}]_i$ response to the Glu pulse. Therefore we concluded that a considerable quantitative difference between the effects of Na^+/NMDG and Na^+/Li^+ replacements on both $[Ca^{2+}]_i$ homeostasis and cell viability resulted mainly from the ability of Li^+ to attenuate the release of endogenous Glu in response to the removal of external Na^+.

Key words: Neurotoxicity; Glutamate; Na^+/Ca^{2+} exchange; Lithium; Cultured neuron

1. Introduction

Until recently it was widely accepted that all the changes in intracellular Ca^{2+} homeostasis caused by the removal of external Na^+ (its replacement with organic cations or Li^+) result from Ca^{2+} influx mediated by the plasma membrane Na^+/Ca^{2+} exchanger (reversed Na^+/Ca^{2+} exchange) [1,2]. Our experiments with cultured rat cerebellar granule cells showed, however, that for nerve cells this notion should be reevaluated [3]. We found that both the increase in basal $[Ca^{2+}]_i$ and the delay in the post-glutamate $[Ca^{2+}]_i$ recovery caused by replacement of external Na^+ with *N*-methyl-D-glucamine (NMDG) resulted mainly from the $[Ca^{2+}]_i$ influx through NMDA-sensitive glutamate (Glu) channels activated by a Na^+-dependent release of endogenous Glu (the so-called reversed Glu uptake) [4,5]. This reversed Glu uptake is mediated by Glu transporters which in normal conditions are responsible for a removal of excessive Glu from the extracellular space. It is established, however, that both the Na^+/Ca^{2+} exchange and Na^+-dependent Glu transport can be also reversed by the Na^+/Li^+ substitution [1,2,4]. Meanwhile in nerve cells this replacement produces only a very small deregulation of $[Ca^{2+}]_i$ homeostasis as compared to that caused by Na^+/NMDG replacement [6,7]. The aim of the present study was to clarify the reason for this surprising difference between the effects of external Na^+ substitution with NMDG and Li^+ on neuronal $[Ca^{2+}]_i$ homeostasis.

The results obtained and some literary data strongly suggest that in contrast to NMDG, Li^+ suppresses the reversed Glu uptake responsible for neuronal $[Ca^{2+}]_i$ increase in response to a removal of external Na^+.

2. Materials and methods

Dissociated cerebellar granule cell cultures were prepared from the cerebella of 7–8 day old Wistar rats using the procedure described in [8]. The cells were grown in MEM (Sigma) containing: 10% fetal bovine serum, 10 mM HEPES, 12 mM $NaHCO_3$, 2 mM L-glutamine, 0.2 U/ml insulin, 0.6% glucose, 25 mM KCl and 2.5 μM Ara-C (36.5°C+5%CO_2). The experiments were carried out on 7–9 day cell cultures. $[Ca^{2+}]_i$ was measured in neurons loaded with the fluorescent dye fura 2/AM (5 μM) for 1 h at 37°C in the above medium. For intracellular pH (pH_i) measurements, the H^+-sensitive dye BCECF/AM (10 μM for 1 h) was used. Than the cells were washed out with a control HEPES-buffered salt solution (HBSS) and placed into an experimental chamber. The chamber was mounted on a Nikon inverted-stage microscope connected with a spectrofluorimeter (SPEX, NJ, USA) equipped with a dual mirror chopping mechanism with a specialized optical configuration to allow rapid alteration (100 Hz) between two excitation wavelengths. The fluorescence of Ca^{2+}-sensitive dye fura 2 was measured using excitation filters of 340 nm and 380 nm and an emission filter at 510 nm. The fluorescence of BCECF was measured using excitation wavelength at 488 nm and emission filter at 535 nm. The buffers for $[Ca^{2+}]_i$ calibration were prepared as described in [9]. The HBSS contained (mM): 145 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 20 HEPES, 5 glucose, 10 sucrose, pH 7.4. In a Na^+-free solution NaCl was replaced with 140 mM NMDG (pH was adjusted with HCl) or 140 mM LiCl (pH was adjusted with LiOH). To study the effect of Li^+ on the $[Ca^{2+}]_i$ response induced

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Abbreviations: $[Ca^{2+}]_i$ and $[Na^+]_i$, cytosolic Ca^{2+} and Na^+ concentration; Fura-2/AM, acetoxymethyl ester of Fura-2; BCECF, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester; Ara-C, cytosine arabinoside; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NMDA, *N*-methyl-D-aspartate; Glu, glutamate; AP-5, 2-amino-5-phosphonopentanoic acid; NMDG, *N*-methyl-D-glucamine; HBSS, HEPES-buffered salt solution; PDC, L-*trans*-pyrrolidine-2,4-dicarboxylate

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by Na^+/NMDG replacement, 20 mM NMDG was replaced by 20 mM LiCl.

