

Original Article

# Brief low $[Mg^{2+}]_o$ -induced $Ca^{2+}$ spikes inhibit subsequent prolonged exposure-induced excitotoxicity in cultured rat hippocampal neurons

Hee Jung Kim<sup>1</sup>, Ji Seon Yang<sup>2</sup>, and Shin Hee Yoon<sup>2,3,\*</sup>

<sup>1</sup>Department of Physiology, College of Medicine, Dankook University, Cheonan 31116, <sup>2</sup>Department of Physiology, College of Medicine, <sup>3</sup>Catholic Neuroscience Institute, The Catholic University of Korea, Seoul 06591, Korea

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## \*Correspondence

Shin Hee Yoon

E-mail: s-hyoon@catholic.ac.kr

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**ABSTRACT** Reducing  $[Mg^{2+}]_o$  to 0.1 mM can evoke repetitive  $[Ca^{2+}]_i$  spikes and seizure activity, which induces neuronal cell death in a process called excitotoxicity. We examined the issue of whether cultured rat hippocampal neurons preconditioned by a brief exposure to 0.1 mM  $[Mg^{2+}]_o$  are rendered resistant to excitotoxicity induced by a subsequent prolonged exposure and whether  $Ca^{2+}$  spikes are involved in this process. Preconditioning by an exposure to 0.1 mM  $[Mg^{2+}]_o$  for 5 min inhibited significantly subsequent 24 h exposure-induced cell death 24 h later (tolerance). Such tolerance was prevented by both the NMDA receptor antagonist D-AP5 and the L-type  $Ca^{2+}$  channel antagonist nimodipine, which blocked 0.1 mM  $[Mg^{2+}]_o$ -induced  $[Ca^{2+}]_i$  spikes. The AMPA receptor antagonist NBQX significantly inhibited both the tolerance and the  $[Ca^{2+}]_i$  spikes. The intracellular  $Ca^{2+}$  chelator BAPTA-AM significantly prevented the tolerance. The nonspecific PKC inhibitor staurosporin inhibited the tolerance without affecting the  $[Ca^{2+}]_i$  spikes. While Gö6976, a specific inhibitor of  $PKC\alpha$  had no effect on the tolerance, both the  $PKC\epsilon$  translocation inhibitor and the  $PKC\zeta$  pseudosubstrate inhibitor significantly inhibited the tolerance without affecting the  $[Ca^{2+}]_i$  spikes. Furthermore, JAK-2 inhibitor AG490, MAPK kinase inhibitor PD98059, and CaMKII inhibitor KN-62 inhibited the tolerance, but PI-3 kinase inhibitor LY294,002 did not. The protein synthesis inhibitor cycloheximide significantly inhibited the tolerance. Collectively, these results suggest that low  $[Mg^{2+}]_o$  preconditioning induced excitotoxic tolerance was directly or indirectly mediated through the  $[Ca^{2+}]_i$  spike-induced activation of  $PKC\epsilon$  and  $PKC\zeta$ , JAK-2, MAPK kinase, CaMKII and the de novo synthesis of proteins.

## INTRODUCTION

Excessive glutamate causes neural dysfunction and degeneration. Excess entry of  $Ca^{2+}$  and the consequent overload of  $Ca^{2+}$  in neuronal cell are an early key step in the excitotoxicity induced by an exposure to excess glutamate in ischemia, epilepsy, and trauma [1-3]. In addition,  $Ca^{2+}$  signals are propagated from the cytosol into the nucleus [4-6], regulate expression of some

genes [7,8] and induce neuroprotection [9].

The stimulation of glutamate receptors has been reported to be required for preconditioning in neurons [10,11]. It has been reported that NMDA preconditioning is able to prevent quinolinic acid-induced neurotoxicity and seizure [12,13]. Calcium signals induced by activation of the glutamatergic receptor during preconditioning may protect against neuronal cell death [14]. Preconditioning by  $Ca^{2+}$  ionophore ionomycin has



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been reported to decrease oxygen-glucose deprivation (OGD)-induced cell death [15].

A reduction in extracellular  $Mg^{2+}$  concentration ( $[Mg^{2+}]_o$ ) to 0.1 mM in neurons elicits an intense pattern of  $[Ca^{2+}]_i$  spikes [16-21]. The low  $[Mg^{2+}]_o$ -induced  $Ca^{2+}$  spikes are synchronized in an active network and induce neuronal cell death [18,21,22]. In addition, the low  $[Mg^{2+}]_o$  in neuronal cells or slice has been characterized as an *in vitro* seizure model [20,23,24]. However, little is known about whether the synaptically-induced  $Ca^{2+}$  spikes by low  $[Mg^{2+}]_o$  directly induce preconditioning in cultured hippocampal neuronal cells.

In the present study, we examined whether hippocampal neurons that are preconditioned by a brief exposure to low  $[Mg^{2+}]_o$  are rendered resistant to subsequent prolonged exposure-induced neuronal cell death 24 h later and whether  $Ca^{2+}$  signals are involved in this process. Low  $[Mg^{2+}]_o$  preconditioning induced excitotoxic tolerance through the  $[Ca^{2+}]_i$  spike-induced activation of PKC $\epsilon$  and PKC $\xi$ , JAK-2, MAPK kinase, CaMKII and the de novo synthesis of proteins.

