The Leading Role of Membrane Ca^{2+}-ATPase in Recovery of Ca^{2+} Homeostasis after Glutamate Shock

T. P. Storozhevykh, V. G. Pinelis, N. P. Vinskaya, A. M. Surin*, and B. I. Khodorov*

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Combined blockade of Na^+/Ca^{2+} exchange, Ca^{2+} uptake by mitochondria and endoplasmic reticulum usually does not prevent recovery of the basal level of intracellular Ca^{2+} after 1-min action of glutamate (100 μM) or K^+ (50 mM). However, replacement of Ca^{2+} with Ba^{2+}, which cannot be transported by Ca^{2+}-ATPase, considerably delayed the decrease in intracellular Ba^{2+} after its rise caused by glutamate or potassium application in all examined cells, which attest to an important role of Ca^{2+}-ATPase in Ca^{2+} extrusion after the action of glutamate or K^+.

Key Words: *neuron; glutamate; neurotoxicity; Ca^{2+}-ATPase; Na^+/Ca^{2+}-exchange*

Intensive stimulation of glutamate (Glu) receptors, *e.g.* caused by cerebral ischemia, induces neuronal damage and death, due to disturbed recovery of the low level of intracellular Ca^{2+} ($[Ca^{2+}]_i$) after termination of Glu exposure [8,12]. It is established that Ca^{2+} is transported from nerve cells by two ATP-dependent membrane systems: Ca^{2+}-ATPase and Na^+/Ca^{2+}-exchanger [3,5,11]. We previously demonstrated that inhibition of Ca^{2+}-ATPase at alkaline pH (pH of bathing solution 8.5) markedly decelerated $[Ca^{2+}]_i$ decrease during recovery after Glu exposure [9]. Here we evaluated the contribution of Ca^{2+}-pump into the recovery of basal $[Ca^{2+}]_i$ after glutamate exposure. To this end two approaches were used: 1) combined inhibition of all Ca^{2+}-regulatory systems except Ca^{2+}-pump (inhibition of Na^+/Ca^{2+}-exchange by replacing Na^+ in incubation saline with Li^+, inhibition of mitochondrial Ca^{2+} uptake with respiration uncoupler dinitrophenol (DNP), and inhibition of Ca^{2+} uptake by endoplasmic reticulum with cyclopiazonic acid, an inhibitor of reticular Ca^{2+}-ATPase); 2) replacement of Ca^{2+}_o in the incubation medium with Ba^{2+}, which can enter the cell via all Ca^{2+} channels, including Glu-dependent channels [2,7,14], but cannot be transported by P-type ATPases [6]. Ba^{2+} can replace Ca^{2+} in Na^+/Ca^{2+}-exchanger, but the efficiency of Ba^{2+} transport is almost 2-fold lower than that of Ca^{2+} [4]. Replacement of Ca^{2+}_0 with Ba^{2+} simulated inhibition of Ca^{2+}-ATPase of the plasmalemma in a Ca^{2+}-containing medium.

MATERIALS AND METHODS

The study was carried out on 7-9-day-old primary culture of cerebellar granular cells isolated from 1-week-old Wistar rat pups [1]. $[Ca^{2+}]_i$ and $[Ba^{2+}]_i$ were measured on a Spex Microfluorimeter (Spex) connected to an inverted microscope (Nikon) and image analysis system (Diamorph). The cells were loaded with Ca^{2+}-sensitive probe Fura-2/AM (4 μM) for 40 min, and then the culture medium was replaced with a buffer containing (in mM): 130.0 NaCl, 5.6 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 5.0 glucose, and 20.0 HEPES (pH 7.4). The cells on coverslips were placed in a 200-μl perfused chamber mounted on microscopic stage. In Na^+-free solutions NaCl was replaced with 130 mM LiCl (pH was adjusted to 7.4 with LiOH). In Ba^{2+}-containing solutions calcium ions were replaced with Ba^{2+}. The

Laboratory of Membranology, Research Center of Children Health, Russian Academy of Medical Sciences; *Institute of General Pathology and Pathological Physiology, Russian Academy of Medical Sciences, Moscow. ***Address for correspondence:*** tstor@mail.ru. Storozhevykh T. P.