Cell survival was assessed by counting survival neurons using trypan blue exclusion staining [10]. In these experiments nerve cells were treated for 30 min with a Na^+-free NMDG or/and Li^+-containing solution, then washed out and transferred to the HBSS medium. 4 h later, 0.4% trypan blue was added to each petri dish with cultured cells and the number of viable (trypan blue excluding) and non-viable (trypan blue containing) neurons was counted. At least three dishes (6–10 fields/dish, 500 cells) were used for each Na^+/NMDG, Na^+/Li^+ or Na^+/NMDG+Li^+ replacements.

Fura 2/AM and BCECF/AM was purchased from Molecular Probes (USA). All the other chemicals were from Sigma or Fisher Chemical (USA).

3. Results and discussion

In agreement with the previous report [3], the replacement of Na^+ with NMDG in the control solution produced a reversible increase of baseline $[Ca^{2+}]_i$ in the majority (74%) of tested cells ($n = 38$). In contrast, Na^+/Li^+ replacement did not normally affect basal $[Ca^{2+}]_i$ (Fig. 1A). A small $[Ca^{2+}]_i$ increase in response to Na^+/Li^+ replacement was observed only in rare cases (8%). Na^+/NMDG replacement just after the termination of a 1 min Glu pulse caused either a delay in $[Ca^{2+}]_i$ recovery (Fig. 1B) or even a secondary increase of $[Ca^{2+}]_i$ (Fig. 2C). In contrast, Na^+/Li^+ substitution following a 1 min pulse produced only a very small delay in $[Ca^{2+}]_i$ decay (Fig. 1B). A qualitatively similar difference between the effects of Na^+/NMDG and Na^+/Li^+ replacements on the post-glutamate $[Ca^{2+}]_i$ dynamics was reported in cultured hippocampal neurons by Koch and Barish [6].

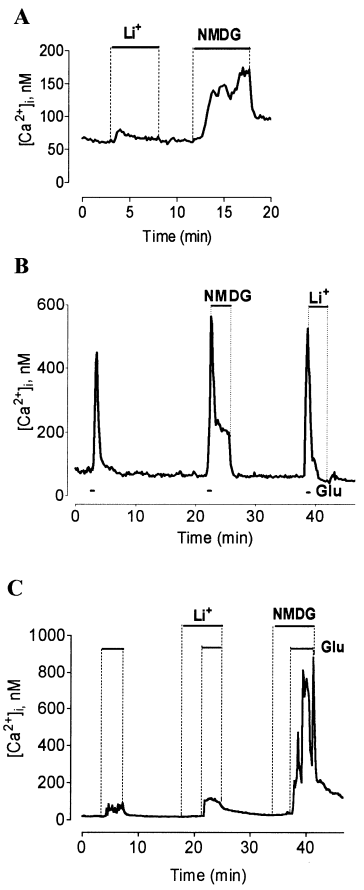

Fig. 1. The difference between the effects of Na^+/NMDG and Na^+/Li^+ replacements on (A) basal $[Ca^{2+}]_i$, (B) $[Ca^{2+}]_i$ following 1 min Glu (100 μM) pulse and (C) the amplitude of the $[Ca^{2+}]_i$ responses evoked by 1 μM Glu applications in individual cultured cerebellar granule cells (representatives of 22 experiments). NMDG = equimolar replacement of external Na^+ with *N*-methyl-D-glucamine. Li^+ = equimolar replacement of external NaCl with LiCl. Glu = 100 μM (B) or 1 μM (C) glutamate in Mg^{2+}-free, 10 μM glycine-containing solution.

