A Doctoral Dissertation

Study on Ca^{2+} signaling-mediated regulation of somatic K^{+} outward currents contributing to neuronal excitability in dissociated hippocampal neurons of rats

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Graduate School

Jeju National University

February, 2015

초대 배양한 흰쥐 해마 신경세포에서 신경세포 흥분성에 관여하는 세포체 칼륨 통로 조절 과정의 칼슘 매개 기전에 대한 연구

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2015년 2월

Study on Ca^{2+} signaling-mediated regulation of somatic K^{+} outward currents contributing to neuronal excitability in dissociated hippocampal neurons of rats

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February, 2015

This thesis has been examined and approved.

Date

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ABSTRACT

In hippocampal neurons, intrinsic excitability (IE) modulated by dynamic synaptic activity is important to determining neuronal functions such as information processing and cognition. Voltage-dependent K^+ channels (K_V channels) showing outward K^+ currents participate in both IE and synaptic excitability by regulating membrane conductance and potential. Therefore, it is necessary to identify the mechanism regulating expression and kinetics of K_V channels. K_V channels consist of two types of currents. One is rapidly inactivated or transient current (A-type current, I_A), which is known to contribute to learning and memory mechanisms by regulating both somatic and dendritic excitability. The other is slowly inactivated or delayed rectifier current (I_{DR}), which participates in neuronal firing.

Recently, the downregulation of somatic I_A channels is systemically targeted by Ca^{2+} influx via synaptic NMDA receptors (NMDARs). However, spatial restriction between synapses and soma in hippocampal neurons strongly suggests that there may be possible cellular links responsible for synaptic Ca^{2+} influx for somatic I_A downregulation. Therefore, in the first theme of the current paper, testing was performed to determine whether two major receptors of endoplasmic reticulum (ER), ryanodine and IP_3 receptors (RyRs and IP_3Rs, respectively), participate in Ca^{2+}-mediated I_A downregulation in dissociated hippocampal neurons. Downregulation of I_A channels was induced by high Ca^{2+} (3.6 mM, for 24 hrs to culture media) or glycine (200 μM), which induces chemical long-term potentiation (LTP), and electrophysiological measurement of the peak of I_A was performed using a whole-cell patch. In results, high Ca^{2+} effect in reducing I_A peak was clearly abolished by antagonists of NMDARs or voltage-dependent Ca^{2+} channels (VDCCs), indicating that somatic I_A downregulation may be responsive to glutamatergic

synaptic activities involving NMDARs and VDCCs. In this cellular processing, blocking RyRs (by Ryanodine, 10 μM) completely abolished I_A downregulation, while blocking IP_3Rs (by 2APB, 100 μM) did not show any effects. In addition, treatment with Ryanodine also resulted in reduced Ca^{2+}-increased activity of protein kinase A (PKA, cyclic AMP-dependent protein kinase), indicating that subsequential signaling cascades including ER and PKA play roles in regulation of somatic I_A. I suggest here that contribution of RyRs to Ca^{2+}-induced Ca^{2+} release (CICR) is required for regulation of neuronal excitability via I_A channel trafficking.

I_{DR} channels act to maintain homeostasis by regulating membrane excitabilities in pathological conditions such as epileptic seizure. However, it is not clear how their kinases or phosphatases are activated during pathological condition. To address this issue, I attempted to confirm mechanisms for regulation of I_{DR} for prevention of overexcitability under high Ca^{2+} condition (3.6 mM, for 24 hrs) in dissociated hippocampal neurons. In results, it was proved that I_{DR} was enhanced by high Ca^{2+} treatment without change of kinetic properties. This increment of I_{DR} was clearly inhibited by nimodipine (VDCCs antagonist, 10 μM) but not APV (NMDARs antagonist, 100 μM), suggesting that the regulation of I_{DR} is targeted by Ca^{2+} influx through VDCCs with the independence on the downregulation of I_A. It is also confirmed that small conductance Ca^{2+}-activated K^+ channels (SK channels) are not involved in I_{DR} upregulation. In addition, results from use of PP2 (1 μM) to block Src family tyrosine kinases (SFKs) showed that the enhancement of I_{DR} is dependent on SFKs activation. These results indicate that SFKs activated by Ca^{2+} influx via VDCCs participate in I_{DR} upregulation to protect neurons from overexcitability conditions.

Consequently, the regulation of K^+ outward currents in local somatic area of neurons is quietly correlated in synaptic activities which occur in distant. However, hippocampal neurons are not questionable to actively reflect the dynamic but systemic signaling

cascades linking synaptic and somatic processings. These successive cellular processings provide a possibility that somatic membrane excitability is directly regulated by synaptic plasticities such as LTP via regulation of K^+ channels as well as Ca^{2+} signaling, determining neuronal excitabilities under various physiological and pathological conditions.

Key words: I_A channels, I_{DR} channels, Ryanodine receptor, VDCC, Synaptic LTP

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Part Ⅰ

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1. Introduction

Hippocampus is extremely important to memory processes by consolidating information. Cellular mechanisms to explain the learning and memory functions are dominantly dependent on synaptic plasticity such as long-term potentiation (LTP) or depression (LTD) (Hebb, 1949; Maren and Baudry, 1995; Yang et al., 2014). These physiological synaptic adaptations to given issues are quietly considerable as a potent model to trigger the initial formation of memory at the cellular level but not enough to support neuronal mechanisms for storage of long-lasting information in the central nervous system (CNS). Many reports have demonstrated that the alteration of neuronal intrinsic excitability (IE), which is an ability to make action potentials (APs) with given inputs, may possibly play a role in cellular information storage (Aizenman and Linden, 2000; Daoudal and Debanne, 2003; Oh et al., 2003; Zhang and Linden, 2003; Xu et al., 2005; Kim and Linden, 2007). Therefore, verification of mechanical or functional links between synaptic and somatic alterations seems to be important for completion of a memory mechanism but difficult because of local restriction of synaptic plasticity (Frick et al., 2004). In this study, I attempted to determine whether and how somatic excitability could be altered by synaptic activities through Ca^{2+} signaling cascades.

Potassium outward currents through various voltage-dependent or independent K^+ channels are crucial factors in regulation of neuronal excitability and in determining the level and pattern of neuronal responsiveness in hippocampus. Total outward K^+ currents are generally classified according to a transient or rapidly inactivating current, known as A-type (I_A) current, and a sustained or slow/non-inactivating current (delayed rectifier K^+ currents, I_{DR}) (Hoffman et al., 1997).

In the first section, I investigated the mechanism to explain Ca^{2+}-mediated somatic I_A channel regulation induced by synaptic activation. The synaptic localization of I_A channels is

important for activity-dependent regulation of glutamatergic transmissions in dendritic processings and synaptic plasticity and their internalization from active spines, which are dependent on N-methyl-D-aspartate receptors (also known as the NMDA receptors or NMDARs) activation (Kim et al., 2007; Hammond et al., 2008). It has been shown that Ca^{2+}-signaling dependent channel trafficking in both dendrites and spines is rapid but locally restricted, because Ca^{2+} influx through NMDARs in active areas is not sufficient to activate total kinases and auxiliary proteins associated with channel trafficking in a whole neuron. However, the internalization of I_A channels from active spines during glycine-induced chemical LTP was also accompanied by the reduction of somatic I_A, which was electrophysiologically observed (Kim et al., 2007). In addition, synaptic LTP of CA1 neurons induced by the paired pulse stimulation resulted in the reduction of somatic I_A, which was lasting at least during LTP (Jung and Hoffman, 2009). It is possible that the activation of synaptic NMDARs may contribute to the regulation of somatic excitability via leading the internalization of somatic I_A channels because either chemical or electrical LTP in hippocampus are mediated with the activation of synaptic NMDARs. However it is still not clear how glutamatergic activation in local synapses affects the trafficking of somatic I_A channels.

Synaptic and extrasynaptic NMDARs in mechanisms of synaptic plasticities observed in mammalian CNS, show distinctive and opposite functions in the modulation of Ca^{2+} signaling and glutamatergic responses. Despite some arguments, synaptic and extrasynaptic NMDARs are commonly thought to participate in LTP and LTD, respectively (Lu et al., 2001). In CA1 neurons, Ca^{2+} influx through synaptic NMDARs contributes to upregulation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (also known as AMPA receptors or AMPARs), resulting in LTP. In particular, the amount of Ca^{2+} entering synaptic sites determines the potentiation level and duration of LTP, indicating the potential role of synaptic NMDARs. Added to NMDAR-dependent Ca^{2+} influx, ryanodine receptors (RyRs)

and inositol-tri-phophate receptors (IP_3Rs) in intracellular Ca^{2+} store are also involved in NMDA-dependent synaptic plasticities of both LTP and LTD (Schiegg et al., 1995; Reyes and Stanton, 1996; Adasme et al., 2011). Furthermore, opening Ca^{2+} stores may be especially considerable as a dominant factor in triggering widespread cellular events following LTP induction because synaptic NMDAR activation increases cytosolic Ca^{2+} levels by opening RyRs and IP_3Rs (Emptage et al., 1999; Nishiyama et al., 2000; Taufiq et al., 2005). These results indicate that Ca^{2+} influx occurring locally in synapses may open Ca^{2+} stores to extend their cellular functions. Therefore, revealing the alteration of outward flow via somatic K^+ channels by synaptic modulations is critically important for understanding how voltage-dependent channels decide neuronal functions in synaptic plasticity for learning and memory mechanism**.**

2. Materials and Methods

2.1. Materials

Minimal essential medium (MEM), Neurobasal medium, fetal bovine serum (FBS), penicillin/streptomycin, B-27 serum-free supplement, and L-glutamine were purchased from Gibco (Gland Island, NY, USA). Nimodipine, 2-Aminoethoxydiphenylborane (2APB), Ryanodine, Tetrodotoxin (TTX), and H89 were purchased from Tocris (Ellisville, MO, USA). Fura-2 acetoxymethyl ester (Fura-2 AM) was purchased from Invitrogen (Carlsbad, CA, USA). All other reagents were purchased from Sigma-Aldrich (St Louis, MO, USA), unless indicated otherwise.

2.2. Experimental animals

Sprague-Dawley (SD) rats were used in this experiment were bred in the animal facility of the Medical School of Jeju National University. The environment of the breeding room was automatically controlled at a temperature of 23 ± 3℃ and 50 ± 10 % humidity. The lighting system was set to repetitively turn on and off for 12 hours a day. Food and water were given ad libitum. Two females and a male SD rats were mated in a cage for breeding.

All experiments and procedures with animals were performed with permission from the Animal Care and Use Committee of Jeju National University.

2.3. Hippocampal primary cultures

Hippocampal primary cultures were prepared from embryonic 20-day SD rats. The

embryos removed from deeply anesthetized pregnant rats were transferred to an ice-cold normal tyrode solution containing the following (in mM): 140 NaCl, 5.4 KCl, 2.3 $MgCl_2$, 10 HEPES, 5 glucose, pH 7.4 adjusted with NaOH. Hippocampi isolated from embryonic rat brains were transferred to ice-cold MEM containing Earle's salts and glutamine with 10% FBS, 0.45% glucose, 1 mM sodium pyruvate, 25 μM glutamate, and antibiotics, and then triturated. For electrophysiological studies and intracellular Ca^{2+} recordings, the cells were counted and seeded on glass coverslips (Fisher Scientific) coated with poly-L-lysine at a density of 9 X 10^4 cells/ml and maintained at 37℃ in 95% air and 5% CO_2. After 7 hours, the whole plating medium was changed to Neurobasal medium containing B-27, and half of the medium was changed twice per week. To examine neuronal viability, dissociated hippocampal neurons were seeded on poly-L-lysine coated 24 well plates at a density of 1.8 X 10^5 cells/ml and 1 μM arabinofuranosyl cytidine (Ara-C) was added to prevent glial proliferation at DIV 4 and 8.

