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NEURAL DEPOLARIZATION TRIGGERS Mg^{2+} INFLUX IN RAT HIPPOCAMPAL NEURONS

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Abstract—Homeostasis of magnesium ion (Mg^{2+}) plays key roles in healthy neuronal functions, and deficiency of Mg^{2+} is involved in various neuronal diseases. In neurons, we have reported that excitotoxicity induced by excitatory neurotransmitter glutamate increases intracellular Mg^{2+} concentration ($[Mg^{2+}]_i$). However, it has not been revealed whether neuronal activity under physiological condition modulates $[Mg^{2+}]_i$. The aim of this study is to explore the direct relationship between neural activity and $[Mg^{2+}]_i$ dynamics. In rat primary-dissociated hippocampal neurons, the $[Mg^{2+}]_i$ and $[Ca^{2+}]_i$ dynamics were simultaneously visualized with a highly selective fluorescent Mg^{2+} probe, KMG-104, and a fluorescent Ca^{2+} probe, Fura Red, respectively. $[Mg^{2+}]_i$ increase concomitant with neural activity by direct current stimulation was observed in neurons plated on an indium-tin oxide (ITO) glass electrode, which enables fluorescent imaging during neural stimulation. The neural activity-dependent $[Mg^{2+}]_i$ increase was also detected in neurons whose excitability was enhanced by the treatment of a voltage-gated K^+ channel blocker, tetraethylammonium (TEA) at the timings of spontaneous Ca^{2+} increase. Furthermore, the $[Mg^{2+}]_i$ increase was abolished in Mg^{2+}-free extracellular medium, indicating $[Mg^{2+}]_i$ increase is due to Mg^{2+} influx induced by neural activity. The direct neuronal depolarization by veratridine, a Na^+ channel opener, induced $[Mg^{2+}]_i$ increase, and this $[Mg^{2+}]_i$ increase was suppressed by the pretreatment of a non-specific Mg^{2+} channel inhibitor, 2-aminoethoxydiphenyl borate (2-APB). Overall, activity-dependent $[Mg^{2+}]_i$ increase results from Mg^{2+} influx through 2-APB-sensitive channels in rat hippocampal neurons.

Key words: Mg^{2+}, KMG-104, fluorescent imaging, depolarization, hippocampal neuron, ITO electrode.

INTRODUCTION

Magnesium ion (Mg^{2+}) plays important roles in numerous biological functions, including cell cycle, channel activity, ATPase activity, metabolic regulation, etc. (Romani, 2007, 2011; de Baaij et al., 2015). In addition, a recent study revealed the second messenger role for Mg^{2+} in the human immune system (Li et al., 2011). In the central nervous system (CNS), elevating brain magnesium content enhances learning and memory (Slutsky et al., 2010). In single neuron level, the intracellular Mg^{2+} concentration ($[Mg^{2+}]_i$) regulates synaptic plasticity at electrical synapses (Palacios-Prado et al., 2013, 2014). Although these reports suggest the possibility that intracellular Mg^{2+} is a key modulator of neural activity, direct investigation of the $[Mg^{2+}]_i$ dynamics in neurons has been limited.

We have studied intracellular Mg^{2+} mobilization induced by neurotransmitters in rat hippocampal neurons (Shindo et al., 2010; Yamanaka et al., 2013). We revealed that high concentration glutamate-induced Ca^{2+} overload in neurons, which is the first step in excitotoxicity, triggers Mg^{2+} release from mitochondria in rat hippocampal neurons (Shindo et al., 2010). Hypoxia is also known to increase $[Mg^{2+}]_i$ *via* transient receptor potential melastatin 7 (TRPM7) channels in rat hippocampal neurons (Zhang et al., 2011). These reports demonstrated Mg^{2+} mobilization in neurons under highly stressed conditions. However, little is known about $[Mg^{2+}]_i$ changes under physiological conditions in healthy neurons.

Previous studies reported, in neurons loaded with mag-fura-2, a Mg^{2+} mobilization elicited by glutamate and veratridine (Brocard et al., 1993) and neuronal depolarizer, a high concentration of KCl (Kato et al., 1998; Gotoh et al., 1999). The $[Mg^{2+}]_i$ imaging, using a conventional Mg^{2+} probe, should be carefully performed in cells with the wide dynamic range of intracellular calcium ion concentration ($[Ca^{2+}]_i$) increase by using Mg^{2+} probes with low selectivity (Stout and Reynolds, 1999). Because these neuronal excitatory stimulations are well-known to trigger a huge Ca^{2+} influx, an interference with

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Abbreviations: $[Ca^{2+}]_i$, intracellular calcium ion concentration; $[Mg^{2+}]_i$, intracellular magnesium ion concentration; 2-APB, 2-aminoethoxydiphenyl borate; Ar, argon; CREB, cAMP response element-binding; FRET, fluorescence resonance energy transfer; HBSS, Hanks' balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IP_3, inositol 1,4,5-trisphosphate; ITO, indium-tin oxide; Mg^{2+}, magnesium ion; ROI, region of interest; S.D., standard deviation; TEA, tetraethylammonium; TRPM7, transient receptor potential melastatin 7; VSFP2.42, voltage-sensitive fluorescent protein.