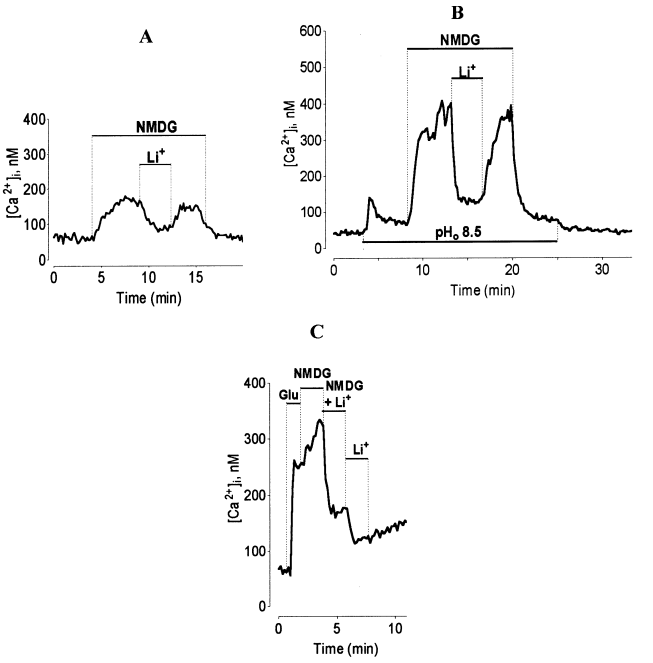

Fig. 2. The inhibitory effect of 20 mM Li^+ on the $[Ca^{2+}]_i$ response induced by Na^+/NMDG replacement at rest (A: pH 7.4, B: pH 8.5) and following a 1 min Glu pulse (C) (representatives of 12 experiments). Glu = 10 μM glutamate in Mg^{2+}-free, 10 μM glycine-containing solution. NMDG = equimolar replacement of external Na^+ with *N*-methyl-D-glucamine. Li^+ = equimolar replacement of external NaCl with LiCl. NMDG+Li^+ = 120 mM NMDG+20 mM LiCl.

Fig. 2A,C demonstrates that the changes in $[Ca^{2+}]_i$ caused by Na^+/NMDG replacement can be also suppressed by the addition of Li^+ (20 mM) to the NMDG-containing solution. In Fig. 2B, the $[Ca^{2+}]_i$ response to Na^+/NMDG substitution was enhanced by an increase in external pH (pH_o) from 7.4 to 8.5. Earlier [3] we proposed that this effect of pH_o elevation is due to both the potentiation of the NMDA receptors activation [11] and the blockade of the plasma membrane Ca^{2+}/H^+ pump. The addition of 20 mM Li^+ to a NMDG solution caused an effective and reversible suppression of this potentiated $[Ca^{2+}]_i$ response.

What is the reason for this similarity in the inhibitory effects of NMDA receptor antagonists and Li^+ on perturbations of $[Ca^{2+}]_i$ homeostasis caused by Na^+/NMDG replacement?

One possibility is that Li^+ added to the external solution may decrease the activation of NMDA channels by endoge-

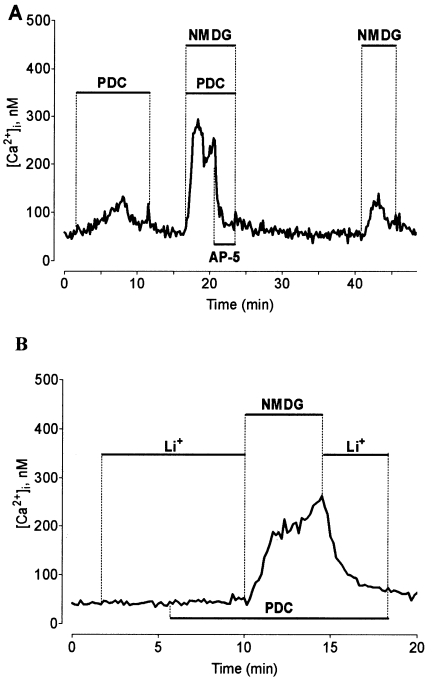

Fig. 3. The difference between the effects of Na^+/NMDG and Na^+Li^+ replacements on the $[Ca^{2+}]_i$ response induced by PDC. A: The NMDA receptor antagonist AP-5 abolishes the $[Ca^{2+}]_i$ response evoked by PDC (200 μM) application combined with Na^+/NMDG replacement (representative of four experiments). B: Na^+/Li^+ replacement in contrast with that of Na^+/NMDG prevents $[Ca^{2+}]_i$ elevation induced by PDC (representative of three experiments).

