Dramatic effects of external alkalinity on neuronal calcium recovery following a short-duration glutamate challenge: the role of the plasma membrane Ca^{2+}/H^+ pump

B. Khodorov[a,*], V. Pinelis[b], O. Vergun[a], T. Storozhevykh[b], D. Fajuk[a], N. Vinskaya[b], E. Arsenjeva[b], L. Khaspekov[c], A. Lyzin[c], N. Isaev[c], N. Andreeva[c], I. Victorov[c]

[a]*Institute of General Pathology and Pathophysiology, Russian Academy of Medical Sciences, Moscow, Russian Federation*
[b]*Institute of Pediatrics, Russian Academy of Medical Sciences, Moscow, Russian Federation*
[c]*Brain Research Institute, Russian Academy of Medical Sciences, Moscow, Russian Federation*

Received 22 June 1995; revised version received 20 July 1995

Abstract **Alkalinization of the external medium has been shown to suppress Ca^{2+} extrusion from neurons due to inhibition of the plasmalemmal Ca^{2+}/H^+ pump. In our experiments on fura-2-loaded rat cerebellar granule cells and mouse hippocampal neurons, an increase in pH_o from 7.4 to 8.5 following a 1-min glutamate or NMDA challenge caused a dramatic delay in $[Ca^{2+}]_i$ recovery which in some cases was accompanied by an additional increase in $[Ca^{2+}]_i$. Normalization of pH_o, or removal of Ca^{2+} from the alkaline solution allowed $[Ca^{2+}]_i$ to decrease rapidly again. External alkalinity did not affect the initial rapid decline in $[Ca^{2+}]_i$ following a 25 mM K^+ pulse. In cerebellar granule cells, the alkaline pH_o considerably increased the $^{45}Ca^{2+}$ uptake both at rest and following a 2-min GLU pulse. A comparison of these effects of alkaline pH_o with those produced by removal of the external Na^+ led us to conclude that the Ca^{2+}/H^+ pump plays a dominant role in the mechanism of the fast Ca^{2+} extrusion from glutamate- or NMDA-treated neurons.**

Key words: Ca^{2+}/H^+ pump; Na^+/Ca^{2+} exchanger; External alkalinity; Cultured neurons

1. Introduction

The role of the plasmalemmal Ca^{2+}/H^+ pump [1] in the mechanism of nerve cells recovery from Ca^{2+}-loads induced by a physiological or abusive stimulation of glutamate receptors is yet not elucidated. To clarify this problem, in the present work we used the experimental approach based on the recent finding that in nerve cells [2,3] as well as in red blood cells [4] the plasmalemmal Ca^{2+}-ATPase exchanges internal Ca^{2+} for external protons and thus can be inhibited by alkaline pH_o. We report here that in cultured rat cerebellar granule cells and mouse hippocampal neurons the alkaline shift of pH_o induces both a dramatic delay in $[Ca^{2+}]_i$ recovery and a considerable increase in $^{45}Ca^{2+}$ uptake following a brief GLU or NMDA challenge. A comparison of these effects of alkaline pH_o with those produced by inhibition of the Na^+/Ca^{2+} exchange leads us to suggest that the Ca^{2+}/H^+ pump plays a dominant role in the mechanism of Ca^{2+} extrusion from nerve cells following a short-duration stimulation of GLU receptors.

*Corresponding author. Fax: (7) (095) 151-9540.

Abbreviations. $[Ca^{2+}]_i$ and $[Ca^{2+}]_o$, the cytosolic and external Ca^{2+} concentrations, respectively; $[Na^+]_i$ and $[Na^+]_o$, the cytosolic and external Na^+ concentrations, respectively; pH_i and pH_o, cytosolic and external pH, respectively; Fura-2/AM, acetoxymethyl ester of fura-2; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TAPS, *N*-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethylether)-*N*,*N*′-tetraacetic 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; SDS, sodium dodecyl sulfate.