## METHODS

### Materials

Materials were purchased from the following companies: fura-2 acetoxymethyl ester (AM) from Molecular Probes (Eugene, OR, USA); Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) from Invitrogen (Carlsbad, CA, USA); the AMPA receptor antagonist 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo [f] quinoxaline-7-sulfonamide (NBQX), the L-type  $Ca^{2+}$  channel antagonist nimodipine, PKC $\epsilon$  translocation inhibitor and PKC $\xi$  pseudosubstrate inhibitor, CaMKII inhibitor KN-62 from Calbiochem (San Diego, CA, USA); the NMDA receptor antagonist D-2-amino-5-phosphonovalerate (D-AP5), the intracellular calcium chelator BAPTA, 1,2-bis(2-aminophenoxy)ethane- $\ast$ N,N,N',N'-tetraacetic acid; the nonspecific PKC inhibitor staurosporin, the JAK-2 inhibitor AG490, the MAPK kinase inhibitor PD98059 and all other reagents from Sigma (St. Louis, MO, USA).  $Mg^{2+}$  free-DMEM (DMEM without  $MgSO_4$ ) was purchased from WelGENE (Korea) as a customer-ordered item.

### Cell culture

Embryonic day 17 hippocampal neurons from adult maternal Sprague-Dawley rats (250~300 g) were grown in primary culture as described previously [21]. All procedures performed on the animals were conducted with the approval of the Catholic Ethics Committee of the Catholic University of Korea. Cells were pelleted and resuspended in 0.814 mM  $[Mg^{2+}]_o$  Dulbecco's modified Eagle's media (DMEM) without glutamine and

supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 U/ml and 100  $\mu$ g/ml, respectively). Dissociated cells were then plated at a density of 50,000 cells/well onto plain 25-mm-round cover glasses (Fisher Scientific) or microetched coverslips (Bellco Biotechnology) that had been coated with poly-L-lysine (0.1 mg/ml). The cells were used between 13.5 and 15.5 days in culture (DIC). During this period, neurons developed extensive neuritic networks and formed functional synapses.

### Induction of preconditioning by 0.1 mM $[Mg^{2+}]_o$ medium in primary hippocampal neurons

In the 0.1 mM  $[Mg^{2+}]_o$  medium-induced preconditioning experiments, at least 100 neurons were counted on microetched coverslips [21]. Hippocampal neurons were exposed to 0.1 mM  $[Mg^{2+}]_o$  medium for 3~5 min and then 24 h later exposed to the same medium for 24 h. 0.1 mM  $[Mg^{2+}]_o$  DMEM were made by adding 0.1 mM  $MgCl_2$  and 0.01 mM glycine into the  $Mg^{2+}$  free-DMEM (WelGENE) just before experiments. Drugs, when included, were added to the 0.1 mM  $[Mg^{2+}]_o$  medium application simultaneously with the cells and given a 5 min exposure. Viable neurons were identified based on morphological criteria; they were phase-bright, had rounded somata, and extended fine processes [21]. Cell death was determined by comparing the number of viable neurons before and after treatment at the same microscopic fields based on the location of the microetched grid coverslips. Viable neurons from at least 5 randomly selected microscopic field on the location of the microetched grid coverslips were counted and averaged. In some experiments, viability was confirmed by demonstrating that cells identified as viable also excluded propidium iodide (50  $\mu$ g/ml).

### Digital Calcium Imaging

To measure intracellular  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ), hippocampal cells were incubated in 5  $\mu$ M fura-2 acetoxymethyl ester (AM) (Molecular Probes) in HEPES-buffered Hanks' Salt Solution (HHSS) containing 0.5% BSA for 45 min at 37°C. The Hank's salt solution was composed of (in millimoles per liter): HEPES, 20; NaCl, 137;  $CaCl_2$ , 1.3;  $MgSO_4$ , 0.4;  $MgCl_2$ , 0.5;  $KH_2PO_4$ , 0.4;  $Na_2H_2PO_4$ , 0.6;  $NaHCO_3$ , 3.0; and glucose, 5.6. The cover glass was then mounted in a flow-through chamber [18] that was superfused at a rate of 1.5 ml/min. Digital calcium imaging was performed as described previously [18].  $[Ca^{2+}]_i$  spikes were induced by HHSS containing 0.1 mM  $MgCl_2$  and 0.01 mM glycine.

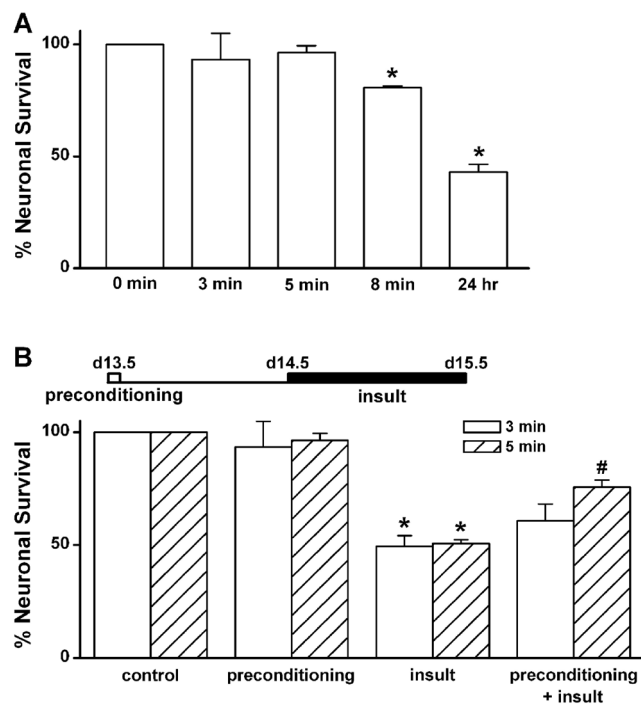
### Statistical Analyses

Data are expressed as means $\pm$ SEM. Significance was determined with a one-way analysis of variance (ANOVA) followed by Bonferroni's test.

