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NO/cGMP/PKG signaling pathway induces magnesium release mediated by mitoK$_{ATP}$ channel opening in rat hippocampal neurons

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ABSTRACT

Intracellular Mg^{2+} concentration ($[Mg^{2+}]_i$) and NO regulate cell survival and death. To reveal the involvement of NO in intracellular Mg^{2+} regulation, we visualized intracellular Mg^{2+} using the fluorescent Mg^{2+} indicator KMG-104-AM in rat hippocampal neurons. Pharmacological experiments using SNAP, 8-Br-cGMP, diazoxide and several inhibitors revealed that the NO/cGMP/Protein kinsase G (PKG) signaling pathway triggers an increase in $[Mg^{2+}]_i$, and that Mg^{2+} mobilization is due to Mg^{2+} release from mitochondria induced by mitoK$_{ATP}$ channel opening. In addition, Mg^{2+} release is potentiated by the positive feedback loop including mitoK$_{ATP}$ channel opening, mitochondrial depolarization and PKC activation.

1. Introduction

Magnesium ion (Mg^{2+}) is involved in a wide variety of biochemical reactions, and a multitude of physiological functions are known to require Mg^{2+} [1,2]. Major intracellular Mg^{2+} store is mitochondria [3], and the basic mitochondrial functions, including ATP synthesis, electron transport chain complex subunits, and oxygen detoxification are affected by intracellular Mg^{2+} [4]. In addition, cytosolic Mg^{2+} is found to be a potent inhibitor of mitochondrial Ca^{2+} uptake, which cause neuronal toxicity, in physiological $[Mg^{2+}]_i$ and $[Ca^{2+}]_i$ [5–7] via a putative Mg^{2+} binding site located on the cytosolic side of the inner mitochondrial membrane [8]. In the nervous system, many Mg^{2+} functions has been implicated in various neuronal diseases, such as migraine, Alzheimer's disease and Parkinson's disease (PD) [9–11]. Especially, many reports suggest the relationship between Mg^{2+} contents and the pathology of PD [9,12–14].

In the brain from patients with PD, neuronal nitric oxide synthase (nNOS) over-expresses [15], and nNOS-gene deficient mice are more resistant to the toxic effect of MPTP [16]. In contrast, NO activates cGMP/PKG signaling cascade, and modulates a number of targets including mitochondrial ATP sensitive potassium channel (mitoK$_{ATP}$) channel [17]. Opening of mitoK$_{ATP}$ channel prevents neuronal damage in vitro [18] and in vivo [19] model of PD. Dietary deficiency of Mg^{2+} in rats induces activation of NO synthase [20]. We demonstrated a gradual decrease in the Mg^{2+} concentration in mitochondria in response to MPP^+, which is an active metabolite of chemical PD inducer MPTP, in differentiated PC12 cells [21]. These previous reports indicate that the complicated regulatory mechanism between NO signal and change in $[Mg^{2+}]_i$ are key players in PD pathology.

However, involvement of NO on Mg^{2+} regulatory mechanism is poorly understood. To reveal the involvement of NO signaling on intracellular Mg^{2+} dynamics, we investigated the change in $[Mg^{2+}]_i$ by using highly sensitive Mg^{2+} fluorescent probe KMG-104 during several pharmacological stimulation and inhibition of hippocampal neurons.

2. Materials and methods

2.1. Cell culture

Primary cultures of hippocampus neurons were prepared from day 18 embryonic Wister rats (Charles River Laboratories Japan,

Abbreviations: $[Mg^{2+}]_i$, intracellular magnesium ion concentration; $[Ca^{2+}]_i$, intracellular calcium concentration; $\Delta\psi_m$, mitochondrial inner membrane potential; mitoK$_{ATP}$ channel, mitochondrial ATP sensitive potassium channel; ROS, reactive oxygen species; PKC, protein kinase C; PKG, protein kinase G

