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Ca^{2+} and acidosis synergistically lead to the dysfunction of cortical GABAergic neurons during ischemia

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ABSTRACT

Cell death in cerebral ischemia is presumably initiated by neural excitotoxicity resulted from the dysfunction of inhibitory neurons in early stage. Molecular processes underlying the ischemic injury of inhibitory neurons remain to be elusive, which we investigated by biochemical manipulations with cellular imaging and patch clamp at GFP-labeled GABAergic cells in cortical slices. Ischemia induces Ca^{2+} elevation, acidosis and dysfunction in GABAergic cells. An elevation of cytoplasmic Ca^{2+} or H^+ impairs the encoding of action potentials in these neurons. The effects of Ca^{2+} and H^+ are additive in nature and occlude ischemic outcomes. Ischemia impairs spike production through prolonging spike refractory periods and raising threshold potentials. Therefore, calcium toxicity and acidosis during ischemia synergistically impair the dynamics of sodium channels and function of cortical GABAergic neurons, which lead to neural excitotoxicity. Our results also suggest that the cocktail therapeutics is needed to prevent neuronal death from ischemia.

1. Introduction

Ischemic neuronal death in the brain is pathologically classified into the necrosis and apoptosis [1,2], and is initiated by neuronal excitotoxicity in the early phase of ischemia [3,4]. Its causative factors presumably include a malfunction of glutamate transporter [5–7], a glutamate-dependent elevation of cytoplasmic Ca^{2+} and free radicals [8–13]. The efforts to correct these deficits have not shown substantial helps to improve stroke patients [14–16]. The alternative mechanisms for neural excitotoxicity are likely present, such as an imbalance between excitation and inhibition from GABAergic neuronal dysfunction [17].

The mechanisms underlying the dysfunction of cortical GABAergic neurons in the early phase of ischemia have not been documented. Hypoxia raises Ca^{2+} levels in cerebellar Purkinje cells for their excitotoxicity [18,19], as well as reduces cellular metabolism and pH [20]. Does ischemia elevate intracellular Ca^{2+} and H^+ in cortical GABAergic neurons? How do Ca^{2+} toxicity and acidosis impair GABAergic neurons quantitatively? To address these issues, we investigated the dynamic changes of cytoplasmic Ca^{2+} and H^+ during ischemia, and the influences of Ca^{2+} toxicity and acidosis on the functions of cortical GABAergic neurons that are genetically labeled by GFP.

2. Methods and materials

2.1. Brain slices

Cortical slices (400 m) were prepared from FVB-Tg(Gad-GFP)45704Swn/J mice (Jackson Lab, USA) that somatostatin-positive GABAergic neurons are genetically labeled by green fluorescent protein (GFP). The use of such mice allows us to be confident that our study is conducted at GABAergic neurons in the CNS. Mice in postnatal days 20–25 were anesthetized by injecting chloral hydrate (300 mg/kg) and decapitated with a guillotine. The slices were sectioned with a Vibratome in the modified and oxygenized (95% O_2/5% CO_2) artificial cerebrospinal fluid (mM: 124 NaCl, 3 KCl, 1.2 NaH_2PO_4, 26 $NaHCO_3$, 0.5 $CaCl_2$, 5 $MgSO_4$, 10 dextrose and 5 HEPES; pH 7.35) at 4 °C, and were held in normal oxygenated ACSF (mM: 124 NaCl, 3 KCl, 1.2 NaH_2PO_4, 26 $NaHCO_3$, 2.4 $CaCl_2$, 1.3 $MgSO_4$, 10 dextrose and 5 HEPES; pH 7.35) 25 °C for 1–2 h before experiments. A slice was transferred to a submersion chamber (Warner RC-26G) that was perfused with normal ACSF at 31 °C for experiments [17]. The procedures are approved by Institutional Animal Care Unit Committee in Beijing, China (approval number B10831).