2. Materials and methods

Primary cerebellar cultures were prepared from the cerebella of 7- to 8-day-old Wistar rats using a procedure described earlier [5]. The cells were grown in minimal Eagle's medium containing 25 mM KCl. The experiments were carried out on 7- to 8-day cell cultures. The hippocampi from C57/Bl mouse embryos of 17–19 day gestational age were dissected, dissociated and cultivated as previously described [6]. The experiments with cultured hippocampal neurons were performed on days 18–22 of cultivation. $[Ca^{2+}]_i$ was measured in individual neurons loaded with the Ca^{2+}-sensitive fluorescent probe, fura-2 acetoxymethyl ester (fura-2/AM) [7]. The neurons were incubated for 1 h at room temperature with 5 μM fura-2/AM in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-buffered salt solution (HBSS) containing (mM) 145 NaCl, 5 KCl, 0.6 Na_2HPO_4, 0.4 KH_2PO_4, 1.8 $CaCl_2$, 1 $MgCl_2$, 20 HEPES, 5 glucose, pH 7.4. Sucrose was added to bring the osmolarity up to 320 mosmol. Prior to the experiment, the cells were washed by HBSS and left for 30 min to allow fura-2 deesterification and equilibration between the Ca^{2+}-bound and Ca^{2+}-free forms. To measure $[Ca^{2+}]_i$, the coverslip with the cell culture was placed into a special chamber and the superfusion of the cells (0.2 ml/min) with HBSS was turned on. The experimental chamber was mounted on Nicon inverted-stage microscope connected to a 'Spex' (USA) spectrofluorimeter equipped with a beam splitter, two excitation monochromators and a dual mirror chopping mechanism with a specialized optical configuration to allow a rapid alternation (100 Hz) between the two fura-2 excitation wavelengths, 340 and 380 nm, or 340 and 360 nm. Excitation bandwidths were set at 3.5 nm. The emitted fluorescence was filtered through a cut-off filter at 505 nm. The $[Ca^{2+}]_i$ was measured by the ratio of fura-2 fluorescence excited at 340 nm to that excited at 380 nm and calibrated according to the procedure described Kiedrowski et al [8].

Accumulation of intracellular $^{45}Ca^{2+}$ was assessed under different experimental conditions by the addition of 1 μCi/ml $^{45}Ca^{2+}$ to each well for 5 min. Then the cultures were washed three times with an ice-cold buffer solution of the following composition (mM): NaCl 154, KCl 5.6, $NaHCO_3$ 3.6, EGTA 2, HEPES 10, pH 7.35, and solubilized in warm (37°C) SDS solution; 1 ml of this solution was added to each well for 1 h; aliquots from each well were used to determine radioactivity and protein.

Fura-2/AM was purchased from Molecular Probes (USA). All the other compounds were purchased from Sigma Chemical Company (USA).

SSDI 0014-5793(95)00894-2

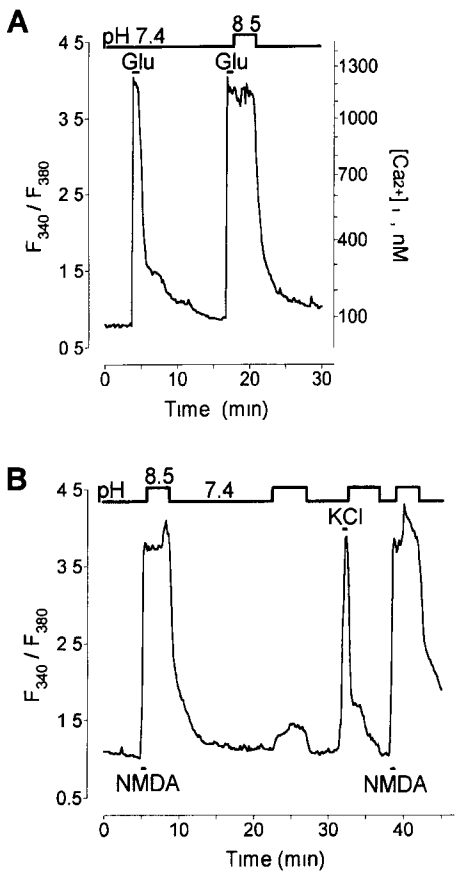

Fig. 1. Effects of pH_o elevation on the time course of $[Ca^{2+}]_i$ recovery in individual cerebellar granule cells (A) and the hippocampal neuron (B) following a GLU- (A) or NMDA- and high-K^+- (25 mM) pulses (B). The cell was treated with 100 μM GLU, or 100 μM NMDA or 25 mM K^+ during the time indicated by small horizontal bars (1-min). Then the cell was washed with HBSS (pH 7.4) or with the alkaline (pH 8.5) buffer. The GLU- and NMDA-solutions were nominally Mg^{2+}-free and contained 10 μM glycine. 20 mM KCl was added to HBSS without a compensatory removal of NaCl. The alkaline solution was buffered with TAPS.