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fluorescent signal from Fura-2 bound to Ca^{2+} or Ba^{2+} was recorded at the emission wavelength of 505±10 nm and two excitation wavelengths of 350 and 390 nm [4]. Relative changes of $[Ca^{2+}]_i$ and $[Ba^{2+}]_i$ were evaluated by the ratio F_{350}/F_{390}. Preliminary tests in a cell-free medium proved reversible binding of Fura-2 with both Ca^{2+} and Ba^{2+}.

The probes and chemicals were from Sigma, Molecular Probe, and Fluka.

RESULTS

Replacement of Ca^{2+}_0 with Ba^{2+} in the incubation medium induced a slow increase in Fura-2 fluorescence in resting neurons, which attested to accumulation of Ba^{2+} in the cytoplasm (Fig. 1, *a*). Blockade of Na^+_0/Ca^{2+}_i-exchange by replacing of Na^+_0 with Li^+ had no effect on the basal $[Ca^{2+}]_i$. Similar replacement in Ba^{2+} solution produced no extra increase in $[Ba^{2+}]_i$, which attested to the absence of intracellular Ba^{2+}-Na^+_0 exchange under these conditions. Removal of Ba^{2+} from the incubation medium (addition of 200 μM EDTA) induced a gradual decrease in $[Ba^{2+}]_i$, which probably resulted from unmasking the direct mode of Na^+/Ba^{2+}-exchange (Na^+_0/Ba^{2+}_i) due to termination of Ba^{2+} entry via ionic channels and reversal (Na^+_i/Ba^{2+}_0) exchange. Figure 1, *b* shows that blockade of Na^+/Ca^{2+}-exchange produced no effect on the basal level of $[Ca^{2+}]_i$ in neurons even when Ca^{2+} uptake by mitochondria was blocked with DNP. However, the following replacement of Ca^{2+}_0 in the medium with Ba^{2+} (Fig. 1, *b*) produced a gradual increase of Fura-2 fluorescence (n=27). Thus, replacement of of Ca^{2+}_0 with Ba^{2+} revealed the key role of plasma membrane Ca^{2+}-ATPase in the control of $[Ca^{2+}]_i$ in resting neurons, which agrees with current views on its role in Ca^{2+}-homeostasis in neurons [3,11].

In the next experimental series, we examined the potency of neurons to restore the initial level of fluorescence after short-term application of 50 mM K^+ or Glu in a Ba^{2+}-containing saline. Replacement of Ca^{2+}_0 with Ba^{2+} markedly decelerated the drop of fluorescence after its rise in response to a 1-min potassium-induced depolarization (Fig. 2, *a*). After 1-min application of 100 μM Glu (together with 10 μM glycine in Mg^{2+}-free saline), no decrease in $[Ba^{2+}]_i$ was observed in 31 of 43 cells (Fig. 2, *b*), and in other cells it was comparable with the drop of $[Ba^{2+}]_i$ observed after K^+ application. Removal of Ba^{2+} from the medium with 200 μM EDTA produced only a minor effect on this process (Fig. 2), which attests to a negligible increment in membrane permeability for Ba^{2+} immediately after termination of neuron stimulation. It should be noted that the blocker of NMDA and AMPA subtypes of Glu-receptor channels memantine (25 μM) and NBQX (75 μM) had no effect on the rate of $[Ba^{2+}]_i$ decrease after glutamate application (Fig. 2, *b*). Thus, the slow rate of $[Ba^{2+}]_i$ decrease was not caused by endogenous Glu.

Therefore, inability of Ca^{2+}-ATPase to transport Ba^{2+} ions from neurons markedly decelerated recovery of basal fluorescence after potassium-induced depolarization and almost completely blocked this process in most cells after application of Glu. Weak dependence of this effect on external Ba^{2+} showed that it is caused namely by a decrease in Ba^{2+} extrusion and not by inward transport of Ba^{2+} across the membrane.