2.4. Measurement of neuronal viability

To assess effects of high Ca^{2+} on cell viability, DIV 9 cultured hippocampal neurons 3.6 mM $CaCl_2$ was treated for 24 hrs, followed by addition of 0.4 mg/ml MTT (3-(4,5-methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide). One hour later, medium was gently removed and DMSO was added to each well to dissolve formazan crystals. The neuronal viability was obtained by reading absorbance at 550 nm using a microplate reader (Model 550, Bio-rad, USA).

2.5. Electrophysiology

Primary dissociated culture neurons of DIV 6-8 were used for patch-clamp recordings of transient A-type K^+ channels. Coverslips containing these young hippocampal neurons were transferred to a recording chamber with a continuous flow of recording solution containing the following (in mM) : 145 NaCl, 5 KCl, 2 $CaCl_2$, 1.3 $MgCl_2$, 10 HEPES, 10 glucose, pH 7.4 with NaOH, and bubbled with 95% O_2 and 5% CO_2. TTX (0.5 μM) was added to the recording solution to block the voltage-dependent Na^+ channels. The patch pipettes (4-6 MΩ) were filled with an internal solution containing the following (in mM) : 20 KCl, 125 K^+-gluconate, 4 NaCl, 10 HEPES, 0.5 EGTA, 4 ATP, 0.3 tris-GTP, and 10 phosphocreatin, and pH 7.2 was adjusted with KOH. During recordings, the series resistance varied between 8~30 MΩ, and recordings where the series resistance varied by more than 10% were rejected. Transient and sustained K^+ currents were digitally separated using a prepulse protocol after subtracting leak currents. Peak currents were measured at +60 mV after 200 ms prepulse to either -120 mV or -20 mV. All electrophysiological data were recorded using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA), and command pulse generation, data acquisition and analysis were performed using Digidata 1322A convertor (Axon Instruments), pClamp 8 (Axon Instruments), and IGOR Pro (Wavemetrics, Lake Oswego, OR) software.

2.6. Intracellular calcium measurements

DIV 9 dissociated culture neurons were loaded with a fluorescent Ca^{2+} indicator Fura-2AM (5 μM) and 0.1% pluronic F127at 37℃ for 45min in a dark incubator with or without ryanodine or 2APB. The loaded neurons on coverslips were transferred to a recording chamber with a continuous flow of recording solution and imaged using an inverted Olympus IX71 microscope (Olympus, Japan). Fluorescence excitation was measured using 340 and 380 nm filter sets and controlled with a high-speed filter switching device (Sutter

Instruments, Lambda DG-4). Digitized fluorescence image were acquired at 6s intervals using a cooled-charged device (CCP) camera (Roper Scientific, USA). To confirm changes of Ca^{2+} levels after enhancing synaptic activity, 20 mM KCl and 100 μM Glycine were added to the recording solution. Ratio images were obtained by acquiring pairs of images at alternate excitation wavelengths (340/380 nm) and analyzed using Metafluor software (Universal Imaging, Sunnyvale, CA, USA).

2.7. PKA activity

DIV 7 hippocampal neurons treated with antagonist of RyRs or IP_3Rs for 24 hrs were lysed with modified radio-immunoprecipitation assay (RIPA) buffer containing protease inhibitors and kept on ice for 15 min. The lysates were centrifuged at 13000 rpm for 15 min at 4℃ and supernatants were collected from the lysates. Protein concentrations were determined using bio-rad protein assay (Bio-Rad, Hercules, CA, USA). PKA activity was measured using a non-radioactive PKA Kinase activity assay kit (Enzo life science, Plymouth Meeting, PA). Kinase assay dilution buffer (50 μl) was added to the PKA substrate pre-coated microplate wells for 10 min and removed. The kinase reaction was then initiated by addition of 0.05 μg proteins and ATP for 30 min at 30°C. After emptying the contents of each well, the microplate was incubated with a phosphosubstrate–specific antibody for 30 min at room temperature, followed by washing four times with wash buffer. The peroxidase-conjugated secondary antibody was added, followed by incubation for 30 min at room temperature. The color was developed with tetramethylbenzidine (TMB) substrate and stopped with stop solution. The intensity of the color was measured at 450 nm.

2.8. Statistical analysis

Data analysis was performed and statistical significance was determined using Excel (Microsoft, USA) software and data were expressed as mean value ± standard error of mean (SEM). The Student's *t*-test was used, and significance was indicated for *p* values less than 0.05 or 0.01.

3. Results

3.1. Ca^{2+} influx through NMDARs and VDCCs can modulate somatic excitability by downregulating I_A channels

In previous study, synaptic activation induced the downregulation of somatic I_A channels (Jung and Hoffman, 2009). However, it is not clear how synaptic excitation affects somatic I_A channels. I tested this issue using DIV6-9 dissociated hippocampal neurons. The neurons are exposed to high Ca^{2+} (3.6 mM $CaCl_2$) for 24 hours to induce synaptic excitability (Jung et al., 2008). Figure 1 shows DIV9 dissociated hippocampal neurons with glia and their viability (A and B). The neuronal viability was determined by measuring mitochondria activity in Ara-C treated neurons. No difference of neuronal viability was observed between control and high Ca^{2+} application groups (Control= 100 ± 3.40 %, n= 48; High Ca^{2+}= 103.71 ± 3.16 %, n= 45, p= 0.43 compared with control), suggesting that high Ca^{2+} treatment did not induce neuronal cell death, at least in this study.

The synaptic activation by high Ca^{2+} treatment clearly reduced the amplitude of the somatic I_A peak (Figure 2). The high Ca^{2+} group showed a significant decrease of approximately 40 % I_A density compared with that of the control group (Control= 193.28 ± 15.08 pA/pF, n = 14; High Ca^{2+}= 113.58 ± 12.03 pA/pF, n = 12, p<0.01). To confirm whether the downregulation of I_A channels was dependent on synaptic activity, antagonists of sources of synaptic Ca^{2+} influx such as NMDARs and VDCCs were applied. In neurons treated with NMDAR antagonist APV under the condition of high Ca^{2+} for 24 hrs, the decrease of I_A peak was completely abolished (APV = 183.72 ± 20.23 pA/pF, n= 7, p= 0.727 and 0.008 compared with control and high Ca^{2+}, respectively). Furthermore, the downregulation of I_A channels also appeared to be dependent on VDCCs in presynapse, as VDCCs antagonist nimodipine diminished the effect of high Ca^{2+} application (Nimodipine = 189.28 ± 14.66

pA/pF, n= 8, *p*= 0.87 compared with control). These results indicate that the activation and enhancement of synaptic transmission can actively regulate intrinsic excitability by decreasing density of I_A channels in soma via Ca^{2+} signaling pathways.

Figure 3 shows the activation and inactivation properties of I_A channels in high Ca^{2+} treated neurons with APV or nimodipine. It was observed that a slight depolarized-shift of I_A inactivation curve was induced by high Ca^{2+} and voltage half (V_h) of I_A inactivation was increased, but it was not significant (Control= -68.87 ± 2.61 mV; High Ca^{2+}= -62.48 ± 1.62 mV, *p*= 0.07). Activation curve of I_A was shifted to the left by high Ca^{2+} and V_h of activation was slightly decreased (Control= -8.68 ± 3.14 mV; High Ca^{2+}= -15.51 ± 2.73 mV, *p*= 0.16). Both NMDARs and VDCCs antagonists did not change activation and inactivation properties of I_A channels (V_h of inactivation: APV= -66.70 ± 2.15 mV; Nimodipine= -65.63 ± 1.23, V_h of activation: APV= -7.54 ± 2.67 mV; Nimodipine= -6.01 ± 1.07). This finding suggests that the downregulation of I_A channels by high Ca^{2+} application may result from the internalization of I_A channels in soma, but not from the alteration of gating kinetics.

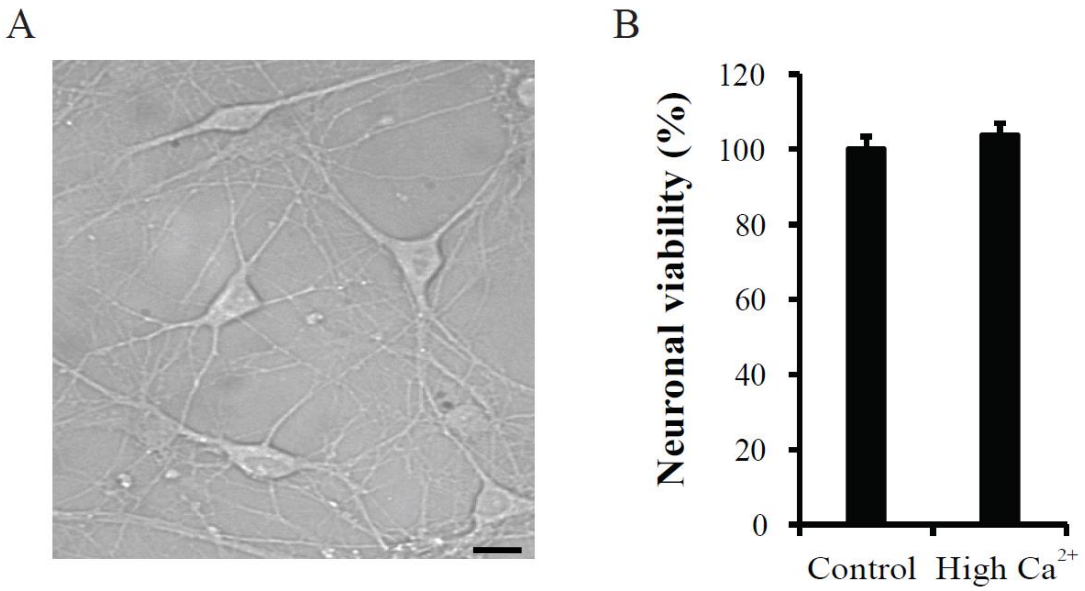


Figure 1. Effects of the high Ca^{2+} treatment on cell viability in cultured hippocampal neurons. A. DIV 9 cultured hippocampal neurons. The morphology was observed by using Metamorph software. Scale bar; 100 μm. B. Neuronal viability was not affected by high Ca^{2+} application. The high Ca^{2+} (3.6 mM $CaCl_2$) was applied to Ara-C exposed hippocampal neurons for 24hrs and neuronal viability was evaluated by using MTT assay. Error bars represent SEM.