3. Results and discussion

3.1. Effect of alkaline pH_o on basal Ca^{2+}

In approximately 70% of resting cells (n = 26), the elevation of pH_o from 7.4 to 8.5 was shown to increase the baseline $[Ca^{2+}]_i$ by about 50% of its initial value (50–80 nM). A return of cells to pH_o 7.4, or a removal of Ca^{2+} from the alkaline solution induced a rapid $[Ca^{2+}]_i$ recovery to its basal level (not illustrated).

3.2. High pH_o-induced changes in the dynamics of $[Ca^{2+}]_i$ recovery following the GLU challenge

Fig. 1 A demonstrates the biphasic time course of $[Ca^{2+}]_i$ recovery following a 1-min GLU (100 μM) pulse at pH_o 7.4 and its dramatic change caused by an increase in pH_o to 8.5. In this case high pH_o nearly abolished $[Ca^{2+}]_i$ recovery. The other patterns of a delayed post-glutamate $[Ca^{2+}]_i$ recovery at pH_o 8.5 were also observed, including an additional $[Ca^{2+}]_i$ increase in response to pH_o elevation (Figs. 1B and 2A). Such a Ca^{2+} response was especially pronounced in the case of a pH_o elevation after a short-duration wash-out of the cells by HBSS at pH_o 7.4. (Fig. 2B). This effect cannot be explained by a delayed activation of NMDA receptors with endogenous excitatory amino acids released from stimulated neurons [9] since in this case, as well as in most of experiments with hippocampal neurons, the alkaline buffer contained the NMDA antagonist, AP-5 (100 μM). However, independent of the pattern of the post-glutamate $[Ca^{2+}]_i$ recovery at pH_o 8.5, the subsequent pH_o lowering to 7.4 caused a rapid decline in $[Ca^{2+}]_i$, followed by the slow phase of recovery.

All the above effects of alkaline pH_o could be readily abolished by removal of Ca^{2+} from the external solution (without or with addition of 50 μM EGTA), suggesting a strong dependence of these effects on Ca^{2+} influx.

Fig. 1B demonstrates a striking difference in the effects of alkaline pH_o on the dynamics of $[Ca^{2+}]_i$ recovery following NMDA and high K^+ (25 mM) pulses applied in sequential order to the same cell. In this and all other experiments of this series (n = 15) pH_o elevation to 8.5 did not affect the first fast phase of $[Ca^{2+}]_i$ recovery following the K^+-induced membrane depolarization. Even a combined pH_o elevation and removal of external Na^+ failed to affect the first fast phase of $[Ca^{2+}]_i$ decline following a high-K^+ pulse (not illustrated).

Thus the effect of Ca^{2+}/H^+ pump inhibition on nerve cell

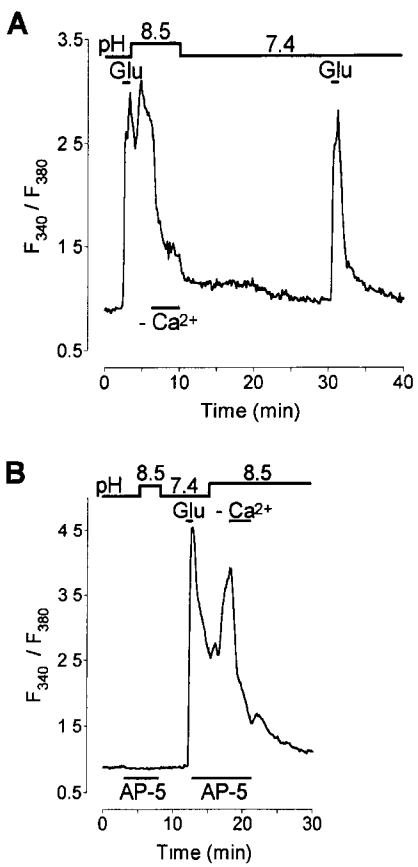

Fig. 2. Rapid decrease in $[Ca^{2+}]_i$ after Ca^{2+} removal from the alkaline solution in the post-glutamate period. (A) cerebellar granule cells; (B) hippocampal neuron The applications of GLU (100 μM) nominally Ca^{2+}-free (–Ca^{2+}) or AP-5-containing (100 μM) solutions are indicated by horizontal bars. Note that in (B) the alkaline solution failed to increase $[Ca^{2+}]_i$ at rest but induced a high Ca^{2+} response in the post-glutamate period.

recovery from Ca^{2+}-load appeared to be strongly dependent on that whether this load was a result from Ca^{2+} influx via GLU receptor channels or via the voltage-sensitive Ca^{2+} channels activated by membrane depolarization.