## RESULTS

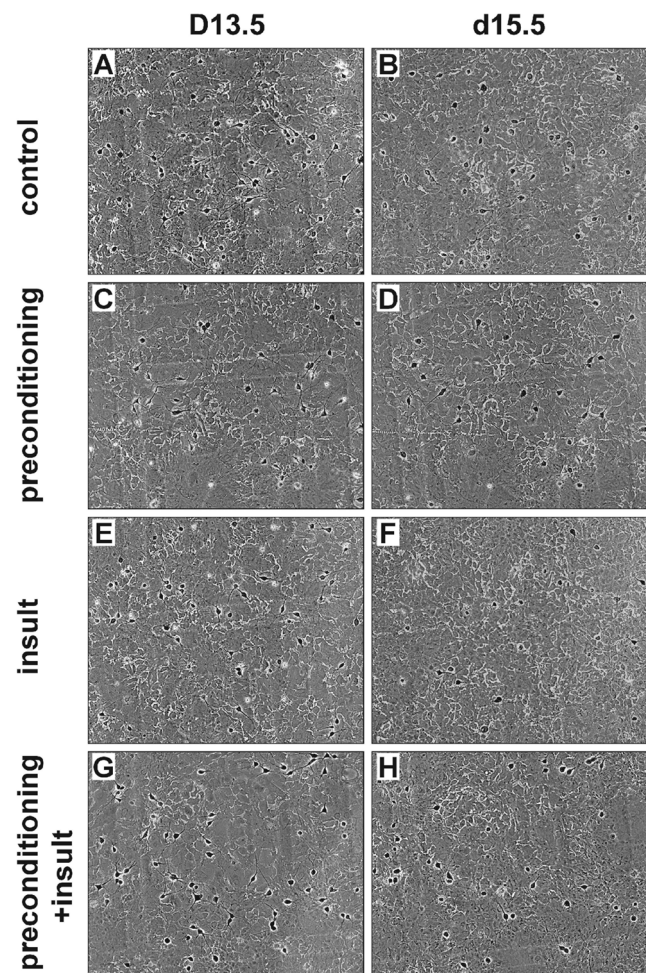
### *In vitro* preconditioning by a brief exposure to low $[Mg^{2+}]_o$ inhibits prolonged exposure-induced neuronal cell death in cultured rat hippocampal neurons

We adapted the low  $[Mg^{2+}]_o$ -induced  $Ca^{2+}$  spiking activity, which produces neuronal cell death when sustained [21], to model *in vitro* preconditioning. To determine the time of 0.1 mM  $[Mg^{2+}]_o$  exposure required to induce preconditioning in cultured rat hippocampal neurons, we replaced the normal DMEM containing 0.814 mM  $[Mg^{2+}]_o$  with 0.1 mM  $[Mg^{2+}]_o$  DMEM for various time periods at day 13.5 (Fig. 1A). When an exposure to 0.1 mM  $[Mg^{2+}]_o$  induces  $[Ca^{2+}]_i$  spikes [19], a brief exposure to the 0.1 mM  $[Mg^{2+}]_o$  media for 3 or 5 min did not elicit cell death when assayed 24 h later (3 min:  $93.4 \pm 11.5\%$  of control,  $n=4$ ; 5 min:  $96.4 \pm 3.1\%$  of control,  $n=20$ , respectively). However, an exposure to the low  $[Mg^{2+}]_o$  for 8 min slightly elicited cell death (8 min:  $79.2 \pm 1.7\%$  of control,  $n=4$ ). Exposure to the low  $[Mg^{2+}]_o$  for 24 h induced marked neuronal death ( $43.1 \pm 3.4\%$  of control,  $n=4$ ).



**Fig. 1. Low  $[Mg^{2+}]_o$ -induced neuronal cell death is inhibited by brief exposure to low  $[Mg^{2+}]_o$ .** (A) Neuronal death is induced by 0.1 mM  $[Mg^{2+}]_o$  in a time-dependent manner. Hippocampal neurons were exposed to 0.1 mM  $[Mg^{2+}]_o$ -containing DMEM for the indicated times (0, 3 min, 5 min, 8 min, and 24 h) at day 13.5. (B) Low  $[Mg^{2+}]_o$ -induced neuronal death is inhibited by a low  $[Mg^{2+}]_o$ -induced preconditioning for 5 min. Hippocampal neurons were preconditioned with 0.1 mM  $[Mg^{2+}]_o$  medium for 3 or 5 min and then 24 h later exposed to the same medium for 24 h. Data are expressed as means  $\pm$  SEM. \* $p < 0.05$  relative to control, # $p < 0.05$  relative to insult (ANOVA with Bonferroni's test).