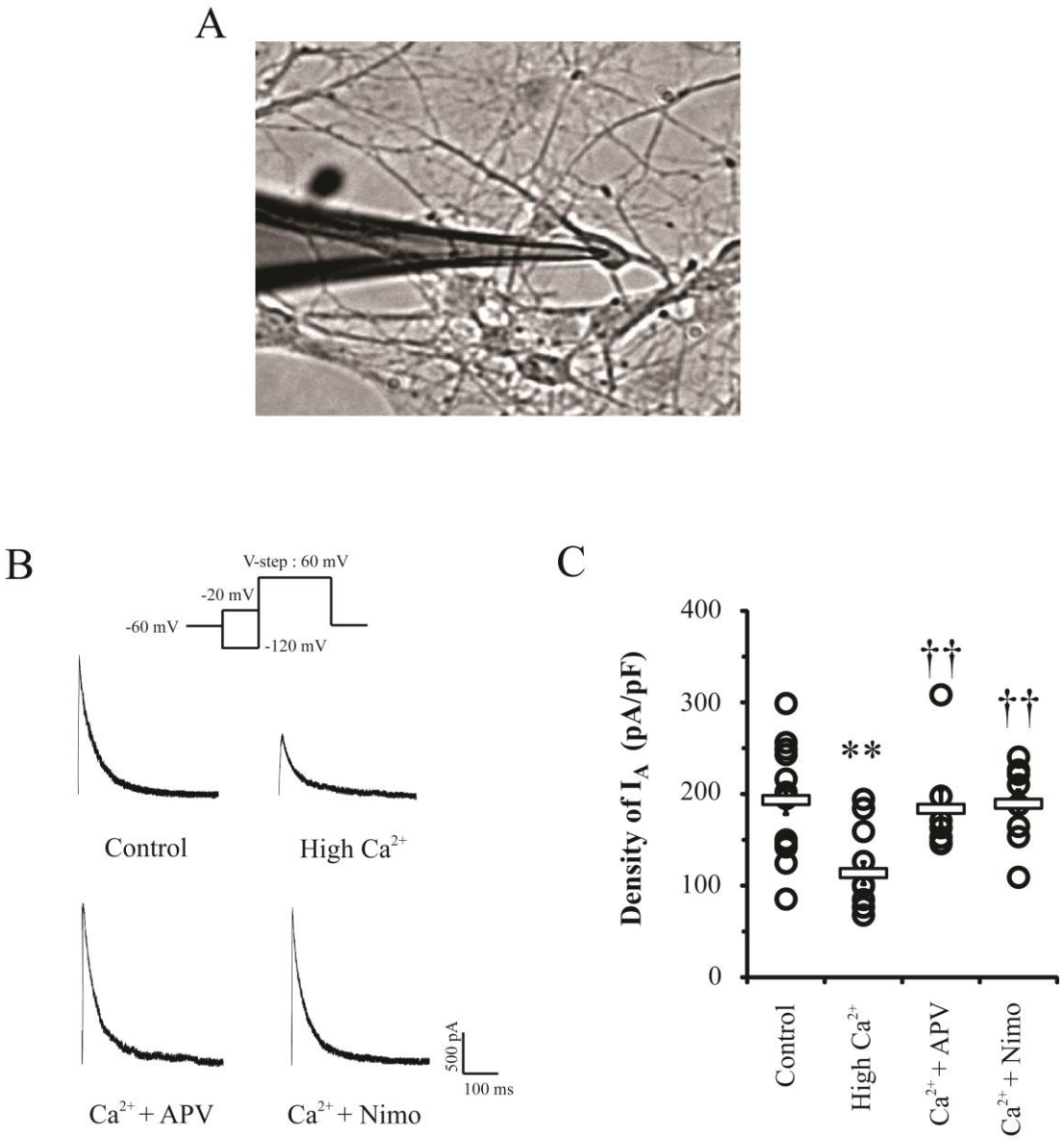


Figure 2. The peaks of somatic I_A were decreased by the enhancement of synaptic Ca^{2+} influx through NMDARs and VDCCs. A. Whole-cell patch clamp in DIV 7 primary hippocampal neurons. The image was obtained by using RS image (Roper Scientific). **B.** Example traces of I_A recorded in dissociated neurons (DIV 6-9) after high Ca^{2+} (3.6 mM) treatment with APV (100 μM) or nimodipine (10 μM) in culture media for 24 hrs. The APV and nimodipine can block NMDARs and VDCCs, respectively. Scale bars; 500 pA, 100 ms. **C.** Individual (circle) and averaged (square) transient current densities. Error bars represent SEM. $p < 0.01$ compared with control* and high Ca^{2+}†.

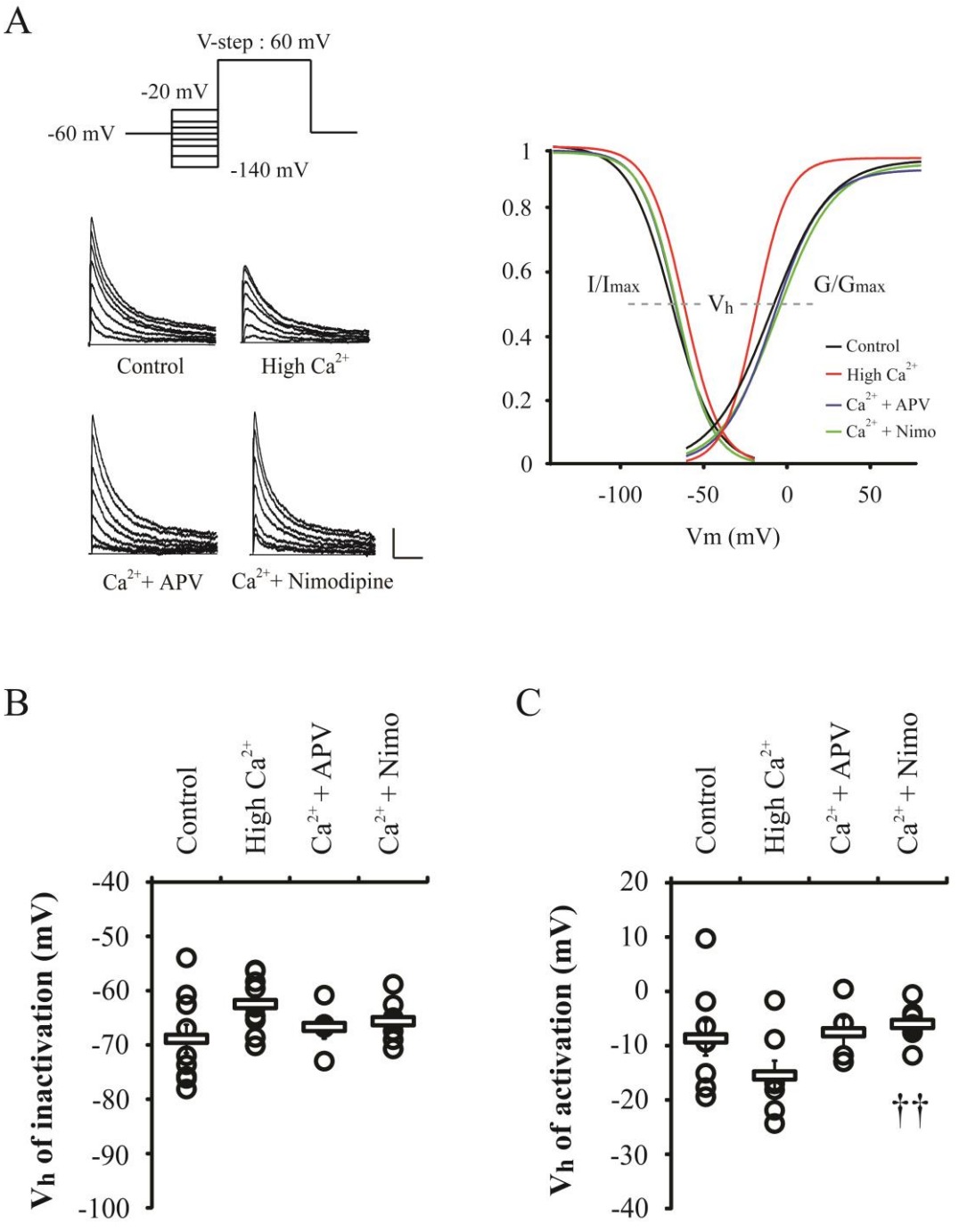


Figure 3. Activation and inactivation properties of I_A channels are not changed by high Ca^{2+} treatment. The high Ca^{2+} (3.6 mM) was treated with APV (100 μM) or nimodipine (10 μM) and then currents kinetics were electrophyically recorded. The inactivation properties were measured at 60 mV after 200 ms prepulse (-140 to -20 mV with 20 or 40 mV steps) and

activation properties at -60 to 80 mV with 20 or 40 mV steps (prepulse: -140 mV for 200 ms). **A.** Examples of inactivation traces and boltzmann fitted gating kinetics of I_A channels. Scale bars 200 pA, 50ms. **B and C.** The voltage half (V_h) values of inactivation and activation properties, indicated with thick gray dotted lines in A. Circles and squares are individuals and averaged V_h value, respectively. Error bars represent SEM. p <0.01 compared with high Ca^{2+} application.

3.2. Ryanodine receptors of intracellular Ca^{2+} store, but not IP_3 receptors, are crucial for I_A downregulation.

In this experiment, a role of ER Ca^{2+} store to find a Ca^{2+} mediator between synapse and soma of neurons was tested by applying 10 μM Ryanodine or 100 μM 2APB under the high Ca^{2+} condition for 24 hrs. Ryanodine and 2APB were used as antagonists of RyRs and IP_3Rs, respectively, which are major Ca^{2+} outflux channels in ER. As shown in Figure 4, the effect of high Ca^{2+} in reducing I_A peaks was completely blocked by Ryanodine application while 2APB did not affect the downregulation of I_A (Ryanodine = 180.63 ± 9.7 pA/pF, p= 0.53; 2APB= 130.34 ± 12.27 pA/pF, p= 0.01). This suggests a possibility that Ca^{2+} influx during synaptic enhancement primarily targets the RyRs to downregulate somatic I_A channels.

Similarly, Ryanodine also prevented the reduction of I_A peaks when chemical LTPs (cLTPs) were induced by 200 μM glycine (Figure 5, before= 100 ± 0 %, 10 min after cLTP= 128.16 ± 6.71 %, p= 0.008 compared with before cLTPs) which significantly reduced I_A peak amplitude by approximately 30 % in the control (before= 100 ± 0 %; 10 min after cLTP= 70.35 ± 6.07 %, p= 0.001) or 2APB group (before= 100 ± 0 %; 10 min after cLTP= 83.53 ± 4.74 %, p= 0.007). Table 1 shows the densities of I_A before and 10 min after cLTP induction. Interestingly, before cLTP induction, density of I_A in Ryanodine-treated neurons was significantly higher than that of the control group (Control= 1.66 ± 0.13 pA/pF; Ryanodine= 2.28 ± 0.28 pA/pF, p= 0.04). This indicates the existence of RyRs' roles to participate in trafficking of somatic I_A channels under the normal condition. These results indicate that Ca^{2+} release from ER via RyRs induced by activation of postsynaptic NMDARs may regulate somatic excitability by acting as cellular linkers between synaptic and somatic areas.

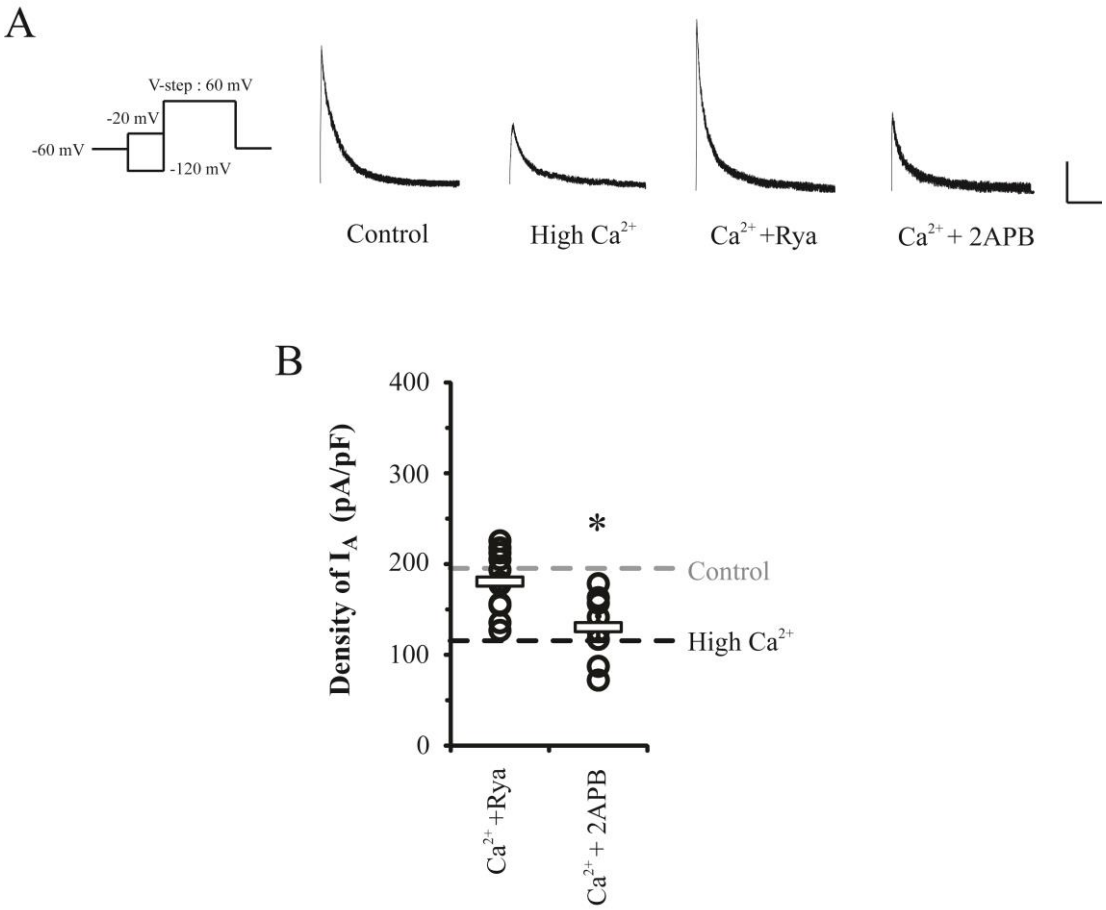


Figure 4. CICR showed the signaling specificity dependent on RyRs but not IP_3Rs in somatic I_A downregulation. Ca^{2+}-induced downregulation of I_A was abolished by Ryanodine (10 μM) to block RyRs of ER, while an IP_3R blocker (2APB, 100 μM) did not show any effects. **A.** Example traces of I_A after high Ca^{2+} treatment with Ryanodine or 2APB to culture media for 24 hrs. Scale bars 500 pA, 100 ms. **B.** Averaged (square bars) densities of I_A with individual values (open circles). Error bars represent SEM. $p < 0.05$