3.3. Role of external Na^+

The kinetics changes in the post-glutamate $[Ca^{2+}]_i$ recovery induced by replacement of external Na^+ by Li^+ or NMDG differ greatly from those produced by the alkaline solution. In agreement with previous reports [8,10,11] Na^+ removal from the post-glutamate solution did not affect the first fast phase of $[Ca^{2+}]_i$ decline but delayed its recovery during its slow phase (Fig. 3A). This result indicates that in contrast to the Ca^{2+}/H^+ pump, the Na^+/Ca^{2+} exchanger is unable to provide fast Ca^{2+} extrusion following a GLU challenge.

In the experiment presented in Fig. 3B, Na^+ was replaced by NMDG in all the external solutions in order to lower the basal $[Na^+]_i$ [12] and to prevent both its increase [8,12] and membrane depolarization caused by stimulation of GLU receptors. Notwithstanding, the increase in pH_o following the GLU pulse retained $[Ca^{2+}]_i$ at a high plateau level, indicating that intracellular Na^+ accumulation is not a prerequisite for the effects of high pH_o on $[Ca^{2+}]_i$ recovery kinetics. Of particular interest is the effect of a subsequent Ca^{2+} removal from the alkaline Na^+-free post-glutamate solution. It can be seen that despite the strong inhibition of both the Ca^{2+} extruding systems, Ca^{2+} removal induced a rapid decrease in $[Ca^{2+}]_i$. It thus becomes

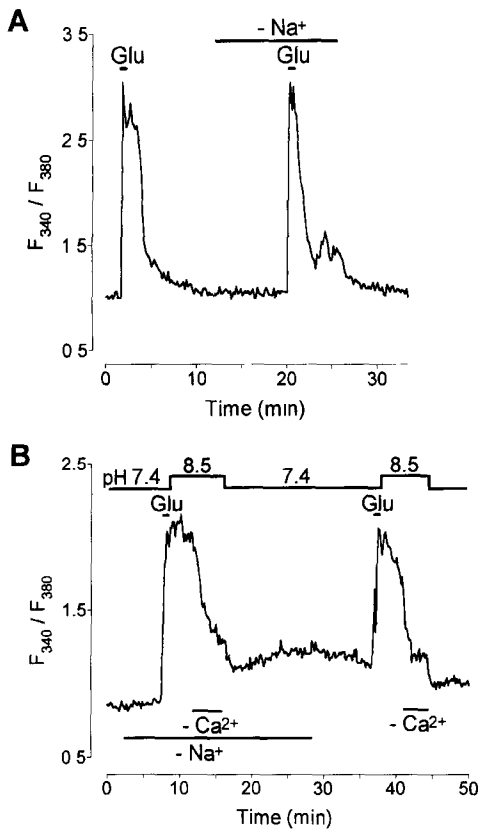

Fig. 3. Effects of removal of external Na^+ on the kinetics of $[Ca^{2+}]_i$ recovery following a 1-min GLU (100 μM) pulse at different pH_o. (A) effect of a replacement of Na^+ by Li^+ ($-Na^+$). (B) effect of a replacement of Na^+ by NMDG. Removal of external Na^+ enhanced the inhibitory effect of alkaline pH_o on $[Ca^{2+}]_i$ recovery (cf. the 1st and 2nd records) but did not impede the fast $[Ca^{2+}]_i$ decrease after removal of Ca^{2+} from the alkaline buffer

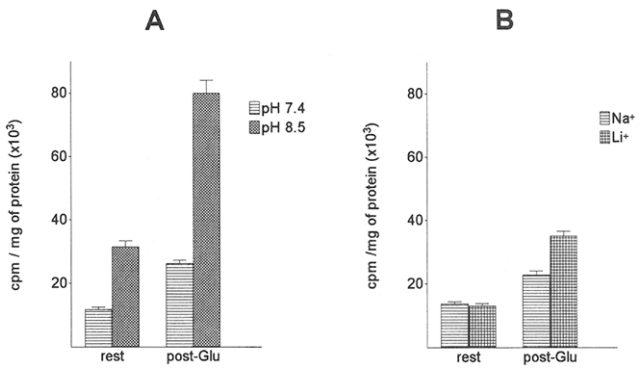

Fig. 4. Effect of pH_o elevation (A) and external Na^+ removal (B) on $^{45}Ca^{2+}$ uptake into cerebellar granule cells at rest and following a 2-min exposure to 100 μM GLU ('rest' and 'post-GLU', respectively). $^{45}Ca^{2+}$ was added to all solutions for 5 min, after which its uptake was measured as described in section 2. Each bar represents the mean ± S.E.M. of 9 (A) and 12 (B) individual experiments performed on sister cultures

evident in the absence of Ca^{2+} influx the intracellular Ca^{2+} buffering systems (Ca^{2+}-binding proteins, mitochondria and endoplasmic reticulum [14]) were capable to clear the cytoplasm from excessive Ca^{2+} even without the assistance of the Na^+/Ca^{2+} exchanger and the plasma membrane Ca^{2+}/H^+ pump.