nous EAA. To test this assumption, we examined the effects of Li^+ on the $[Ca^{2+}]_i$ response induced by a small (1 μM) concentration of Glu (Fig. 1C). As seen, replacement of Na^+ with Li^+ did not reduce the Glu-induced $[Ca^{2+}]_i$ elevation, while Na^+/NMDG substitution greatly increased the $[Ca^{2+}]_i$ response elicited by the same small Glu concentration. This was evidently due to the fact that in a Na^+-free medium the Ca^{2+} influx induced by small Glu concentrations was not counterbalanced by Na^+/Ca^{2+} exchange-mediated Ca^{2+} extrusion. This observation indicates that the $[Ca^{2+}]_i$ response induced by Na^+/NMDG replacement should be considered as a result of reversed Glu uptake combined with Na^+/Ca^{2+} exchange inhibition.

Another explanation of the inhibitory effect of Li^+ on the $[Ca^{2+}]_i$ response induced by Na^+/NMDG replacement may be that Li^+ may antagonize the reversed Glu uptake. To test this possibility, we used L-*trans*-pyrrolidine-2,4-dicarboxylate (PDC), a competitive inhibitor of Glu uptake with low affinity for Glu receptors [12]. It was found that at high concentrations ($\geq$ 200 μM) PDC is able to stimulate the Na^+-dependent release of endogenous EAA both in nerve and glial cells [13,14]. In Fig. 3A, 200 μM PDC alone produced only a small reversible increase in the baseline $[Ca^{2+}]_i$. However, in combination with Na^+/NMDG replacement PDC evoked a significant increase in $[Ca^{2+}]_i$ which was effectively abolished by the competitive NMDA receptor inhibitor AP-5 (100 μM). This evidently indicates that the $[Ca^{2+}]_i$ response under consideration was induced by the Ca^{2+} influx through the NMDA receptor channels activated by endogenous EAA. In Fig. 3B, PDC application was combined alternatively with Na^+/Li^+ and Na^+/NMDG replacements. At the beginning, the Na^+/Li^+ substitution alone did not affect the baseline $[Ca^{2+}]_i$. The addition of PDC to an Li^+-containing (Na^+-free) solution failed to increase $[Ca^{2+}]_i$. However, further replacement of Li^+ by NMDG evoked a pronounced $[Ca^{2+}]_i$ response which was immediately abolished after a return of the cell to the Li^+-containing medium. Thus, Li^+ cancelled the $[Ca^{2+}]_i$ increase produced by the release of endogenous EAA.

Koch and Barish [6] believe that the difference between the effects of Na^+/NMDG and Na^+/Li^+ replacements on $[Ca^{2+}]_i$ homeostasis results mainly from the fact that the former replacement, in contrast with the latter, blocks the Na^+/H^+ antiport and thus decreases pH_i. Previously [3] we found that the blockade of NMDA receptors by AP-5 application abolished the effects of the Na^+/NMDG replacement on $[Ca^{2+}]_i$ but not on pH_i. Similar results were obtained in current experiments after the addition of 20 mM Li^+ to a NMDG Na^+-free medium: Li^+ suppressed the increase in $[Ca^{2+}]_i$ but did not attenuate pH_i reduction caused by the replacement of Na^+ with NMDG (data not shown).

Finally, we examined the effect of Li^+ on delayed neuronal death induced by a removal of Na^+ from the external solution. Earlier [3] it was shown that a 30 min exposure of cerebellar granule cells in a Na^+-free NMDG-containing solution induced delayed neuronal death comparable to that caused by Glu (100 μM) application. Fig. 4 shows that in contrast with Na^+/NMDG replacement, substitution of external Na^+ with Li^+ did not affect cell viability. It can also be seen that the addition of 20 mM Li^+ to a NMDG solution noticeably decreased the percentage of dead cells counted 4 h after this 30 min treatment. Thus, Li^+ proved to be able to protect nerve cells against the toxic effect of Na^+/NMDG replacement.