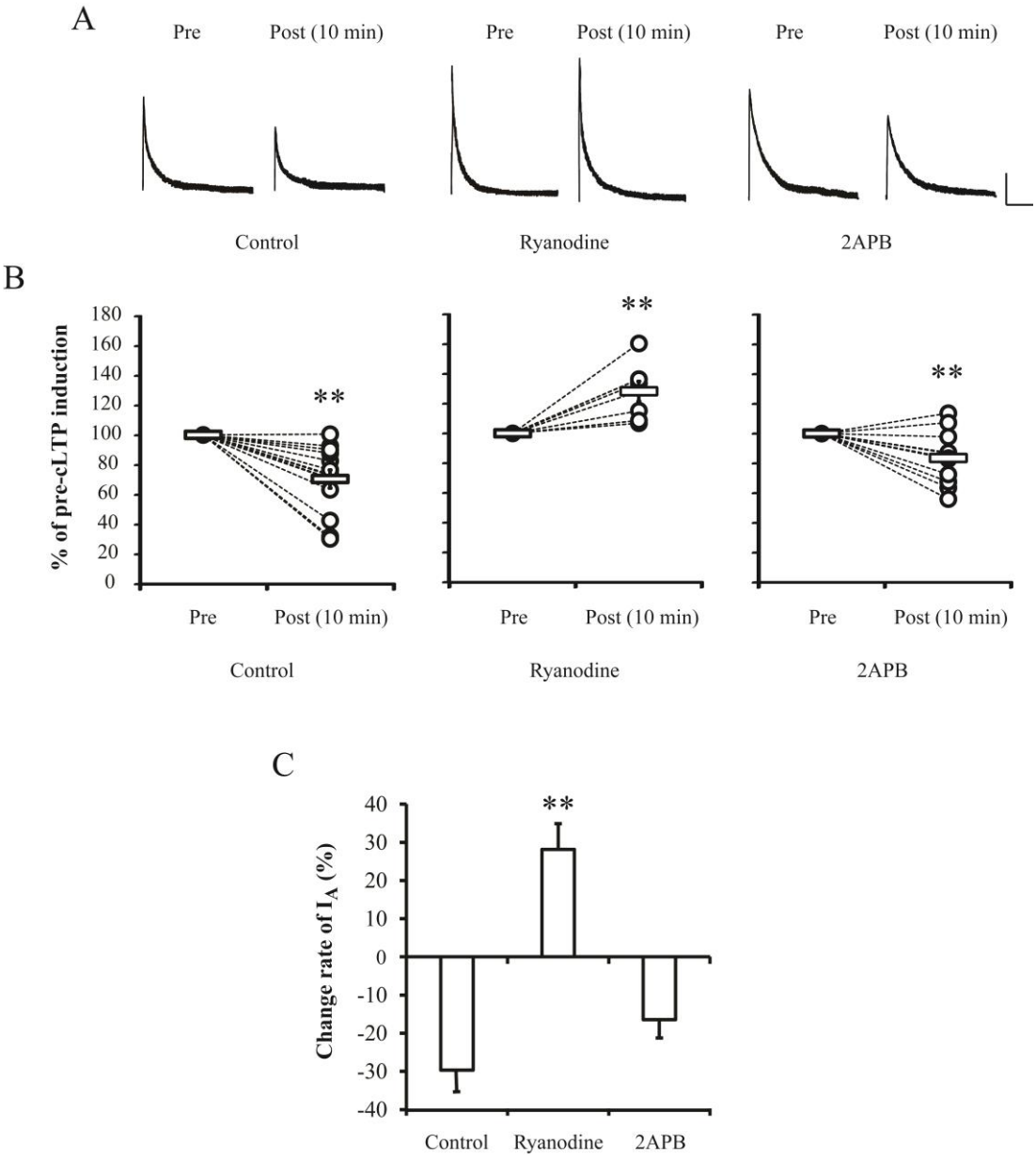


Figure 5. Downregulation of somatic I_A induced by chemical LTP resulted from RyRs activation. Ryanodine completely blocked the downregulation of I_A shown in case of chemical LTPs (cLTPs) which were induced by adding 200 μM glycine to recording solution. **A.** Example traces of I_A before and after 10 min of cLTP induction. Scale bars 500 pA, 100 ms. **B.** Normalized changes of I_A peaks induced by cLTP. Open circles and square bars indicate individuals and averaged values, respectively. **C.** Change rate of I_A peak after cLTPs induction. Error bars represent SEM. $p < 0.01$

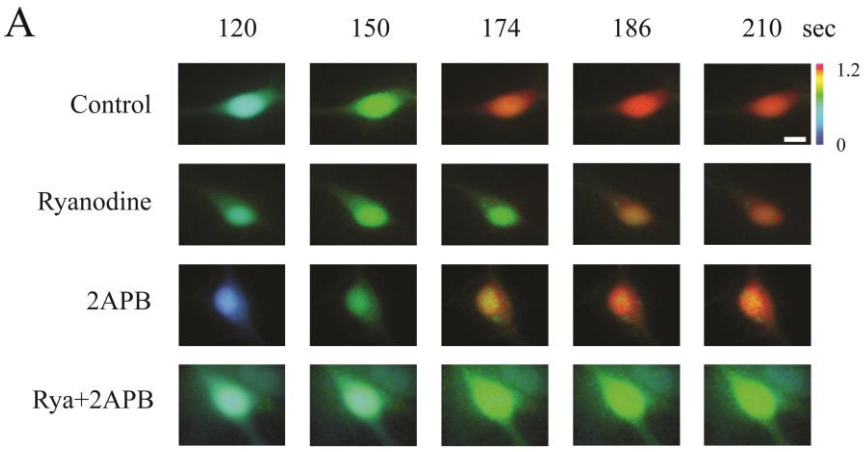
Table 1. The changes of A-type currents induced by cLTP

	Control	Ryanodine	2APB
before cLTP	1.66 ± 0.13	2.28 ± 0.28 †	1.49 ± 0.11
10 min after cLTP	1.17 ± 0.16 **	2.82 ± 0.26 **	1.30 ± 0.17 **

※ P < 0.05, 0.01 compared with before cLTP* and contol †

3.3. RyRs of ER more rapidly release Ca^{2+} than IP_3Rs in dissociated hippocampal neurons.

Intracellular Ca^{2+} levels were measured to evaluate how RyRs of ER, not IP_3Rs, are involved in I_A downregulation. To observe changes of somatic free Ca^{2+} level after synaptic enhancement, 20 mM KCl and 100 μM glycine were applied to cultured neurons loaded with fluorescent Ca^{2+} indicator Fura-2AM (Figure 6). Intracellular Ca^{2+} levels in Ryanodine-treated neurons were increased slowly, compared with control and 2APB treated neurons (Figure 6.D, Control= 196.86 ± 4.26 sec, n= 42; Ryanodine= 216.63 ± 4.45 sec, n= 38; 2APB= 201.6 ± 4.89, n= 40; Ryanodine with 2APB= 214.7 ± 3.9, n= 46). However, no significant changes of Ca^{2+} wave peak and the level of late phase (5 min after synaptic activation) were observed among each group (Peak of F1/F0: Control= 2.17 ± 0.06; Ryanodine= 2.11 ± 0.08; 2APB= 2.24 ± 0.08, 5 min after synaptic activation; Control= 1.71 ± 0.04, Ryanodine= 1.72 ± 0.06, 2APB= 1.76 ± 0.06). These results suggest that the increasing rate of intracellular Ca^{2+} levels is more important than the total amount for regulation of neuronal excitability.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120
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174
186
210 sec
1.2
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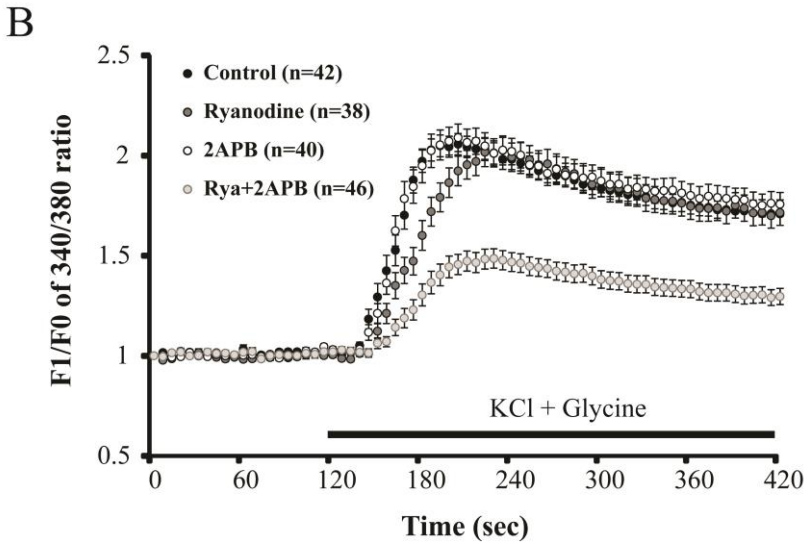
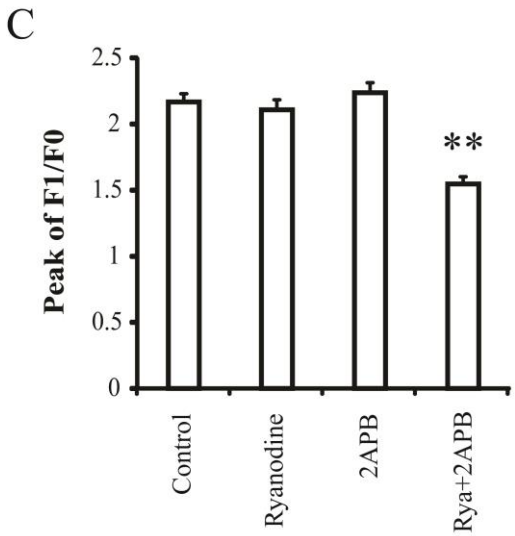
Control
Ryanodine
2APB
Rya+2APB


B
Control (n=42)
Ryanodine (n=38)
2APB (n=40)
Rya+2APB (n=46)
F1/F0 of 340/380 ratio
KCl + Glycine
Time (sec)