GFP-labeled GABAergic neurons in layers II–IV of cerebral cortex were selected for our study. These neurons show a round or ovary-like soma and tree branch-like dendrites under DIC microscope (Nikon FN-E600), and their morphology can be seen under fluorescent microscopy with excitation wavelength at 488 nm and emission wavelength at 525 nm. The neurons were studied by whole-cell recording or two-photon cellular imaging.

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2.2. In vitro ischemia and biochemical manipulation

To simulate the artery occlusion and intracranial anastomotic circulation during *in vivo* ischemic stroke, we reduced the perfusion rate to cortical slices from 2 ml/min to 0.2 ml/min for 6 min [17,19]. Intracellular Ca^{2+} was elevated by infusing adenophostin-A [21]. Acidosis was mimicked by filling pipette solution at pH 6.5 [22]. Spike patterns are sensitive merit for monitoring the ischemic dysfunction of central neurons.

2.3. Electrophysiology

Action potentials were recorded by AxonClamp-2B and their signals were inputted into pClamp-9 (Axon Instrument INC, CA, USA). Transient capacitance was compensated, and output bandwidth filter was 3 kHz. Standard pipette solution includes 150 K-gluconate, 5 NaCl, 0.4 EGTA, 4 Mg-ATP, 4 Na-phosphocreatine, 0.5 Tris-GTP and 10 HEPES (mM), and its pH was adjusted to 7.4 by 2 M KOH. Fresh pipette solution was used with osmolarity in 295–305 mOsmol. Pipette resistance was 6–8 M.

Neuronal intrinsic properties in our study include refractory period after each spike and threshold potential. Spike absolute refractory period is measured by injecting two depolarization pulses (3 ms in duration and 5% above threshold in intensity) into the neurons, in which pulse one induces spike one at 100% firing probability and inter-pulse intervals are adjusted to have pulse two inducing spike two at 50% probability. The duration between spike one and two is defined as ARP (Fig. 4A and [23]. Threshold potentials (Vts) for sequential spikes are the voltages of initiating spikes [23].

Data were analyzed if the recorded neurons had resting membrane potential negatively more than −60 mV. The criteria for the acceptation of experimental data also included less than 5% changes in resting membrane potential, spike amplitude, and input resistance. The values of inter-spike intervals (ISI), ARP and Vts are presented as mean ± se. Comparisons between groups are done by *t*-test.

2.4. Cellular imaging

To load indicative dyes for Ca^{2+} and pH into neurons in slices, AM esters of Ca^{2+} dye (Fluo-3-AM) and pH indicator (Snarf-4F-AM) were dissolved in DMSO and 20% Pluronic F-127 (2 g Pluronic F-127 in 10 ml DMSO) to have its stock solution at ~1 mM, which were diluted in ACSF to yield the final concentrations of dyes at 10 μM. The loading solutions were added into the slice incubation chamber for 30 min, and washed out with the oxygenated ACSF. A slice was transferred to a submersion chamber and perfused with the oxygenated ACSF at 2 ml/min for cellular imaging experiments.

Ca^{2+} and pH images of GFP-labeled GABAergic neurons were taken by using a confocal laser scanning microscope (Olympus FV-1000, Olympus, Tokyo Japan), which is equipped by two-photon imaging. A laser beam (488 nm) was given for Fluo-3 and Snarf-4F excitation; and emission wave spectra were 515–553 nm for Ca^{2+}-binding Fluo-3 and 550–620 nm for low pH Snarf-4F, respectively. In addition, a laser in 910 nm from two-photon system was used to excite GFP, which was scanned by 495–515 nm for cellular imaging. Scanning system is mounted onto an upright microscope (Olympus BX61WI) with water immersion objectives (40X, 0.8NA). Average power delivered to brain slices was <10 mW. These parameters set for laser beam and photomultiplier tube were locked throughout experiments in order to have consistent condition for the comparisons before and after the ischemia. Images were viewed and analyzed with Fluoviewer. Data are presented as the changes in fluorescent intensity.