3.4. Changes in $^{45}Ca^{2+}$ uptake induced by high pH_o

In parallel experiments with sister cultures of cerebellar granule cells, we studied changes in the $^{45}Ca^{2+}$ uptake caused by high pH_o (Fig. 4A) or Na^+ replacement with Li^+ (Fig. 4B). Measurements were performed both at rest and following a 2-min GLU (100 μM) application. It can be seen that at rest $^{45}Ca^{2+}$ uptake remained practically unchanged after removal of external Na^+ but increased about twofold at pH_o 8.5. In the post-glutamate period $^{45}Ca^{2+}$ uptake at pH_o 8.5 was about 2.5 times greater than that measured in Na^+-free medium at pH 7.4.

To explain these results, it is tempting to suggest that high pH_o does not simply inhibit Ca^{2+} extrusion mediated by the Ca^{2+}/H^+ pump, but it reverses the mode of operation of this pump. Within the framwork of such a hypothesis it is easy to interprete most of the other effects of external alkalinity, including the: (i) reversible increase in the baseline $[Ca^{2+}]_i$; (ii) dramatic delay in $[Ca^{2+}]_i$ which is sometimes accompanied by an additional $[Ca^{2+}]_i$ increase; and (iii) a high dependence of all effects of high pH_o on external $[Ca^{2+}]_i$. It is not clear, however, how to explain from this standpoint the finding that alkaline pH_o does not induce similar changes in the dynamics of $[Ca^{2+}]_i$ recovery following a K^+-induced membrane depolarization.

Although the reversibility of the purified plasma membrane Ca^{2+}/H^+ pump is well established [1], further studies are required to clarify the role of pH_o or pH_i changes in determining the mode of its operation.

In conclusion, the plasmalemmal Ca^{2+}/H^+ pump of mammalian central neurons plays an important role not only in the regulation of the baseline $[Ca^{2+}]_i$ [13] but also in the processing of its recovery after an elevation induced by Ca^{2+} influx via NMDA (but not via the voltage-operated) ionic channels.

Acknowledgements. We are grateful to R. Thomas and V. Skulachev for valuable critical comments. The work is supported by grant MBA 300 from ISP and by Russian Foundation of Basic Research.

References

[1] Carafoli, E. (1991) Physiol. Rev. 71, N1, 129–153.
[2] Benham, C., Evans, M. and McBain, C. (1992) J. Physiol. 455, 567–583.
[3] Schwiening, C.J., Kennedy, H.J. and Thomas, R C. (1993) Proc. R. Soc. Lond. 253, 285–289.
[4] Niggli, V., Sigel, E. and Carafoli, E. (1982) J Biol. Chem. 257, 2350–2356.
[5] Andreeva, N., Khodorov, B., Stelmashuk, E., Cragoe, E. and Victorov, I. (1991) Brain Res. 548, 322–325.
[6] Khaspekov, L., Kida, E., Victorov, I and Mossakowsky, M (1989) J. Neurosci. Res. 22, 150–157.
[7] Grynkiewicz, G., Poenie, M. and Tsien, R. (1985) J. Biol. Chem. 260, 3440–3450.
[8] Kiedrowski, L., Brooker, G., Costa, E and Wroblewski, J. (1994) Neuron 12, 1–20.
[9] Hartley, D., Kurth, M., Bjerkness, L., Weiss, J.H. and Choi, D. (1993) J. Neurosci. 13, 1993–2000.
[10] White, R.J and Reynolds, I.J. (1995) J. Neurosci. 15, 1318–1328.
[11] Koch, R. and Barish, E. (1994) J. Neurosci. 14, N5, 2585–2593.
[12] Pinelis, V., Segal, M , Greenberger, V. and Khodorov, B. (1994) Bioch. Mol. Biol. Int. 32, N3, 475–482
[13] Blaustein, M. (1988) Trends Neurosci 11, 438–443.
[14] Miller, R. (1991) Progress Neurobiol. 37, 255–285.