C
Peak of F1/F0
**
Control
Ryanodine
2APB
Rya+2APB

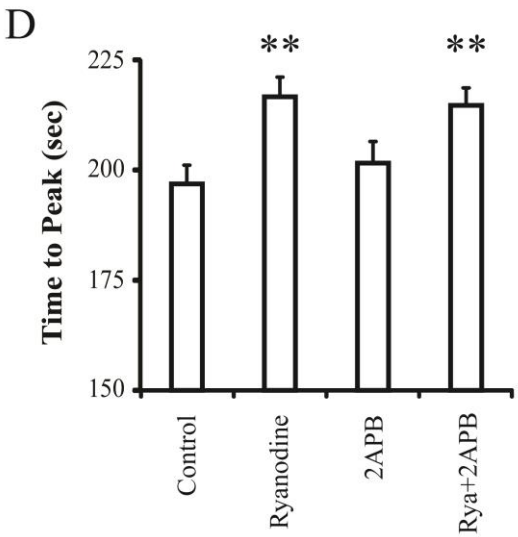

D
Time to Peak (sec)
**
**
Control
Ryanodine
2APB
Rya+2APB

Figure 6. RyRs induce faster enhancement of $[Ca^{2+}]_i$ than IP_3Rs in hippocampal neurons. Intracellular Ca^{2+} levels were more rapidly increased through RyRs of ER, compared with IP_3Rs. Ca^{2+} levels were recorded in cultured hippocampal neurons by loading fluorescent Ca^{2+} indicator Fura-2AM (5 μM). High K^+ (20 mM) and glycine (100 μM) were treated to induce neurotransmitter release and NMDARs activation in synaptic sites. **A.** Cultured hippocampal neurons loaded with fura-2AM before and after high K^+ and glycine treatment. Ryanodine and 2APB were added to culture media for 45 min before recording. Scale bars 50 μm. **B.** Averaged F1/F0 of 340/380 ratio**. C and D.** Averaged peak of F1/F0 and time to peak. Error bars represent SEM. $p < 0.01$

3.4. Activation of RyRs independently downregulates I_A without synaptic activity

It is clear that RyRs of Ca^{2+} stores are targeted by the activation of synaptic NMDA receptors to regulate somatic excitability from the above. Those results suggest one more question; 'Can active RyRs independently induce I_A downregulation?'. To answer this question, RyRs agonists, Caffeine (5 mM) and 4CmC (50 μM), were hired. Caffeine was added to internal solution to prevent effects of adenosine receptors in plasma membrane and 4CmC was applied extracellularly to culture media for 24 hrs. The density of somatic I_A was significantly reduced by either caffeine or 4CmC without the enhancement of synaptic transmission (Figure 7, caffeine= 131.17 ± 13.1 pA/pF, n= 11, *p*= 0.008; 4CmC= 110.88 ± 9.85 pA/pF, n= 8, *p*= 0.001). In addition, 4CmC effect was also observed in cases in which 100 μM APV or 0.5 μM TTX was added to abolish synaptic transmission(APV= 139.35 ± 8.93 pA/pF, n=11, TTX= 146.43 ± 13.96 pA/pF, n=10, $P<0.05$). These results indicate that opening RyRs of ER is necessary and sufficient for I_A downregulation.

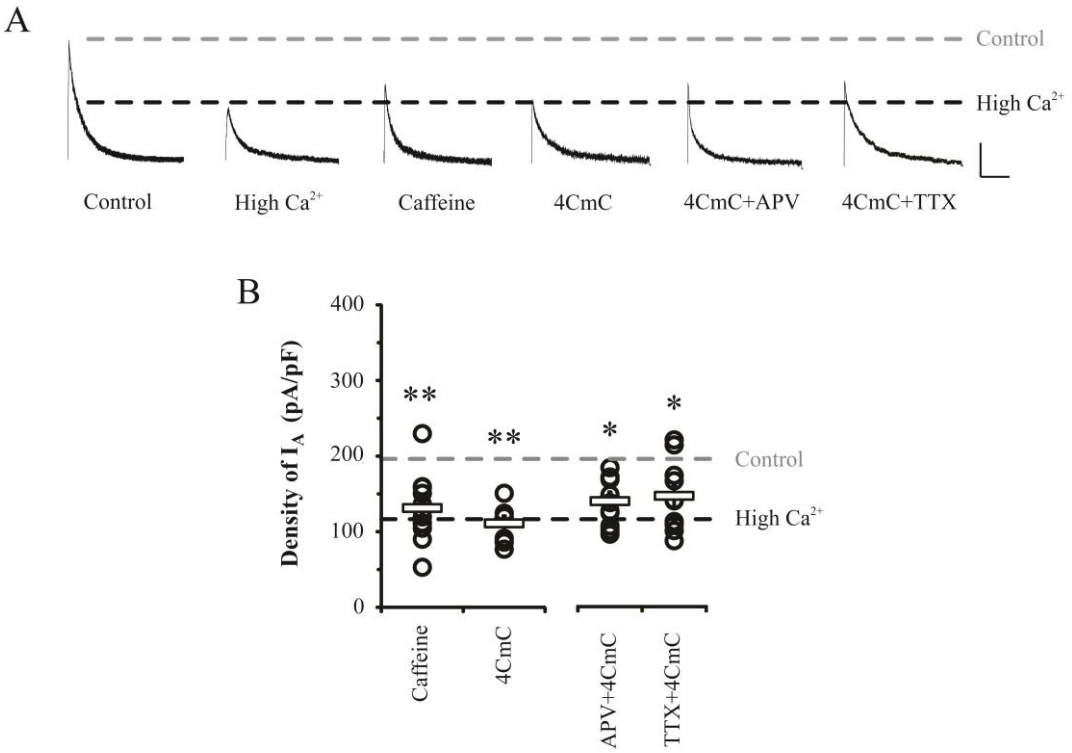


Figure 7. Downregulation of I_A by opening RyRs did not require synaptic activity. Caffeine or 4CmC treatment to open Ca^{2+} store via activating RyRs alone induced the downregulation of I_A without synaptic activities, as reduced I_A channel densities were not restored by APV (100 μM) or Na^+ channel blocker TTX (0.5 μM). **A**. Example traces of I_A currents after caffeine (5 mM) or 4CmC (50 μM) treatments with/without APV and TTX to culture media for 24 hrs in pyramidal neurons. Scale bars 500 pA, 100 ms. **B**. Averaged density of I_A (square bars) with individual values (open circles). Error bars represent SEM. $p < 0.05$ or 0.01 compared with control.

3.5. RyRs-mediated signaling absolutely requires PKA phosphorylation for I_A downregulation.

It is well known that the downregulation of I_A channels is mediated by PKA activity (Hoffman and Johnston, 1998; Schrader et al., 2002). Therefore, in the present study, PKA effects were also tested by observation of I_A changes after treatment with PKA antagonist and a solid phase enzyme-linked immune-absorbent assay (ELISA). As shown in Figure 8, effects of high Ca^{2+} and 4CmC on density of somatic I_A were significantly abolished by adding PKA antagonist H89 (10 μM). H89 restored somatic I_A peaks up to control levels under conditions of high Ca^{2+} (232.32 ± 27.49 pA/pF, p<0.001 compared with high Ca^{2+}) as well as 4CmC application (182.13 ± 19 pA/pF p<0.01), while it did not affect I_A densities in normal condition (215.60 ± 19.24 pA/pF, p= 0.39). In addition, the effects of RyRs and IP_3Rs on PKA activity were observed by using ELISA under high Ca^{2+} condition (Figure 9). In neurons treated with high Ca^{2+}, PKA activity was slightly but not significantly increased (Control= 100 ± 1.89 %, high Ca^{2+}= 108.28 ± 3.71, P= 0.18). However, Ryanodine-treated neurons showed a significant reduction of PKA activity under high Ca^{2+} condition (Ryanodine= 76.38 ± 6.8 %, p= 0.05 and 0.03 compared with control and high Ca^{2+}, respectively), while 2APB did not affect the degree of active PKA (2APB= 108.28 ± 2.4 %). These results indicate that RyRs-mediated I_A downregulation absolutely involves PKA signaling for regulation of somatic excitability in neurons.

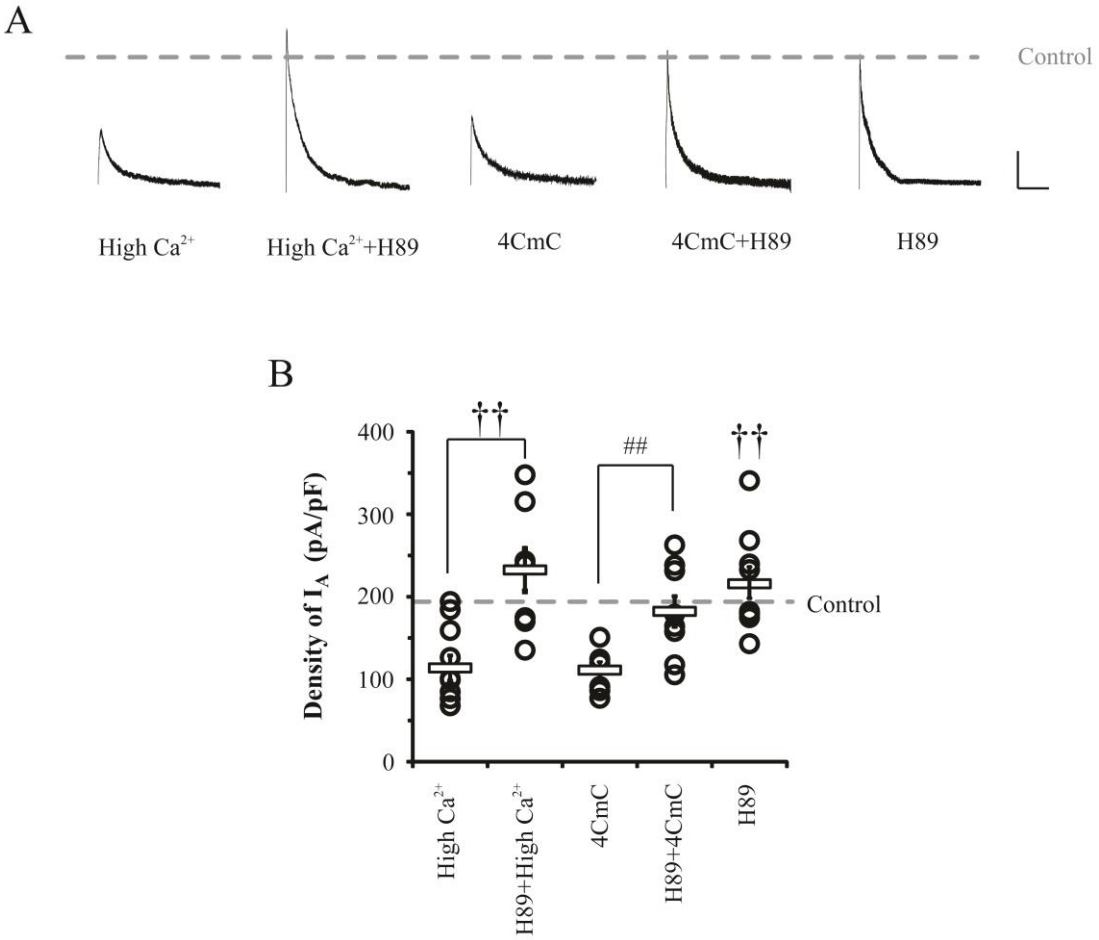


Figure 8. RyRs-mediated I_A downregulation required the activation of PKA. The activation of PKA was crucial for neuronal excitability via I_A downregulation in both synaptic dependent and independent conditions. **A**. Example traces of I_A after H89 (10 μM, PKA inhibitors) treatment to culture media for 24hrs in various conditions. Scale bars 500 pA, 100 ms. **B.** Averaged densities of I_A (square bars) with individual values (open circles). Error bars represent SEM. $p < 0.05$ or 0.01