This result should be compared to that obtained by other authors in studies of low Na^+-induced neurotoxicity. Thus, Takahashi and Hashimoto [5] showed that a 30 min exposure of cultured hippocampal slices to a 3.6 mM Na^+ choline-containing solution led to the almost complete degeneration of neurons in the CA1 region within 24 h. This low-Na^+ neurotoxic effect (see also [15]) was significantly decreased by the NMDA receptor channel antagonist MK-801 (1–3 μM). In parallel experiments, it was established that 30 min exposure of slices to 3.6 mM Na^+ caused an about 40-fold increase in the extracellular Glu concentration. In studies with co-cultures of rat hippocampal neurons and glia, Volterra et al. [13] showed that the PDC-induced Glu release and delayed neuronal death can be fully prevented by the replacement of external Na^+ with Li^+. The replacement of Na^+ with choline

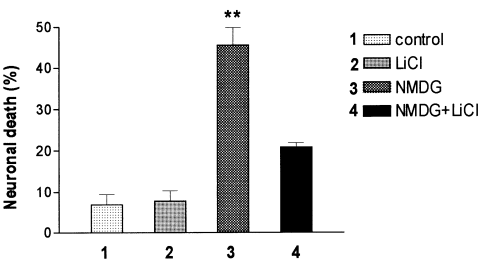

Fig. 4. Li^+ (20 mM added to NMDG-containing solution) protects nerve cells against delayed death caused by Na^+/NMDG replacement. The percentage of non-viable cells was counted 4 h after 30 min exposure in Na^+-free NMDG and/or Li^+-containing solutions. Note that Na^+/Li^+ replacement in contrast with that of Na^+/NMDG does not produce any increase in the percentage of dead cells. $^{**}P < 0.05$ in comparison with control.

failed to inhibit Glu release and did not protect nerve cells against the PDC-induced injury. Na^+/Li^+ replacement also prevented the PDC-induced depolarization of the neuronal membrane.

All these data are in good agreement with our suggestion that Li^+ is able to suppress Na^+-dependent release of Glu ('reversed Glu uptake') and thus to abolish both the Ca^{2+} influx through NMDA channels and delayed neuronal death caused by the removal of Na^+ from the external solution.

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References

[1] Blaustein, M. (1996) FEBS Lett. 431, 215–218.
[2] Reeves, J. (1990) in: Intracellular Calcium Regulation (Bonner, F., Ed.), pp. 306–307, Alan R. Liss, New York.
[3] Storozhevykh, T., Grigortsevich, N., Sorokina, E., Vinskaya, N., Vergun, O., Pinelis, V. and Khodorov, B. (1998) FEBS Lett. 431, 215–218.
[4] Attwell, D., Barbour, B. and Szatkowski, M. (1993) Neuron 11, 401–407.
[5] Takahashi, M. and Hashimoto, M. (1996) Brain Res. 735, 1–8.
[6] Koch, R.A. and Barish, M.E. (1994) J. Neurosci. 14, 2585–2593.
[7] Khodorov, B., Pinelis, V., Vergin, O., Storozhevykh, T., Fajuk, D., Vinskaya, N., Arsenjeva, E., Khaspekov, L., Lyzin, A., Isaev, N., Andreeva, N. and Victorov, I. (1995) FEBS Lett. 371, 249–252.
[8] Andreeva, N., Khodorov, B., Stelmashuk, E., Cragoe, E. and Victorov, I. (1991) Brain Res. 22, 150–157.
[9] Kiedrowski, L., Brooker, G., Costa, E. and Wroblewski, J.T. (1994) Neuron 12, 295–300.
[10] Dubinsky, J.M. (1993) J. Neurosci. 13, 623–631.
[11] Traynelis, S.F. and Cull-Candy, S.G. (1990) Nature 345, 347–350.
[12] Bridges, R., Stanley, M., Anderson, M., Cotman, C. and Chamberlin, A. (1991) J. Med. Chem. 34, 717–725.
[13] Volterra, A., Bezzi, P., Rizzini, B.L., Trotti, D., Ullensvang, K., Danbolt, N.C. and Racagni, G. (1996) Eur. J. Neurosci. 8, 2019–2028.
[14] Griffiths, R., Dunlop, J., Gorman, A., Senior, J. and Grieve, A. (1994) Biochem. Pharmacol. 47, 267–274.
[15] Parsons, J., Wallis, A., Panizzon, L. and Wasterliain, C. (1992) Brain Res. 595, 141–144.