In order to determine whether a brief exposure to 0.1 mM  $[Mg^{2+}]_o$  protects cells from excitotoxicity induced by a second prolonged exposure to the same insult, cells were preconditioned with 0.1 mM  $[Mg^{2+}]_o$  medium for 3 or 5 min and then 24 h later exposed to the same medium for 24 h (Fig. 1B). Low  $[Mg^{2+}]_o$  preconditioning for 3 min did not significantly inhibit the prolonged exposure-induced neuronal death ( $60.8 \pm 7.3\%$ ,  $n=4$ ). However, preconditioning by the exposure for 5 min significantly inhibited neuronal death ( $75.7 \pm 3.0\%$ ,  $n=20$ ). Therefore, we used a paradigm of a 5 min preconditioning and a 24 h main insult to study the characteristics of low  $[Mg^{2+}]_o$ -induced tolerance in excitotoxicity. Fig. 2 shows representative photographs of effects of the preconditioning on low  $[Mg^{2+}]_o$ -induced neuronal death. In control cells (medium exchange only),  $50.5 \pm 1.3\%$  of the cells died



**Fig. 2. The preconditioning by brief exposure to a 0.1 mM  $[Mg^{2+}]_o$  medium for 5 min protects cells against prolonged exposure-induced excitotoxicity.** Representative photographs show the same field of cultured rat hippocampal neurons before (left) and 24 h after a 5 min preconditioning and 24 h insult (right) in control (A, B), preconditioning only (C, D), insult (E, F) and preconditioning+insult (G, H). Neurons (identified by a light halo around the soma and long fine processes) grew on a bed of non-neuronal cells that formed a mosaic beneath them.

(Fig. 2A and B). This value is similar to the previous observations that  $50.7 \pm 1.7\%$  of control cells may die by extrapolation for 48 h since  $22 \pm 2\%$  of hippocampal neurons die in a 24 h culture under similar culture conditions [18]. A similar proportion of the preconditioned cells without an insult to control cells disappeared as the result of the preconditioning (Fig. 2B & D). However, the low  $[Mg^{2+}]_o$ -induced neuronal cell death was markedly inhibited by the preconditioning (insult:  $49.4 \pm 4.8\%$  of control,  $n=4$ ; preconditioning + insult:  $75.7 \pm 3.0\%$  of control,  $n=4$ ) (Fig. 2F & H).

### Roles of $[Ca^{2+}]_i$ spikes in low $[Mg^{2+}]_o$ -induced preconditioning effects

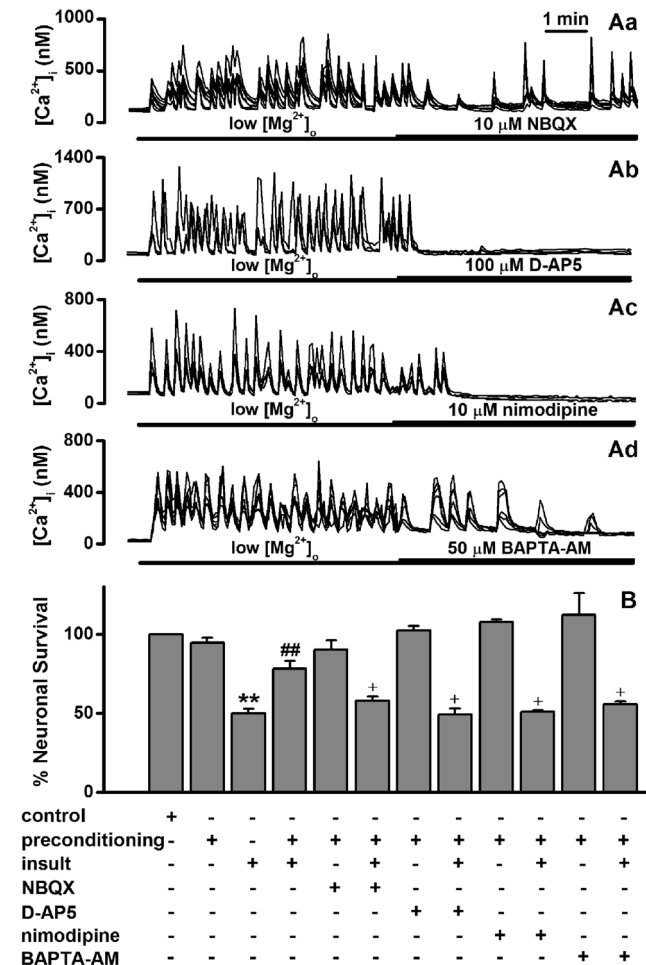
Low  $[Mg^{2+}]_o$  can elicit an intense pattern of  $[Ca^{2+}]_i$  spikes that are dependent on glutamatergic synaptic transmission [19]. We examined whether the low  $[Mg^{2+}]_o$ -induced  $[Ca^{2+}]_i$  spikes are involved in the low  $[Mg^{2+}]_o$  preconditioning-induced excitotoxic tolerance. An exposure of hippocampal neurons to 0.1 mM  $[Mg^{2+}]_o$  induced synchronized  $[Ca^{2+}]_i$  spikes within 30 s at day 13.5 (Fig. 3A). Treatment with the AMPA receptor antagonist NBQX (10  $\mu$ M) partly decreased the frequency and amplitude of the  $[Ca^{2+}]_i$  spikes (Fig. 3Aa). The NMDA receptor antagonist D-AP5 (100  $\mu$ M) and the L-type voltage-gated  $Ca^{2+}$  channel antagonist nimodipine (10  $\mu$ M) completely blocked the  $[Ca^{2+}]_i$  spikes (Fig. 3Ab and Ac). In addition, BAPTA-AM (50  $\mu$ M) gradually reduced both the frequency and amplitude of the  $[Ca^{2+}]_i$  spikes (Fig. 3Ad). BAPTA-AM (50  $\mu$ M) has been reported to slightly inhibit the  $[Ca^{2+}]_i$  response to higher concentration of glutamate (500  $\mu$ M) [25].