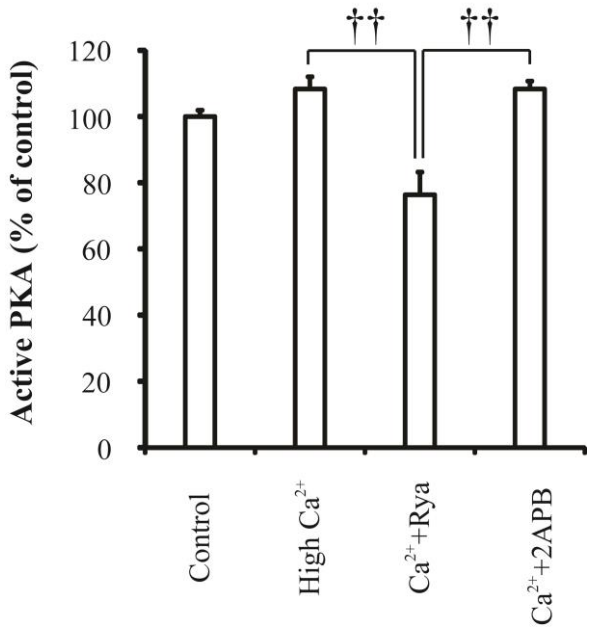


Figure 9. Active PKA was reduced by blocking RyRs in cultured hippocampal neurons. Normalized levels of PKA activities in each group were measured by using Elisa Assay. Error bars represent SEM. $p < 0.01$

Table 2. Whole-cell parameters of cultured hippocampal neurons

	control	High Ca^{2+} (3.6 mM)						5 mM	50 μM 4CmC					H89
	(n = 14)	Ca^{2+} (n = 12)	APV (n = 7)	Nimo (n = 8)	Ryano (n = 11)	2APB (n = 8)	H89 (n = 7)	caffeine (n = 11)	4CmC (n = 8)	APV (n = 11)	Nimo (n = 9)	TTX (n = 10)	H89 (n = 8)	(n = 9)
WC (pF)	10.51 ± 0.25	10.57 ± 0.52	10.74 ± 0.50	11.07 ± 0.47	9.9 ± 0.43	9.31 ± 0.55*	10.07 ± 0.37	9.25 ± 0.31**	9.86 ± 0.54	10.8 ± 0.49	10.64 ± 0.54	10.97 ± 0.43	9.63 ± 0.51	9.18 ± 0.44*
RS (MΩ)	11 ± 0.69	13.53 ± 0.77*	11.09 ± 1.03	9.43 ± 0.38	12.15 ± 0.88	10.49 ± 1.10	12.11 ± 0.82	13.45 ± 0.52*	12.61 ± 0.94	12.02 ± 0.42	11.83 ± 0.49	11.47 ± 0.44	13.42 ± 1.24	12.27 ± 1.28
RMP (mV)	-41.64 ± 3.14	-43.33 ± 1.68	-38.43 ± 4.55	-41.63 ± 3.80	-38.64 ± 1.60	-43.38 ± 3.60	-42.43 ± 3.36	-28.18 ± 3.53*	-32.13 ± 1.59*	-36.36 ± 1.34	-43 ± 1.98	-38 ± 1.98	-41.88 ± 3.15	-38.44 ± 2.10

* Statistically indicates $p < 0.05$.
** Statistically indicates $p < 0.01$.

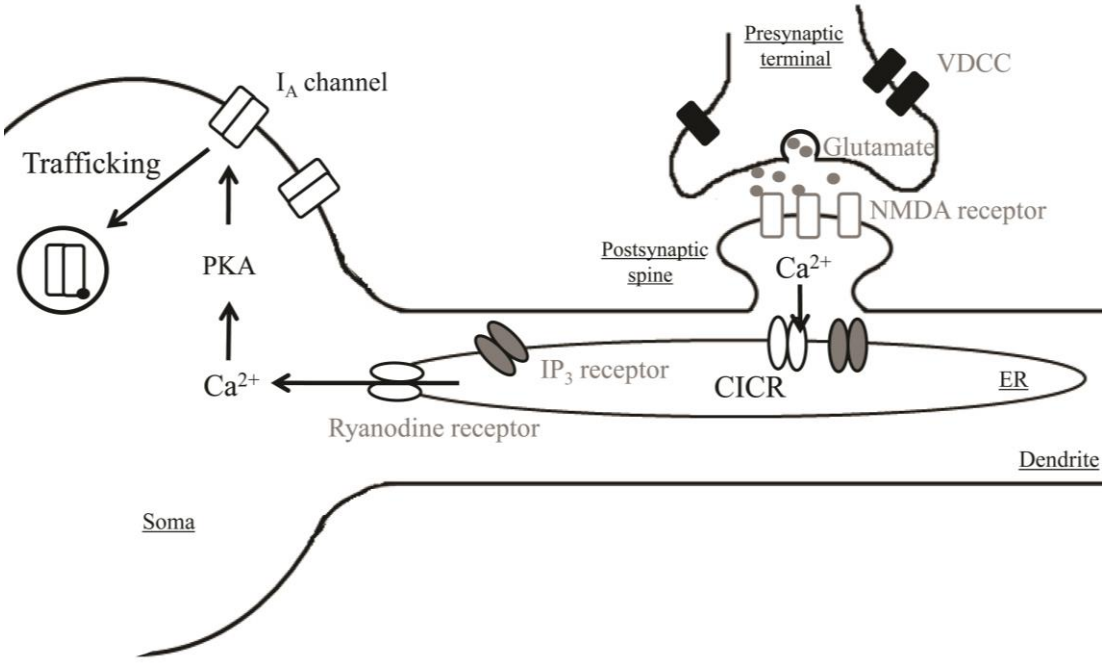


Figure 10. A simplified model of Ca^{2+}-mediated downregulation of somatic I_A channels induced by synaptic activation. The enhancement of Ca^{2+} influx through synaptic NMDARs acts as a modulator to activate CICR via opening RyRs of ER. Opened RyRs more rapidly releases Ca^{2+} from ER than IP_3Rs and then Ca^{2+} phosphorylates PKA . Subsequentially, the activation of PKA induces the downregulation of I_A channels in soma. Consequentially, synaptic activation possibly regulates intrinsic excitability by internalizing somatic I_A channels.

4. Discussion

A number of studies have investigated regulatory mechanisms of I_A channels modulating intrinsic excitability and synaptic plasticity since 1961 (Hagiwara, 1961; Kang et al., 2014). Downregulation of I_A channels is mediated by intracellular Ca^{2+} signaling cascades activated by Ca^{2+} influx through NMDARs of active postsynaptic sites. This I_A downregulation is absolutely required for the formation of synaptic plasticity induced by synaptic specific protocols and enhancement of intrinsic excitability (Ramakers and Storm, 2002; Watanabe et al., 2002; Jung et al., 2008; Jung and Hoffman, 2009). However, it is not clear how synaptic activity influence somatic I_A channels. In this study, it was confirmed that synaptic activity participates in somatic excitability by regulating I_A channels via activating RyRs of Ca^{2+} store and PKA signaling in hippocampal neurons. I_A channel density of plasma membrane under the condition of high Ca^{2+} was significantly reduced and these changes were dramatically blocked by postsynaptic NMDARs antagonist (Figure 2). This finding indicates that postsynaptic Ca^{2+} influx through NMDARs is required for downregulation of I_A channels. VDCCs participating in neurotransmitter release in presynaptic terminals (Tomizawa et al., 2002; Uriu et al., 2010) also seem to be crucial regulating I_A channels, as shown in experiments using VDCCs antagonist, nimodipine. This suggests that I_A channels are internalized from somatic membrane by Ca^{2+} influx through postsynaptic NMDARs activated by glutamate released by presynaptic VDCCs activation.

How does synaptic activity influence somatic excitability by downregulating I_A channels? For this issue, ER Ca^{2+} store was focused as a Ca^{2+} mediator to make the correlation between synapse and soma of neurons. ER Ca^{2+} stores play important roles in dynamic neuronal functions such as neuronal excitability, neurotransmitter release, and synaptic plasticity by regulating intracellular Ca^{2+} signaling (Sharma and Vijayaraghavan, 2003; Verkhratsky,

2005). ER has two major Ca^{2+} release channels, one is a Ca^{2+}-gated Ca^{2+} channels known as RyRs that mainly plays important roles in CICR, and the other is $InsP_3$-gated Ca^{2+} channels referred to as IP_3Rs. Previous studies have showed that RyRs correlated with both synaptic (LTP and LTD) and somatic modification in normal and pathologic condition such as Alzheimer's disease (Kumar and Foster, 2005; Thibault et al., 2007; Grigoryan et al., 2012). In the present study, RyRs participated in downregulation of I_A channels by releasing Ca^{2+} more quickly than IP_3 receptors (Figure 4-6). Miyazaki and Ross (2013) recently reported that RyRs mediate Ca^{2+} "sparks" by spontaneous events without IP_3Rs activation and IP_3Rs induce Ca^{2+} "puff" by a synaptic tetanus in hippocampal neurons. They also demonstrated that rise time of Ca^{2+} via RyRs was faster than that of IP_3Rs, consistent with results observed in the current study. It is considered that rapid Ca^{2+} increase in soma through RyRs occurs because opened RyRs by binding Ca^{2+} activate surrounding RyRs in ER. However, it is still not clear how rapid efflux of Ca^{2+} through RyRs modulates somatic excitability by regulation of I_A channels. RyRs agonists such as caffeine and 4CmC significantly reduced the density of somatic I_A without synaptic enhancement (Figure 7), indicating that the activation of RyRs is necessary and sufficient for modulation of neuronal excitability via downregulation of I_A channels. In addition, blocking the RyRs enhanced the density of somatic I_A before induction of synaptic plasticity while inactivated IP_3Rs did not change it (Table 1). The result shows that RyRs also participate in turn-over of I_A channels in normal condition as well as synaptic plasticity.

In previous studies, the trafficking of I_A channels was dependent on PKA activation induced by Ca^{2+} signaling (Hoffman and Johnston, 1998; Schrader et al., 2002). To test whether increased Ca^{2+} levels by RyRs activation regulate the neuronal excitability by phosphorylation of PKA, H89, selective inhibitor of PKA, was treated with high Ca^{2+} or 4CmC. RyRs-mediated I_A downregulation was confirmed to result from PKA activation, supported by measurement of active PKA (Figures 8 and 9).

Table 2 shows whole-cell parameters of neurons recorded in the present study. The values of whole cell capacitance (WC) under various conditions were similar to those of control, but differences were observed in 2APB-, caffeine- and H89-treated neurons. To compensate for difference of WC, density of I_A channels was analyzed in all recordings. Resting membrane potentials (RMPs) were also not affected under various conditions except in the case of RyRs activation. It is considerable that elevated RMPs in caffeine- and 4CmC-treated neurons may be due to over-efflux of Ca^{2+} from ER.

In conclusion, it is possible that the synaptic enhancement via activation of NMDARs and VDCCs directly induces the downregulation of I_A channels by releasing sufficient Ca^{2+} through RyRs of ER (Figure 10). Here, I show that the RyRs act as a linker between synapses and soma and then participate in PKA signaling to regulate the trafficking of I_A channels.

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Part Ⅱ

Ca^{2+} influx through VDCCs prevents neuronal hyperexcitability by increasing K^{+} currents through delayed rectifier channels

1. Introduction

Neuronal intrinsic excitability (IE) reflecting action potentials (APs) is mainly determined by voltage-dependent K^+ and Na^+ channels (K_V and Na_V channels) and is important in neuronal functions (Misonou et al., 2005a). Kv channels consisting of I_A or I_{DR} participate in the repolarization phase of APs and determine the resting membrane potentials and conductance (Dominik Oliver et al., 2004).