3. Results

3.1. Ischemia impairs the function and Ca^{2+}/pH homeostasis of cortical GABAergic neurons

The effect of ischemia on the functions of cortical GFP-GABAergic neurons was examined by whole-cell current clamp. Depolarization pulses (>100 ms) were injected into these neurons to evoke action potentials, and inter-spike-intervals (ISI) were measured to quantify the capability of encoding spikes, spike capacity [21,23]. Spike capacity was measured under control and after reducing perfusion rate [17,19].

Fig. 1 shows the effect of ischemia on spike capacity of GABAergic neurons. Ischemia prolongs ISIs (Fig. 1A). Values for ISI_{1-2} up to ISI_{4-5} under control (open symbols) are 7.4 ± .35, 8 ± .34, 9.28 ± .37 and 10.5 ± .4 ms, whereas those under ischemia (filled in Fig. 1B) are 11.13 ± .8, 13.3 ± 1.4, 15.6 ± 1.5 and 17.6 ± 1.35 ms. ISI values for corresponding spikes under such two conditions are statistically different ($p < 0.01$, $n = 18$). Ischemia impairs the capability of encoding spikes at GABAergic neurons.

The effect of ischemia on Ca^{2+} and pH homeostasis was studied by two-photon cellular imaging, in which Fluo-3 and Snarf-4F were applied to measure Ca^{2+} and pH, respectively. Fig. 2A shows Ca^{2+} levels in a GFP-GABAergic neuron (yellow arrow) under control (top panels) and ischemia (bottom), i.e., ischemia elevates cytoplasmic Ca^{2+}. Fig. 2B illustrates H^+ levels in this GFP-GABAergic cell (a yellow arrow) under control (top panels) and ischemia (bottom), i.e., ischemia lowers cytoplasmic pH. Fig. 2C shows dynamic changes of Ca^{2+} (left panel) and pH (right) levels in GFP-GABAergic cells ($n = 12$) under control (0 min) and ischemia (horizontal arrows). Fig. 2D confirms the measurements of Ca^{2+} (Fluo-3) and pH (Snarf-4F) in a GFP-GABAergic cell (light green) as showed in an overlap of three images. Thus, ischemia impairs the homeostasis of Ca^{2+} and pH in cortical GABAergic neurons. We then examined how high cytoplasmic Ca^{2+} and acidosis work together to impair GABAergic cells.

3.2. Ca^{2+} and H^+ overloads during ischemia additively impair GABAergic neurons

To examine the effects of Ca^{2+} or H^+ overload on neuronal impairment, we added adenophostin-A (100 nM) into pipette solu-

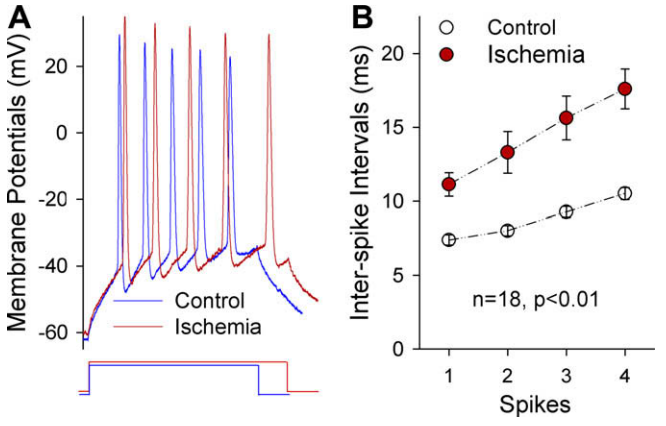

Fig. 1. The ischemia deteriorates the capability of encoding sequential spikes at cortical GABAergic neurons that are genetically labeled with eGFP in FVB-Tg(GadGFP)45704Swn/J mice. Depolarization pulses (>100 ms) were injected into these neurons in cortical slices to evoke sequential spikes, and inter-spike-intervals were measured to quantify spike capacity. (A) Compared with control (blue line), the ischemia (perfusion rate was reduced from 2 ml/min to 0.2 ml/min) prolongs inter-spike intervals. (B) Illustrates quantitative data about inter-spike intervals under the control (open symbols) and ischemia (filled symbols; $p < 0.01$, $n = 18$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)