In subsequent experiments, we determined the effects of these  $[Ca^{2+}]_i$  changes on low  $[Mg^{2+}]_o$  preconditioning-induced excitotoxic tolerance (Fig. 3B). Treatment with NBQX during the 5 min period of preconditioning significantly inhibited the tolerance. D-AP5 and nimodipine markedly inhibited the tolerance to a greater extent, respectively. Moreover, BAPTA-AM significantly inhibited the tolerance. All these data are consistent with an evidence that  $[Ca^{2+}]_i$  spikes are involved in low  $[Mg^{2+}]_o$  preconditioning-induced excitotoxic tolerance.

### Roles of protein kinase C in low $[Mg^{2+}]_o$ preconditioning-induced excitotoxic tolerance

In subsequent experiments, we attempted to determine how low  $[Mg^{2+}]_o$ -induced  $[Ca^{2+}]_i$  spikes induce the low  $[Mg^{2+}]_o$  preconditioning-induced excitotoxic tolerance in hippocampal neurons. An increase in  $[Ca^{2+}]_i$  can directly and indirectly activate PKC isozymes. PKC has been reported to play a role in brain ischemic preconditioning [14,26,27]. In order to study the role of PKC in low  $[Mg^{2+}]_o$  preconditioning-induced excitotoxic tolerance, we screened four PKC inhibitors, staurosporin, Gö6976, a PKC $\epsilon$  translocation inhibitor, and a PKC $\xi$  pseudosubstrate inhibitor, which alone does not affect  $[Ca^{2+}]_i$  spikes (Fig. 4A). Treatment

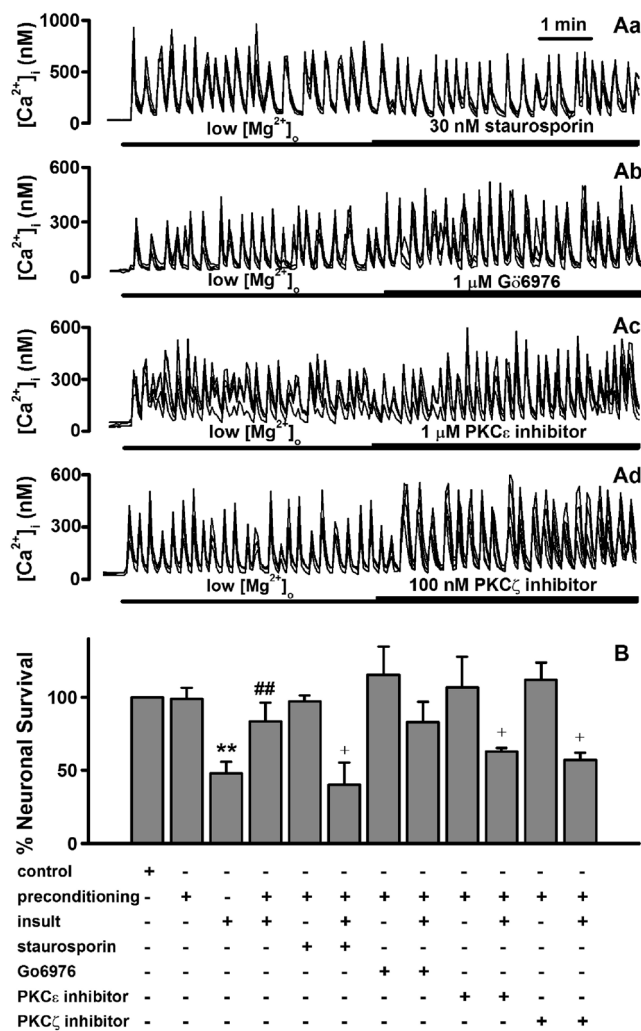
with staurosporin (30 nM), a non-specific inhibitor of PKC, during the preconditioning significantly reduced the tolerance (Fig. 4B). To investigate which specific PKC isozymes are involved in the preconditioning, the specific PKC inhibitors were used (Fig. 4B). Gö6976 (1  $\mu$ M), a specific inhibitor of PKC $\alpha$  had no effect on the tolerance. However, both the specific PKC $\epsilon$  translocation inhibitor peptide (1  $\mu$ M) and PKC $\xi$  pseudosubstrate inhibitor (100 nM) significantly inhibited the tolerance.



**Fig. 3. Low  $[Mg^{2+}]_o$ -induced tolerance is mediated through  $Ca^{2+}$  signaling.** (A) While NBQX inhibited low  $[Mg^{2+}]_o$ -induced  $[Ca^{2+}]_i$  spikes (Aa), D-AP5, nimodipine blocked the spikes (Ab-Ac). Ad, BAPTA-AM gradually inhibited the spikes.  $[Mg^{2+}]_o$  was reduced to 0.1 mM for the entire imaging period and drugs were added by superfusion at the times indicated by the horizontal bars. (B) While each treatment with D-AP5 and nimodipine during preconditioning markedly inhibited the low  $[Mg^{2+}]_o$ -induced tolerance, NBQX and BAPTA-AM moderately inhibited the tolerance. Neurons were exposed to 0.814 mM  $[Mg^{2+}]_o$  DMEM (control,  $n=10$ ) or 0.1 mM  $[Mg^{2+}]_o$  DMEM without ( $n=4$ ) or with NBQX ( $n=10$ ), D-AP5 ( $n=8$ ), nimodipine ( $n=4$ ) and BAPTA-AM ( $n=4$ ) for 5 min, and 24 h later exposed to 0.1 mM  $[Mg^{2+}]_o$  medium for following 24 h. Data are expressed as means  $\pm$  SEM. \*\* $p < 0.01$  relative to control, # $p < 0.01$  relative to insult, + $p < 0.05$  relative to preconditioning+insult (ANOVA with Bonferroni's test).