In mammalian neurons, Kv2.1 channels are a major component of I_{DR} channels exhibiting sustained outward K^+ current (Du et al., 2000; Malin and Nerbonne, 2002; Pal et al., 2003). This subtype acts as a rheostat to homeostatically depress AP firings by keeping single APs short and limiting high frequency AP firing and prevent neurotoxicity on the basis of these actions (Lien and Jonas, 2003; Surmeier and Foehring, 2004). Previous studies have demonstrated that the increase of intracellular Ca^{2+} levels under pathologic conditions such as epileptic seizures, neuromodulatory stimuli, and ischemia leads to enhancement of I_{DR} channels (Misonou et al., 2004; Misonou et al., 2005b; Park et al., 2006). Activation of calcineurin induced by the increased intracellular Ca^{2+} changes I_{DR} properties by dephosphorylating $K_V2.1$ channels. It has also been reported that $K_V2.1$ channel clustering is disrupted and then the threshold for I_{DR} activation is decreased with alteration of activation kinetics by calcineurin-dependent dephosphorylation of K_V 2.1 channels (Misonou et al., 2005a; Mohapatra and Trimmer, 2006). However, it was not observed that changes of activation properties of I_{DR} were induced by high Ca^{2+} application.

Regulation of I_{DR} channels is dependent on phosphorylation and many phosphorylation sites such as serine, threonine and tyrosine, existing on $K_V2.1$ channels (Park et al., 2006). Previous studies have shown that the mutation of threonine sites does not affect $K_V2.1$

activity while serine/threonine kinase PKA changes only the activation kinetics of I_{DR} channels but not membrane expression of $K_V2.1$ (Jonas and Kaczmarek, 1996; Murakoshi et al., 1997).

In the current study, I demonstrated mechanisms of I_{DR} upregulation to protect neurons under the condition of high Ca^{2+}. The increase of outward K^+ currents through I_{DR} channels can prevent neuronal hyperexcitability, which leads to abnormal responses and neuronal cell damage in severe cases.

2. Materials and methods

2.1. Materials

Materials for cell culture, including MEM, Neurobasal medium and FBS, were purchased from Gibco (Gland Island, NY, USA). Nimodipine and tetrodotoxin (TTX) were purchased from Tocris (Ellisville, MO, USA). All other reagents were purchased from Sigma-Aldrich (St Louis, MO, USA), unless indicated otherwise.

2.2. Hippocampal primary cultures

Twenty-day embryos were removed from anesthetized pregnant SD rats and transferred to an ice-cold normal tyrode solution. The hippocampi were isolated from embryonic rat brains and transferred to ice-cold plating medium containing the following: MEM, 10% FBS, 0.45% glucose, 1 mM sodium pyruvate, 25 μM glutamate and penicillin/streptomycin, and then triturated. The cells were seeded on poly-L-lysine coated coverslips at a density of 9 X 10^4 cells/ml and incubated at 37℃ in 95% air and 5% CO_2. After 7 hours**,** the whole plating medium was changed with Neurobasal medium containing B-27, and half of the medium was changed twice for a week.

All experiments and procedures with animals were performed with permission from the Animal Care and Use Committee of Jeju National University.

2.3. Electrophysiology

To record sustained K^+ currents in the dissociated hippocampal neurons, DIV 6-8 neurons seeded on coverslips were transferred to a recording chamber with a continuous flow of recording solution containing 0.5 μM TTX to block the voltage-dependent Na^+ channels and bubbled with 95% O_2, 5% CO_2. The patch pipettes (4-6 MΩ) were filled with an internal solution and the series resistance varied between 8~30 MΩ. Recordings where series resistance varied by more than 10% were rejected. Sustained K^+ currents were measured at +60 mV after prepulse at -20 mV for 200 ms. All electrophysiological data were acquired using an Axopatch200B amplifier (Axon Instruments), and command pulse generation, data acquisition and analysis were performed using Digidata 1322A convertor (Axon Instruments), pClamp 8 (Axon Instruments) and IGOR Pro (Wavemetrics) software.

2.4 Statistical analysis

Data analysis was performed and statistical significance was determined using Excel (Microsoft) software and sigma plot, and then data were represented as mean value ± SEM. The Student's *t*-test was used, and statistical difference between groups was indicated for *p* values of < 0.05 or 0.01.

3. Results

3.1. Enhancement of I_{DR} under the high Ca^{2+} condition did not include any changes of kinetic properties in hippocampal neurons derived from DIV 6-9.

In this part, the mechanisms for regulation of I_{DR} channels after inducing neuronal overexcitability were investigated. The high Ca^{2+} (3.6 mM $CaCl_2$) was used to induce overexcitability by increasing the intracellular Ca^{2+} level. Figure 11 shows that the density of I_{DR} was significantly elevated by high Ca^{2+} application (Control= 112.62 ± 10.28 pA/pF, n= 13; high Ca^{2+}= 171.05 ± 18.30 pA/pF, n= 12, p = 0.02), reflecting a possibility that upregulating I_{DR} channels may suppress the neuronal overexcitability via increasing K^+ outflux under high Ca^{2+} condition.

However, the activation kinetics of I_{DR} channels were not changed under high Ca^{2+} condition despite the enhancement of current density (Figure 12). The density as well as increase rate of I_{DR} also positively reflected the increase rates of command potentials. At +80 mv injection, significant changes of I_{DR} were observed between control and high Ca^{2+} treated neurons (Control= 139.82 ± 15.62 pA/pF; High Ca^{2+}= 226.79 ± 17.34 pA/pF, p= 0.02). The increase rate of I_{DR} was obtained by dividing the density of high Ca^{2+} I_{DR} with the control value. This parameter was enhanced up to 60 % from -40 to 80 mV injection range compared with the control group. Activation properties of I_{DR} channels are shown in Figures 12 D and E. Left-shifted pattern of activation curve by high Ca^{2+} according to slightly increased voltage half (V_h) value of activation was observed but not significant (V_h of activation: Control= 12.38 ± 2.62 mV; High Ca^{2+}= 22.21 ± 4.4 mV, p= 0.1). This indicates that upregulation of I_{DR} channels under high Ca^{2+} may not be dependent on their dephosphorylation by calcineurin activated by Ca^{2+} signaling.

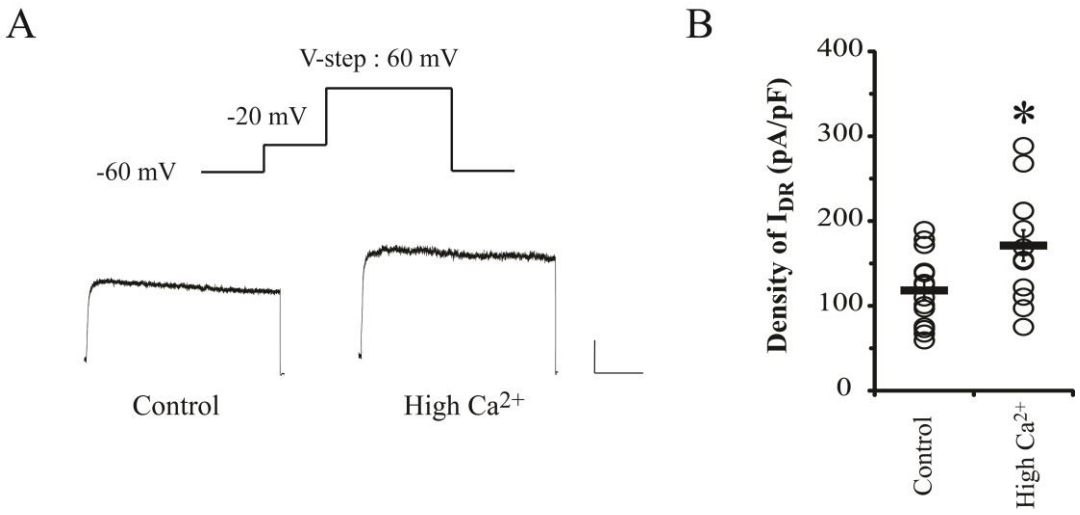

Figure 11. I_{DR} are increased by high Ca^{2+} application in dissociated hippocampal neurons. A. Example traces of I_{DR} after high Ca^{2+} (3.6 mM $CaCl_2$) treatment for 24 hrs. Scale bars 500 pA, 100 ms. **B.** Individuals (circles) and averaged (square bars) densities of I_{DR}. Error bars represent SEM. $p<0.05$.

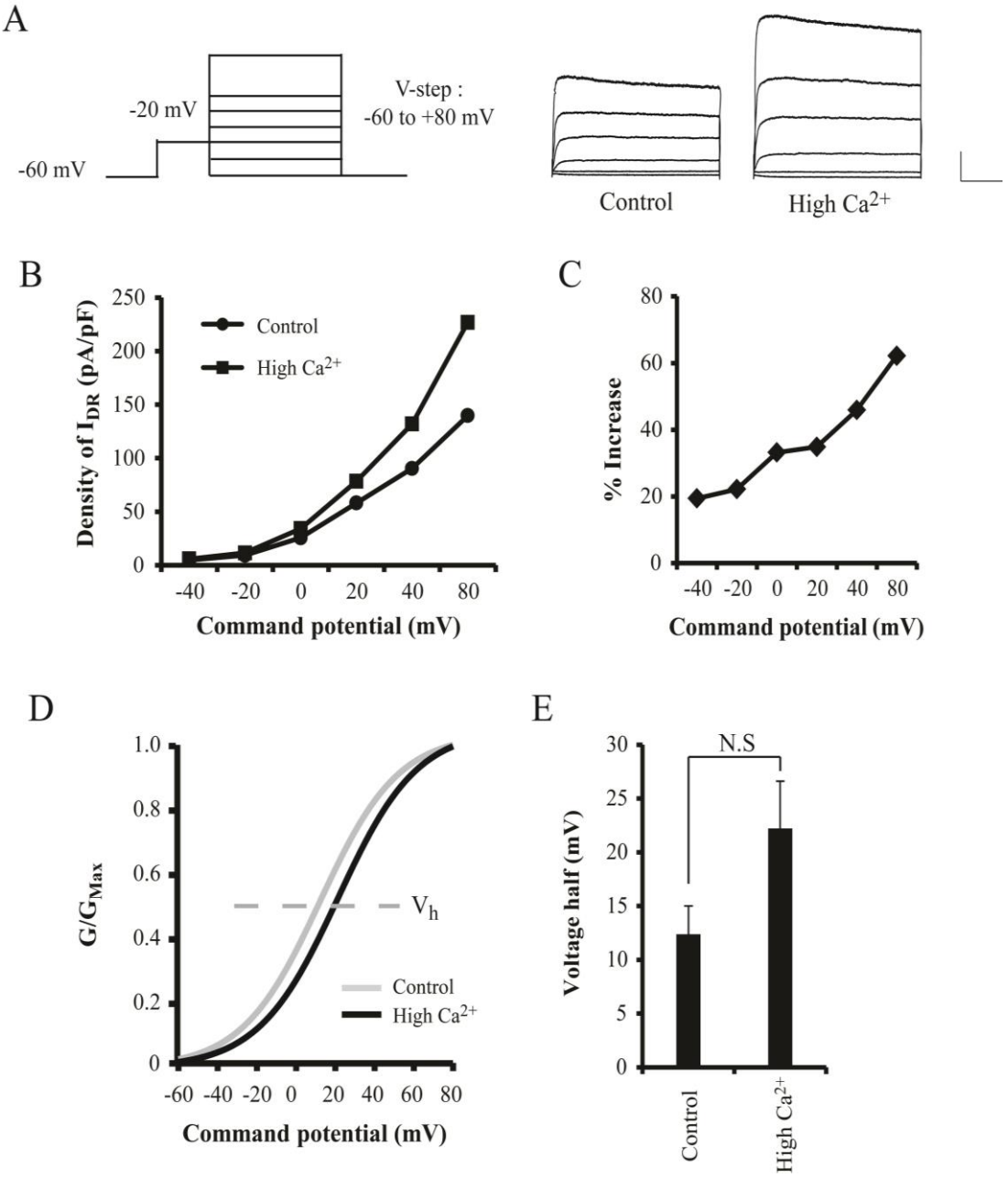

Figure 12. Upregulating I_{DR} by high Ca^{2+} does not involve significant alteration of activation properties in hippocampal neurons. The activation properties were measured at -60 to 80 mV with 20 or 40 mV steps after prepulse injection (-20 mV for 200 ms). **A.** Example activation traces of I_{DR}. Scale bars 500 pA, 100 ms. **B.** The density of I_{DR} at each command potential (-40 to 80 mV). **C.** The increase rate of I_{DR} in high Ca^{2+} condition

compared with control group. The density of I_{DR} was increased with the increment of command potentials. **D and E**. Boltzmann fitted gating kinetics of I_{DR} activation and its voltage half (V_h) (thick gray dotted line in D). The significant change of the activation curves was not observed. Error bars represent SEM.