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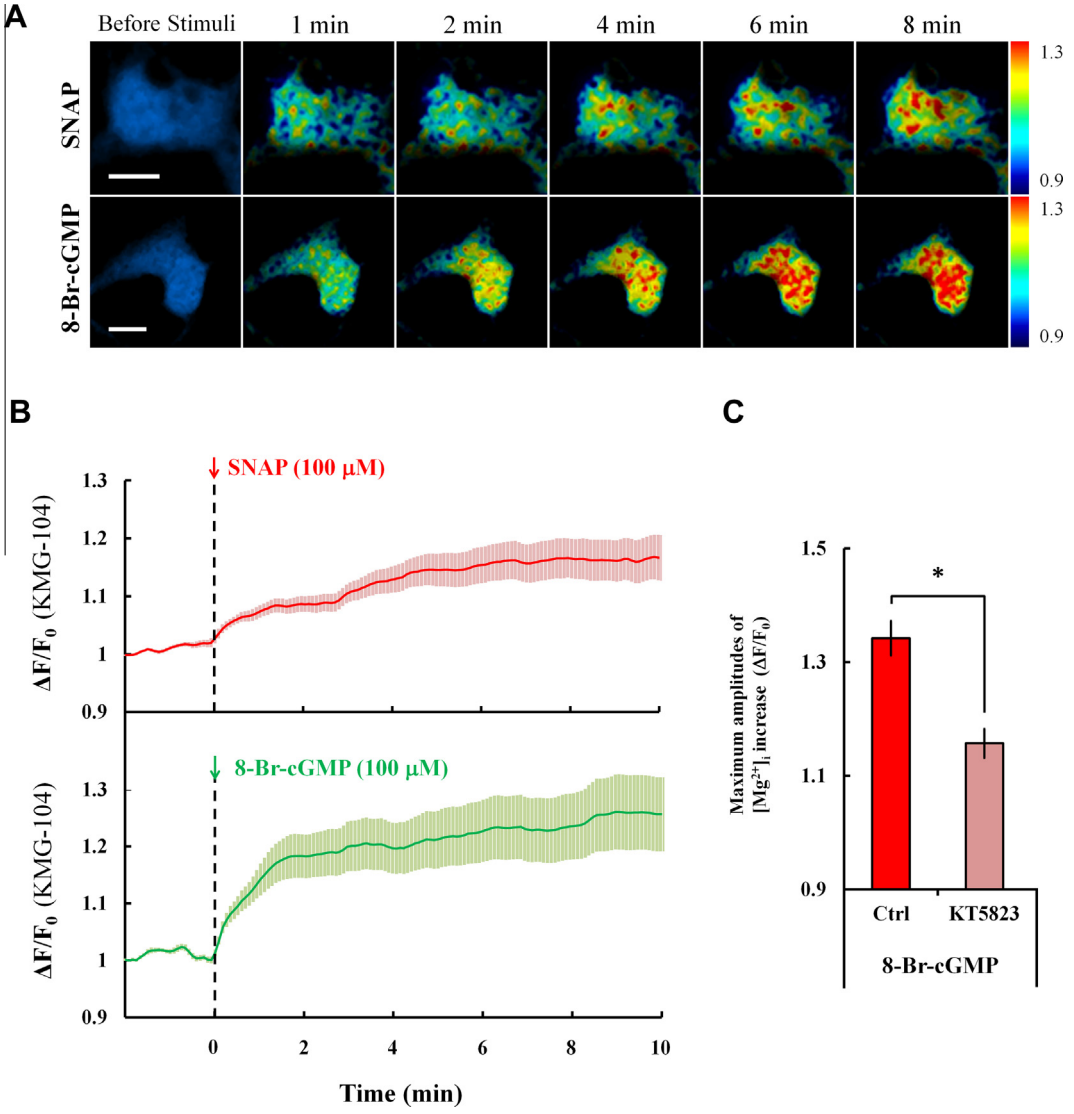

Fig. 1. Nitric oxide donor SNAP (100 μM) and PKG activator 8-Br-cGMP (100 μM) induced an increase in $[Mg^{2+}]_i$ in cultured hippocampal neurons. Changes in $[Mg^{2+}]_i$ was visualized with KMG-104-AM. (A) Pseudo-colored images of hippocampal neurons. Pseudo-colored images are presented for KMG-104 at the times indicated above when stimulating with SNAP and 8-Br-cGMP. Colored bars indicate F/F_0. Scale bars indicate 50 μm. (B) Averaged time courses of changes in $[Mg^{2+}]_i$. Bath application of 100 μM SNAP ($n = 116$, $N = 7$) and 100 μM 8-Br-cGMP ($n = 132$, $N = 8$) induced an increase in $[Mg^{2+}]_i$. Error bars indicate S.E.M. (C) Comparison of the maximum amplitudes of the 8-Br-cGMP-induced change in $[Mg^{2+}]_i$ under normal ($n = 132$, $N = 8$) and PKG-inhibited conditions ($n = 57$, $N = 3$). PKG inhibitor KT5823 (5 μM) inhibited 8-Br-cGMP-induced increase in $[Mg^{2+}]_i$. Error bars indicates S.E.M. $P < 0.01$.

Tokyo, Japan). The hippocampal neurons were extirpated and submerged in ice-cold PBS. The neurons were dissociated using a dissociation solution (Sumitomo Bakelite Co., Tokyo, Japan), and the cells were plated on a poly-D-lysine-coated (Sigma–Aldrich, St. Louis, MO, USA) glass-bottomed dishes (Iwaki, Tokyo, Japan). The neurons were cultured in a neurobasal medium supplemented with B-27, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (Invitrogen, Carsbad, CA). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2. Cells were cultured for a minimum of 4 days before experimental use.

2.2. Dye loading

For intracellular Mg^{2+} imaging, hippocampal neurons were incubated with 5 μM KMG-104-AM for 30 min at 37 °C. KMG-104-AM was designed and synthesized as a highly selective fluorescent Mg^{2+} indicator by our group [22]. Cells were then washed twice with Hanks' balanced salt solution (HBSS) at pH 7.4 (adjusted with NaOH) containing the following: NaCl (137 mM); KCl (5.4 mM); $CaCl_2$ (1.3 mM); $MgCl_2$ (0.5 mM); $MgSO_4$ (0.4 mM); Na_2HPO_4 (0.3 mM); KH_2PO_4 (0.4 mM); $NaHCO_3$ (4.2 mM); D-glucose (5.6 mM); HEPES (5 mM). Further incubation was carried out for 15 min to allow complete hydrolysis of the acetoxymethyl ester of KMG-104-AM in the cells. In some experiments, the cells were incubated for 15 min in HBSS without Mg^{2+} (Mg^{2+}-free) during the hydrolysis of acetoxymethyl ester. In addition, we used HBSS containing the following inhibitors: 500 μM 5-HD, 50 μM glibenclamide, 5 μM KT5823, and 10 μM Go6983 respectively. These inhibitors are obtained from Sigma–Aldrich (St. Louis, MO, USA) and Tocris Bioscience (Ellisville, MO, USA). For imaging of $\Delta\psi_m$, 25 nM $\Delta\psi_m$ indicator TMRE (Invitrogen) was loaded, and 2.5 nM TMRE was remained in HBSS even during measurement to compensate the dye leakage from mitochondria.

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