### Low [Mg<sup>2+</sup>]<sub>o</sub> preconditioning-induced excitotoxic tolerance through activation of other protein kinases

The Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway has been reported to play an essential role in the development of preconditioning in myocardial ischemia [28-30], suggesting that the JAK-STAT pathway could be involved in seizure preconditioning in

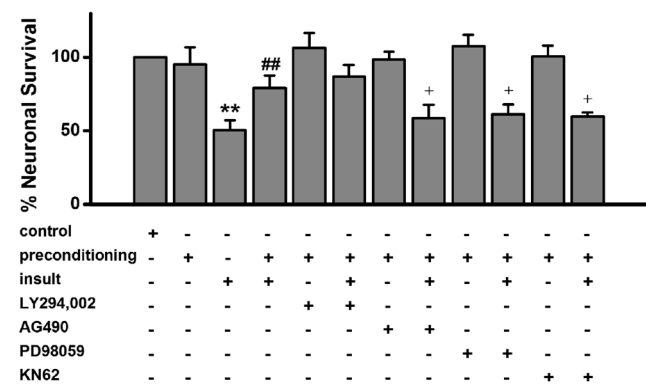


**Fig. 4. Low [Mg<sup>2+</sup>]<sub>o</sub>-induced tolerance is mediated through activation of certain forms of PKC.** (A) Low [Mg<sup>2+</sup>]<sub>o</sub>-induced [Ca<sup>2+</sup>]<sub>i</sub> spikes are not affected by staurosporin (Aa), Gö6976 (Ab), the specific PKCε translocation inhibitor (Ac) and the PKCζ pseudosubstrate inhibitor (Ad). The [Mg<sup>2+</sup>]<sub>o</sub> was reduced to 0.1 mM for the entire imaging period and drugs were added by superfusion at the times indicated by the horizontal bars. (B) Low [Mg<sup>2+</sup>]<sub>o</sub>-induced tolerance is mediated through certain forms of PKC. Neurons were exposed to 0.814 mM [Mg<sup>2+</sup>]<sub>o</sub> DMEM (control, n=4) or 0.1 mM [Mg<sup>2+</sup>]<sub>o</sub> DMEM without (n=4) or with staurosporin (n=4), Gö6976 (n=4), PKCε translocation inhibitor (n=4), and PKCζ pseudosubstrate inhibitor (n=4) for 5 min, and 24 h later exposed to low [Mg<sup>2+</sup>]<sub>o</sub> medium or control medium for 24 h. Data are expressed as mean±SEM. \*\*p<0.01 relative to control, ##p<0.01 relative to insult. +p<0.05 relative to preconditioning+insult (ANOVA with Bonferroni's test).

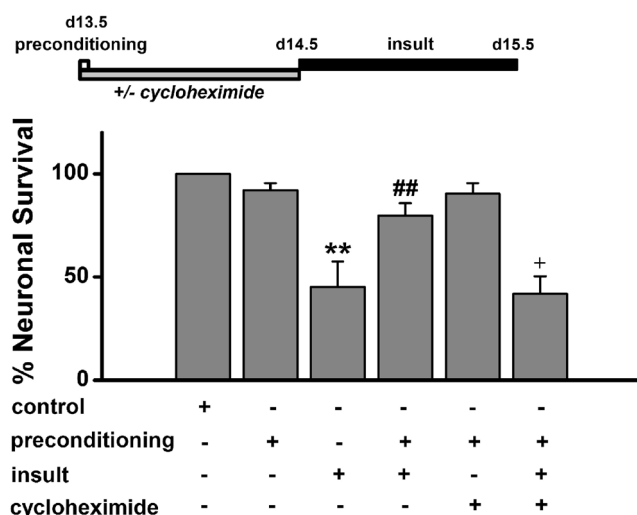
hippocampal neurons. As shown in Fig. 5, treatment with the specific JAK-2 inhibitor AG490 during the preconditioning significantly inhibited the tolerance. However, AG490 alone had no effect on the [Ca<sup>2+</sup>]<sub>i</sub> spikes (Data are not shown) as well as on neuronal viability (Fig. 5). Phosphoinositide-3kinase (PI3-kinase) [31], mitogen activated protein kinase (MAPK) kinase [26] and Ca<sup>2+</sup>/calmodulin dependent protein kinase II (CaMKII) [32] have been reported to reduce ischemic preconditioning effects in an oxygen-glucose deprivation (OGD) model. Treatment with the PI3-kinase inhibitor LY294,002 did not significantly inhibit the tolerance. However, both the MAPK kinase inhibitor PD98059 and the CaMKII inhibitor KN-62 significantly inhibited the tolerance (Fig. 5). LY294002 (5 μM), AG490 (3 μM), PD98059 (3 μM) or KN-62 (5 μM) alone had no effect on [Ca<sup>2+</sup>]<sub>i</sub> spikes (Data are not shown) and low [Mg<sup>2+</sup>]<sub>o</sub>-induced neuronal death (Fig. 5).

### Effects of protein synthesis inhibitor on low [Mg<sup>2+</sup>]<sub>o</sub> preconditioning-induced excitotoxic tolerance

To examine whether the synthesis of de novo proteins is necessary for low [Mg<sup>2+</sup>]<sub>o</sub> preconditioning -induced excitotoxic tolerance, we treated cells for 24 h with the protein synthesis inhibitor cycloheximide (0.5 μg/ml) from beginning of preconditioning to immediately prior to prolonged insult in order to block preconditioning-induced protein synthesis (Fig. 6). Cycloheximide abolished the tolerance (41.9±8.5%, n= 6) while it alone had no effect on neuronal survival. This finding indicates that de novo protein synthesis is necessary in low [Mg<sup>2+</sup>]<sub>o</sub> preconditioning -induced excitotoxic tolerance.