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Mg^{2+} measurement of change in $[Ca^{2+}]_i$ cannot be neglected. Therefore, to trace the intracellular Mg^{2+} dynamics without Ca^{2+} interference, we have developed imaging techniques of intracellular Mg^{2+} dynamics in living cells (Suzuki et al., 2002; Komatsu et al., 2005; Shindo et al., 2011; Fujii et al., 2014). Comparing to conventional Mg^{2+} probes such as mag-fura-2 (K_d for Mg^{2+} and Ca^{2+} of 1.9 mM and 25 μM), mag-fura-5 (K_d for Mg^{2+} and Ca^{2+} of 2.3 mM and 28 μM), and mag-indo-1 (K_d for Mg^{2+} and Ca^{2+} of 2.7 mM and 35 μM), KMG-104 is a highly selective Mg^{2+} probe (K_d for Mg^{2+} and Ca^{2+} of 2.1 mM and 7.5 mM) (Trapani et al., 2010). Because the physiological $[Ca^{2+}]_i$ change ranges from 10 nM to 100 μM, KMG-104 can detect exactly the neuronal $[Mg^{2+}]_i$ dynamics without interference from Ca^{2+} signal. In differentiated PC12 cells, we previously reported a high K^+ induced $[Mg^{2+}]_i$ increase using the highly selective Mg^{2+} probe KMG series (Suzuki et al., 2002). However, the precise interaction between Mg^{2+} mobilization and neural activity is still limited. Therefore, the present study aimed to exactly explore the detailed relationship between neural activity and $[Mg^{2+}]_i$ dynamics.

EXPERIMENTAL PROCEDURES

Cell preparation

Hippocampal neurons were isolated from embryonic day 18 (E18) Wister rat embryos (Charles River Laboratories Japan, Tokyo, Japan), and immediately put into ice-cold Dulbecco's phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} (NISSUI PHAMACEUTICAL, Tokyo, Japan). The dissected hippocampi were dissociated using Nerve Cell Dissociation Medium (Sumitomo Bakelite, Tokyo, Japan), and the cells at a density of 4.0×10^5 cells/mL were plated on poly-D-lysine-coated (Sigma–Aldrich, St. Louis, MO, USA) glass-bottomed dishes (Iwaki, Tokyo, Japan). The hippocampal neurons were cultured in neurobasal medium supplemented with B-27, 2 mM L-glutamine, 50 U/mL penicillin and 50 mg/mL streptomycin (Life Technologies, Carlsbad, CA, USA). Cultures were fed at 37 °C in a humidified atmosphere of 5% CO_2 for a minimum of 5 days before experimental use. The medium was changed every 3–4 days.

Dye loading

For simultaneous imaging of intracellular Mg^{2+} and Ca^{2+}, primary hippocampal neurons were incubated in a medium with 5 μM KMG-104-AM and 5 μM Fura Red-AM (Life Technologies) for 30 min at 37 °C in 5% CO_2 in a humidified atmosphere. The cells were gently washed twice with 1.0 mL of Hanks' balanced salt solution (HBSS) at pH7.4 (adjusted with NaOH) that consisted of (in mM) 137 NaCl, 5.4 KCl, 1.3 $CaCl_2$, 0.5 $MgCl_2$, 0.4 $MgSO_4$, 0.3 Na_2HPO_4, 0.4 KH_2PO_4, 4.2 $NaHCO_3$, 5.6 D-glucose, 5.0 HEPES. Then, further incubation was carried out for 15 min to allow for complete de-esterification of AM esters. In the case of Mg^{2+}-free condition, HBSS was replaced by HBSS without $MgCl_2$ and $MgSO_4$ in the above-mentioned procedures.

Cell transfection

For intracellular ATP level and membrane potential measurement, neurons were transfected with the plasmids coding ATeam1.03 or VSFP2.42. The transfection was performed one day prior to the live cell imaging by using Lipofectamine™ 2000 Transfection Reagent (Life Technologies). The 100 μL transfection complexes including 4.0 μL transfection reagent and 2.0 μg plasmid DNA in Opti-Mem (Life Technologies) were added into cells cultivated at a medium volume of 1.0 mL. The cells were incubated for 6 h with transfection complexes, subsequently the Opti-Mem including transfection complexes was replaced by the cell culture medium.

Fluorescent imaging

Fluorescent imaging was conducted with a confocal laser scanning microscope system (FluoViewFV1000; Olympus, Tokyo, Japan) mounted on an inverted microscope (IX81; Olympus) equipped with a ×20 objective and an argon (Ar) laser. For the measurements of KMG-104 and Fura Red, the cells were illuminated with the excitation wavelengths at 488 nm from an Ar laser, and the signals from KMG-104 and Fura Red were separated using a 560-nm dichroic mirror. The fluorescence images were obtained by detecting the signals at 500–560 nm for KMG-104 and 630–730 nm for Fura Red, respectively. Region of interest (ROI) was located on the cell body of each neuron. In each ROI, the fluorescent intensity was spatially averaged using FluoView software package (Olympus).

Fluorescence imaging during stimulation through indium-tin oxide (ITO) electrode was performed using a fluorescence microscope ECLIPSE TE300 (Nikon, Tokyo, Japan) equipped with a ×10 objective (S Fluor, Nikon), a 505-nm dichroic mirror, and a 535/55-nm barrier filter. A Xe lamp (150 W) with a monochromator unit was used for 488-nm excitation for KMG-104 and Fura Red, and fluorescence was measured with a CCD camera (HiSCA, Hamamatsu Photonics, Shizuoka, Japan). The fluorescence was calculated as the mean intensity over a ROI on the cell body of each cell using the software package, Aquacosmos (Hamamatsu Photonics). The fluorescence change was defined as:

$$\frac{F}{F_0} = \frac{F_t - F_0}{F_0}$$

where F_t and F_0 are the fluorescent intensity at time t and 0, respectively.

Patterning of ITO electrode

ITO electrodes were designed to provide simultaneous current stimulations for a large number of neurons during fluorescent observation in the present study. The detailed design and fabrication methods of ITO electrodes were described in our previous study (Tanamoto et al., 2015). ITO electrodes were fabricated by using a photolithographic technique described previously: creating photomasks and etching