After application of Glu under conditions of DNP-induced depolarization of mitochondria and blockade of Na^+/Ca^{2+}-exchange and Ca^{2+} entry into endoplasmic

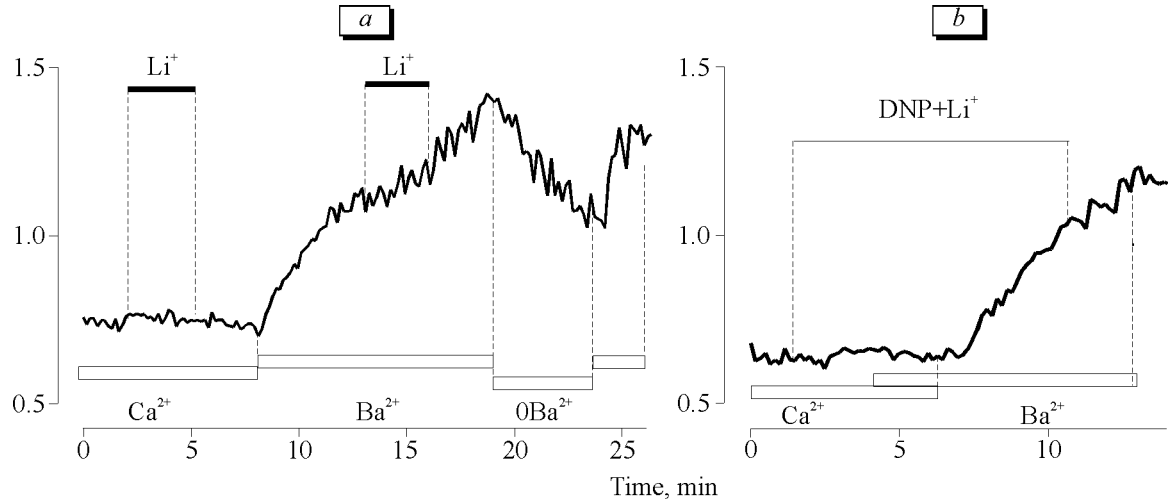

Fig. 1. Fluorescence F_{350}/F_{390} in cerebellar granular cells at rest in Ca^{2+}- and Ba^{2+}-containing medium. *a*) blockade of Na^+/Ba^{2+}-exchange by replacement of Na^+_0 with Li^+. $0Ba^{2+}$ denotes application of Ba^{2+}-free saline with 200 μM EDTA (n=37 in 4 experiments). *b*) replacement of Ca^{2+} with Ba^{2+} under conditions of mitochondrial depolarization with dinitrophenol (DNP) and blockade of cytoplasmic exchange of Ba^{2+} for Na^+_0 by replacement of Na^+_0 with Li^+ (n=27 in 3 experiments).

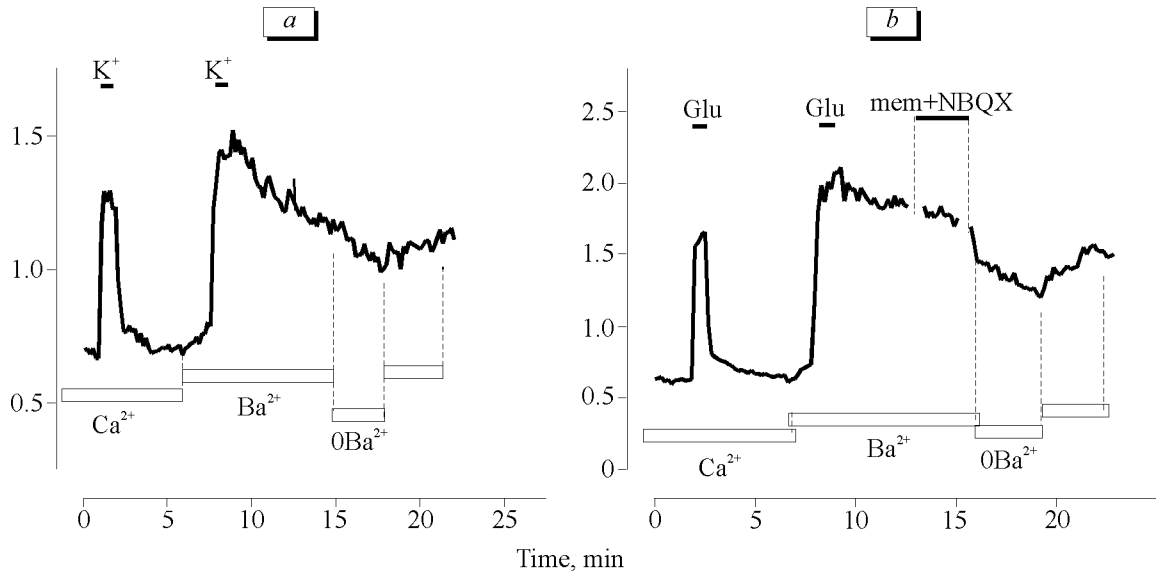

Fig. 2. Restoration of F_{350}/F_{390} fluorescence after exposure to K^+ (*a*) and Glu (*b*). *a*) replacement of Ca^{2+} with Ba^{2+} in the external medium (*n*=31); *b*) blockade of Glu-dependent Ca^{2+} channels with 25 μM memantine and 75 μM NBQX (mem+NBQX).