**Fig. 5. Low [Mg<sup>2+</sup>]<sub>o</sub>-induced tolerance is mediated through activation of the JAK-2, MAPK kinase and CaMKII but not the PI3-kinase.** Low [Mg<sup>2+</sup>]<sub>o</sub>-induced tolerance is affected by AG490, PD98059 and KN-62 but not by LY294,002. Neurons were exposed to 0.814 mM [Mg<sup>2+</sup>]<sub>o</sub> DMEM (control, n=8) or 0.1 mM [Mg<sup>2+</sup>]<sub>o</sub> DMEM without (n=4) or with LY294,002 (n=4), AG490 (n=8), PD98059 (n=4) or KN-62 (n=6) for 5 min, and then exposed 24 h later to 0.1 mM [Mg<sup>2+</sup>]<sub>o</sub> medium or control medium for 24 h. Data are expressed as means±SEM. \*\*p<0.01 relative to control, ##p<0.01 relative to insult. +p<0.05 relative to preconditioning+insult (ANOVA with Bonferroni's test).



**Fig. 6. Low  $[Mg^{2+}]_o$ -induced tolerance by de novo synthesis of proteins.** Hippocampal neurons were exposed to 0.814 mM  $[Mg^{2+}]_o$  DMEM (control, n=4) or 0.1 mM  $[Mg^{2+}]_o$  DMEM without or with cycloheximide for 5 min, and then exposed to 0.814 mM  $[Mg^{2+}]_o$  DMEM (n=4) with or without cycloheximide (n=4) for 24 h before an exposure to 0.1 mM  $[Mg^{2+}]_o$  medium for 24 h. Data are expressed as mean±SEM. \*\*p<0.01 relative to control, ##p<0.01 relative to insult. †p<0.05 relative to preconditioning+insult (ANOVA with Bonferroni's test).

## DISCUSSION

The findings herein show that *in vitro* preconditioning by a brief exposure to 0.1 mM  $[Mg^{2+}]_o$  protected cells from subsequent prolonged exposure-induced excitotoxicity in hippocampal neurons. The low  $[Mg^{2+}]_o$  preconditioning-induced excitotoxic tolerance was suppressed by the inhibition of  $[Ca^{2+}]_i$  spikes. Furthermore, the tolerance was inhibited by the inhibition of PKC $\epsilon$ , PKC $\zeta$ , JAK-2, MAPK, and CaMKII without affecting the  $[Ca^{2+}]_i$  spikes during the preconditioning. In addition, the low  $[Mg^{2+}]_o$ -induced tolerance was blocked by inhibition of de novo protein synthesis.

Although a sustained exposure to low  $[Mg^{2+}]_o$  for 24 hr is known to be harmful, the present study indicates that a brief exposure to low  $[Mg^{2+}]_o$  can also lead to adaptations that protect neurons from prolonged exposure to low  $[Mg^{2+}]_o$  (tolerance). We defined the duration of a brief, nonlethal preconditioning exposure. A paradigm of a 5 min preconditioning and a 24 h main insult was optimal to study the characteristics of the tolerance in our *in vitro* model. A brief exposure of hippocampal neurons to low  $[Mg^{2+}]_o$  for 5 min, which induces synaptically synchronized  $[Ca^{2+}]_i$  spikes, inhibited subsequent prolonged exposure-induced excitotoxicity. The low  $[Mg^{2+}]_o$ -induced  $Ca^{2+}$  spikes are synchronized in an active network and induce neuronal cell death [18,21,22]. The low  $[Mg^{2+}]_o$  in neuronal cells or slice has been characterized as an *in vitro* seizure model [20,23,24]. All these data suggest a possibility that the brief synaptically-induced  $Ca^{2+}$  spikes by seizure-inducing low  $[Mg^{2+}]_o$  directly induce

preconditioning and prevent seizure as well as excitotoxicity. In fact, it has been reported that one or more brief seizure episodes to prolonged seizures render neurons resistant to subsequent more severe insults *in vivo* [33,34] and *in vitro* [35].

D-AP5 or nimodipine completely inhibited the low  $[Mg^{2+}]_o$ -induced  $[Ca^{2+}]_i$  spikes and NBQX significantly inhibited it, thus confirming previously reported results [18]. D-AP5 and nimodipine markedly, and NBQX slightly inhibited the tolerance, which depends on their inhibition of  $[Ca^{2+}]_i$  spikes. Moreover, the chelation of intracellular  $Ca^{2+}$  with BAPTA-AM inhibited the tolerance. These observations are indirectly supported by other studies that activation of the NMDA receptor is involved in the preconditioning effects [11,12,14,26]. This study is directly supported by others' results that the NMDA receptor and  $Ca^{2+}$  are implicated in the induction of anoxia-induced tolerance in cortical slices [36] and ischemia-induced tolerance in hippocampal slices [14,15].  $Ca^{2+}$  spikes have also been reported to induce effectively the expression of specific genes [7]. Moreover, nuclear  $Ca^{2+}$  and the cAMP response element-binding protein family have been reported to mediate a late phase of activity-dependent neuroprotection [9]. Ischemic preconditioning in heart has been reported to be attenuated by the inhibition of voltage-dependent  $Ca^{2+}$  [37]. However, ischemic preconditioning of the cortical neurons was not inhibited by the removal of extracellular  $Ca^{2+}$  or the inhibition of the AMPA receptor [38]. All these data suggest that preconditioning effects are dependent on the preconditioning methods and the location of the tissue used in the study.

Cellular signaling through  $Ca^{2+}$ , which is partly mediated by protein kinases such as PKC, CaMKII, and other kinases, has been previously described in ischemic cell death [39]. The intracellular signaling triggered by preconditioning stimuli is achieved by post-translational modifications, primarily the phosphorylation of key proteins [10,11,32]. In this study, inhibition of a novel PKC $\epsilon$  or an atypical PKC $\zeta$  during the preconditioning decreased the low  $[Mg^{2+}]_o$ -induced tolerance. The translocation of PKC and the phosphorylation of several membrane proteins are mediated through NMDA receptor-mediated  $Ca^{2+}$  influx [40]. *In vitro* hippocampal neurons express various PKC isoforms including PKC $\epsilon$  and PKC $\zeta$  [41]. Ischemic or NMDA-induced neuroprotection was the results of the activation of PKC $\epsilon$  during preconditioning in hippocampal slices [14,26] and neuronal cultures [27]. These results suggest that the low  $[Mg^{2+}]_o$  preconditioning-induced excitotoxic tolerance is mediated through the  $Ca^{2+}$ -induced activation of PKC $\epsilon$  and PKC $\zeta$ , although PKC activity has been reported not to be involved in ischemic preconditioning in cortical cultures [38]. Since PKC $\epsilon$  and PKC $\zeta$  are  $Ca^{2+}$ -insensitive PKCs, low  $[Mg^{2+}]_o$ -induced  $[Ca^{2+}]_i$  spikes may indirectly induce the activation of PKC $\epsilon$  and PKC $\zeta$  during the low  $[Mg^{2+}]_o$  preconditioning.

Although CaMKII plays a key role in mediating some of the biochemical events that lead to cell death following an

ischemic insult [42], treatment with KN-62 inhibited the low  $[Mg^{2+}]_o$ -induced tolerance without affecting  $[Ca^{2+}]_i$  spikes in the present study. This observation is directly supported by a report that treatment with KN-62 during ischemic preconditioning suppressed the development of subsequent tolerance in rat cortical cultures [32]. In addition, CaMKII- $\alpha$  knockout mice are more susceptible to cerebral ischemia [43]. These data suggest that the activation of CaMKII is involved in  $Ca^{2+}$ -mediated tolerance as well as neuronal cell death. On the other hand, treatment with KN62 during NMDA preconditioning did not significantly reduce the quinolinic acid-induced seizure [13].

JAK signaling is important in ischemic preconditioning in the heart [28]. The inhibition of JAK-2 with AG490 abolishes erythropoietin-induced tolerance against cerebral ischemia [31]. In this study, AG490 inhibited the low  $[Mg^{2+}]_o$ -induced tolerance. Although the issue of how  $[Ca^{2+}]_i$  spikes activate JAK-2 remains unknown, these data strongly suggest that JAK-2 is involved in low  $[Mg^{2+}]_o$ -induced tolerance.

PI3-kinase has been implicated in anti-apoptotic signaling in various cell types, including neurons [44]. LY294,002 did not block the low  $[Mg^{2+}]_o$ -induced tolerance, consistent with a previous report indicating that PI3-kinase activity is not required for preconditioning in an OGD model [11]. Ischemic preconditioning has also been linked to the phosphorylation of extracellular signal-regulated protein kinases (ERKs) [45]. Ras, Raf, MEK and ERK are required for the development of neuronal preconditioning induced by OGD [11]. Preconditioning by  $Ca^{2+}$  ionophore ionomycin has been reported to increase the MAP kinase p42/44 after OGD [15]. In our study, PD98059 inhibited the low  $[Mg^{2+}]_o$ -induced tolerance. These observations are supported by *in vivo* observations of phosphorylation of MEK and ERK in the rat hippocampus in global cerebral ischemic preconditioning [45] and the inhibition of *in vitro*  $Mg^{2+}$ -free preconditioning with U0126 [10]. In contrast to our result, Tauskela et al. [32] reported that treatment with MAPK kinase inhibitors had no effect on OGD-induced preconditioning effects in rat cortical neurons.

Calcium ions have the ability to propagate to the nucleus. The activity of the transcription factors Elk-1, CREB, and MEF-2 is induced by calcium [8]. Moreover,  $Ca^{2+}$  spikes efficiently induce the expression of certain specific genes [7]. These results support the possibility that low  $[Mg^{2+}]_o$ -induced  $[Ca^{2+}]_i$  spikes induce the de novo synthesis of proteins. In fact, low  $[Mg^{2+}]_o$ -induced tolerance was blocked by treatment with cycloheximide, suggesting that the tolerance is dependent on the synthesis of de novo proteins. However the issue of which proteins are synthesized by  $[Ca^{2+}]_i$  spikes remains to be elucidated. In conclusion, a brief low  $[Mg^{2+}]_o$  preconditioning induces subsequent prolonged exposure-induced excitotoxicity. The low  $[Mg^{2+}]_o$  preconditioning-induced excitotoxic tolerance was directly or indirectly mediated through the  $[Ca^{2+}]_i$  spike-induced activation of PKC $\epsilon$ , PKC $\xi$ , JAK-2, MAPK kinase, CaMKII and

the de novo synthesis of proteins, which is involved in seizure as well as excitotoxicity.

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