reticulum, only Ca^{2+}-pump in plasmalemma remained active (Fig. 3). After 1-min application of Glu (20 μM), rapid recovery of basal $[Ca^{2+}]_i$ level occurred in 70% cells (28 of 39). Since combined application of Glu and DNP decreased the content of ATP in neurons to 30% of the initial value [1], it can be suggested that the drop in ATP was most pronounced in those neurons (*n*=11), where Glu irreversibly enhanced $[Ca^{2+}]_i$. In those neurons where the basal level of $[Ca^{2+}]_i$ recovered, the replacement of $Ca^{2+}{}_0$ with Ba^{2+} resulted in a dramatic delay in extrusion of Ba^{2+} from cells after the action of Glu even in sodium saline where $Na^+{}_0/Ba^{2+}{}_i$ exchange was possible (Fig. 3).

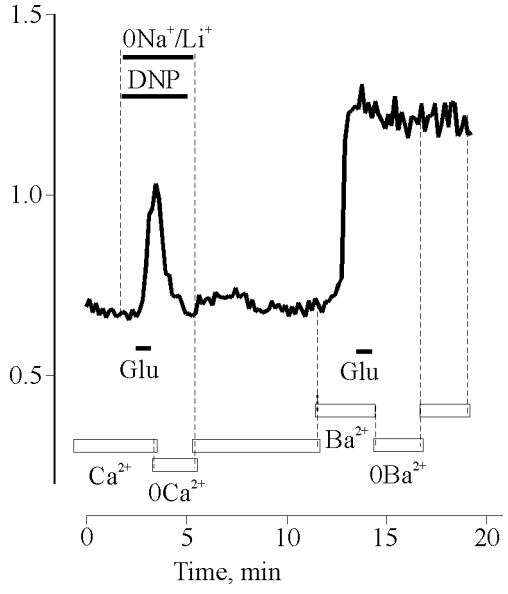

Fig. 3. Recovery of basal $[Ca^{2+}]_i$ in cerebellar cells (*n*=28) after exposure to 20 μM Glu (+10 μM glycine, $0Mg^{2+}$, 100 sec) under conditions of blockade of $Na^+{}_0/Ca^{2+}{}_i$-exchange by replacement of $Na^+{}_0$ for Li^+ and depolarization of mitochondria induced by dinitrophenol (DNP). All solutions contained 10 μM CPA, an inhibitor of Ca^{2+}-ATPase in sarcoplasmic reticulum. After the first Glu application, Ca^{2+} was replaced with Ba^{2+}.

The present data suggest that Ca^{2+} pump of the plasma membrane plays the leading role in Ca^{2+} release from neurons after short-term application of K^+ or Glu. Neither inhibition of Na^+/Ca^{2+}-exchange, nor block of Ca^{2+} uptake by mitochondria and endoplasmic reticulum disturbed recovery of the basal level of fluorescence in neurons to the same extent as replacement of $Ca^{2+}{}_0$ with Ba^{2+}. Therefore, the current view that the major work needed for Ca^{2+} release from the neurons after their stimulation is performed by Na^+/Ca^{2+}-exchange, while Ca^{2+}-pump is responsible only for "fine" adjustment of $[Ca^{2+}]_i$ near the resting value [3,5,11], was not supported in this study. Probably, only Na^+/Ca^{2+}-exchange can restore the basal level of $[Ca^{2+}]_i$ after potent Glu shock in neurons with damaged Ca^{2+}-ATP system. Evidently, Glu-induced increase in $[Na^+]_i$, which persists for a long time after Clu application [10,13], inhibits Na^+/Ca^{2+}-exchange, which depends on sodium gradient across the membrane [5]. Under these conditions, Ca^{2+} is extruded by Ca^{2+} pump, which compensates dysfunction of other cellular systems responsible for the control of internal calcium level. Thus, Ca^{2+} overload after hyperstimulation of Clu receptors in neurons most probably results from disturbance in the work of plasma membrane Ca^{2+}-ATPase.

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