3.2. Upregulation of I_{DR} under high Ca^{2+} condition is mediated with voltage-dependent Ca^{2+} channels.

A number of ion channels are regulated by Ca^{2+} signaling-mediated phosphorylation or dephosphorylation. There are two major Ca^{2+} influx pathways opened by the depolarization of membrane potentials; one is VDCC and the other is NMDAR. To confirm their contribution to I_{DR} upregulation, APV (100 μM) or nimodipine (10 μM) with high Ca^{2+} was applied to culture media for 24 hrs. As shown in Figure 13, Nimodipine, VDCCs antagonist, critically blocked the increase of I_{DR} by high Ca^{2+} treatment, while APV treatment did not show any effects on I_{DR} alteration (APV = 151.55 ± 12.56 pA/pF, Nimodipine = 96.03 ± 9.34 pA/pF, P= 0.48, 0.008 respectively, compared with high Ca^{2+}). In addition, each increase pattern of I_{DR} shown in APV- or nimodipine-treated neurons was respectively similar to the pattern of high Ca^{2+}- or non-treated neurons (Figure 14. B, at the 80 mV injection, APV= 192.53 ± 17.34, Nimodipine= 132.05 ± 15.06). However, effects of both antagonists on the activation kinetics of I_{DR} channels were not observed (V_h of activation in APV group= 10.11 ± 1.91 and in Nimodipine group= 14.92 ± 1.63, *p*= 0.55 and 0.45 respectively). These results indicate that Ca^{2+} influx through VDCCs may modulate the density of I_{DR} channels in a whole neuron.

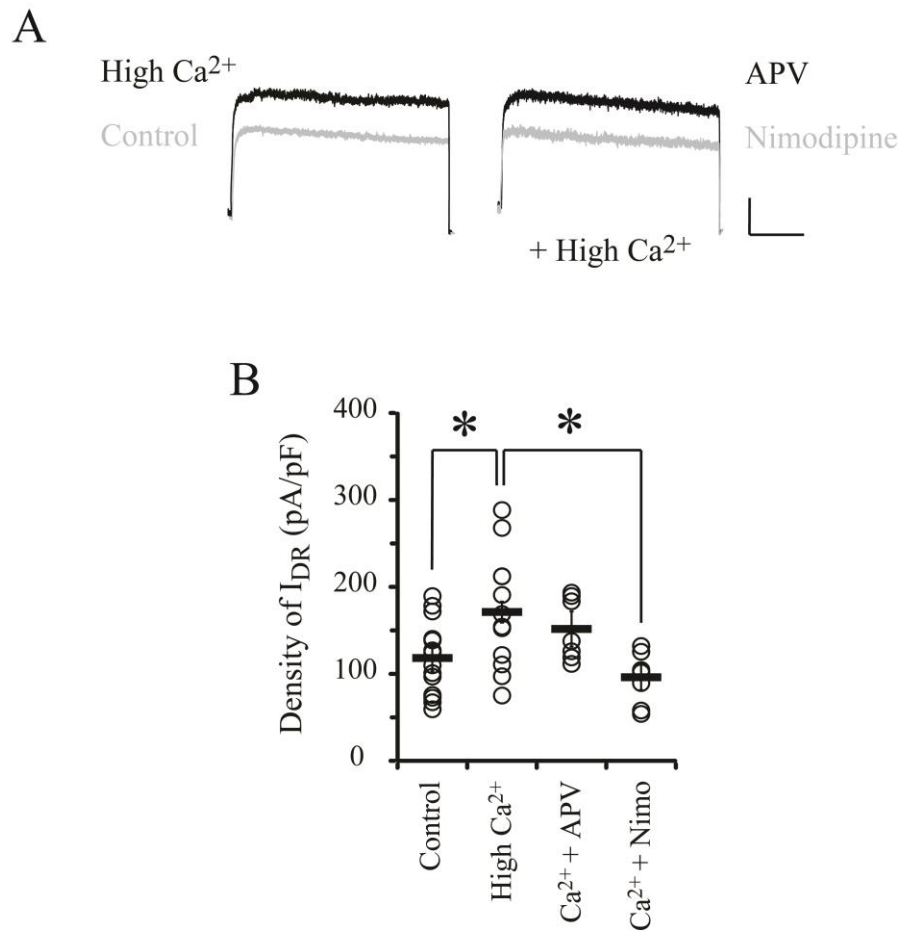

Figure 13. I_{DR} upregulation to prevent neuronal overexcitation requires VDCCs. VDCC antagonist nimodipine completely blocked the effect of high Ca^{2+} on density of I_{DR}, but NMDARs antagonist APV did not. **A.** Example traces of I_{DR} after high Ca^{2+} treatment with 100 μM APV or 10 μM nimodipine. Scale bars 500 pA, 100 ms. **B.** Square bars and open circles indicate averaged and individual densities of I_{DR}, respectively. Error bars represent SEM. $p<0.05$.

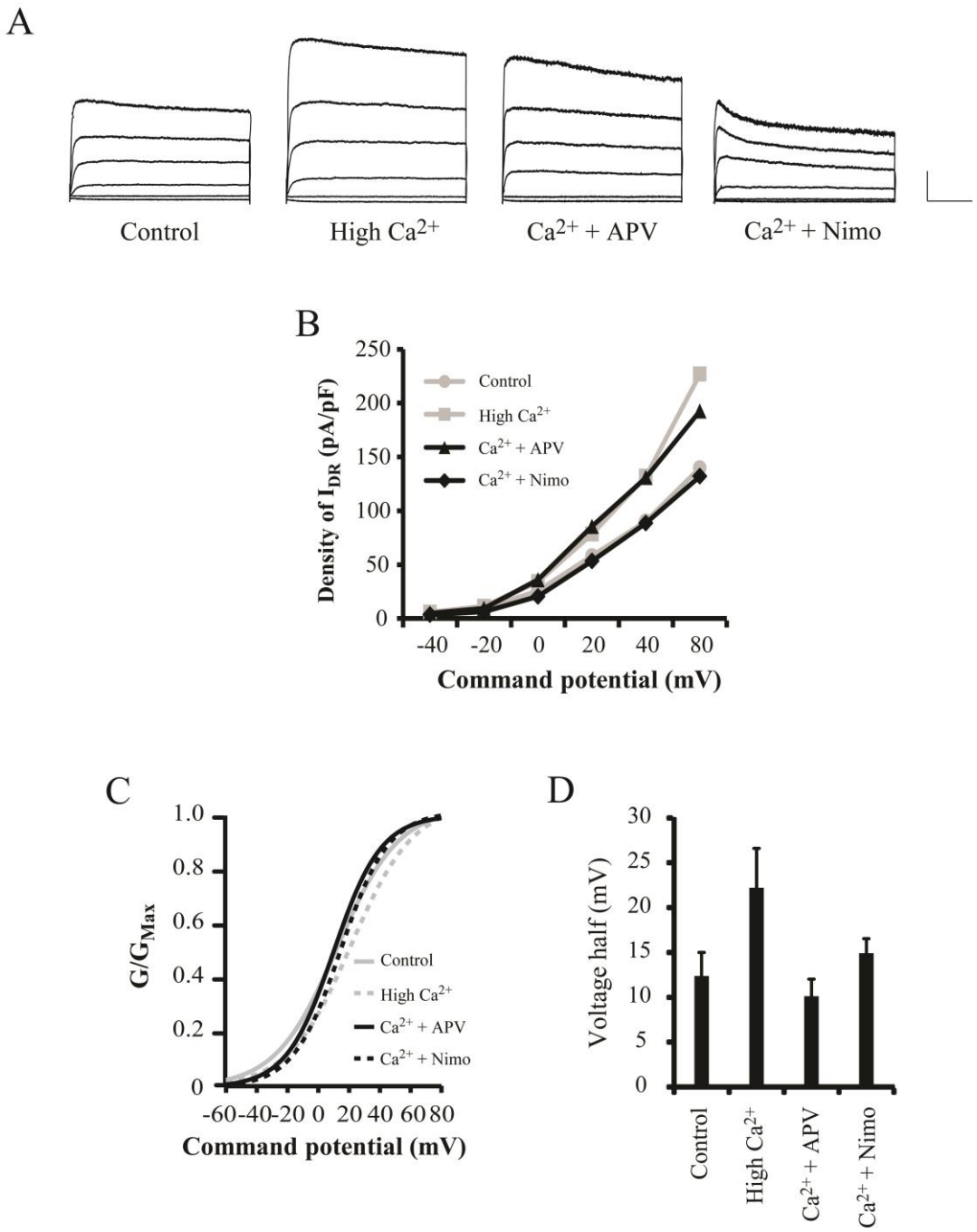

Figure 14. The blockade of I_{DR} upregulation by nimodipine does not exhibit any changes of I_{DR} activation property. A. Example traces of I_{DR} induced by injecting command potentials from -40 mV to 80 mV. Scale bars 500 pA, 100 ms. **B.** Changes of I_{DR} densities according to each command potential. The increase pattern of I_{DR} density in nimodipine-treated neurons is closely similar to control group and also the density change of

I_{DR} in APV-treated neurons resembled those of high Ca^{2+}-treated neurons. **C and D.** Boltzmann fitted activation curves of I_{DR} channels and their V_h values. These activation curves were not shifted and therefore, V_h values did not show any significant differences in statistical analysis. Error bars represent SEM.

3.3. Nimodipine, alone, does not affect the density of I_{DR} in dissociated hippocampal neurons.

Previous studies have reported effects of VDCCs blockers on sustained K^+ currents (Rampe et al., 1993; Perchenet and Clement-Chomienne, 2000; Choe et al., 2003). To examine effects of nimodipine in the present study, culture media was treated with nimodipine alone for 24 hrs. Figure 16 shows that the density of I_{DR} was never changed by nimodipine treatment (Nimodipine= 104.98 ± 15.24, p= 0.7 and 0.63 compared with control and nimodipine with high Ca^{2+}, respectively), suggesting that Ca^{2+} influx via VDCCs can decrease the neuronal hyperexcitability by regulating the expression of I_{DR} channels as there were no drug-originated side effects.

3.4. Upregulation of I_{DR} channels is independent of Ca^{2+} signaling-mediated regulation of I_A channels.

In the current study, RyRs antagonist Ryanodine (10 μM) did not interrupt high Ca^{2+} effects on I_{DR} channels (Figure 17, Ryanodine= 180.98 ± 27.97 pA/pF, p= 0.04 and 0.78 compared with the control and high Ca^{2+}, respectively). The result indicates that upregulation of I_{DR} channels is not relevant to Ca^{2+} efflux from RyRs of ER while I_A channels are regulated by RyR-mediated Ca^{2+} signaling. Upregulation of I_{DR} channels by high Ca^{2+} was also observed regardless of PKA activation, showing sensitivity to H89 (10 μM) treatment (H89= 192.84 ± 36.08 pA/pF, p= 0.03 and 0.58 compared with the control and high Ca^{2+}, respectively). These results suggest that I_{DR} channels are regulated by Ca^{2+} signaling separately from Ca^{2+} signaling-mediated downregulation of I_A channels

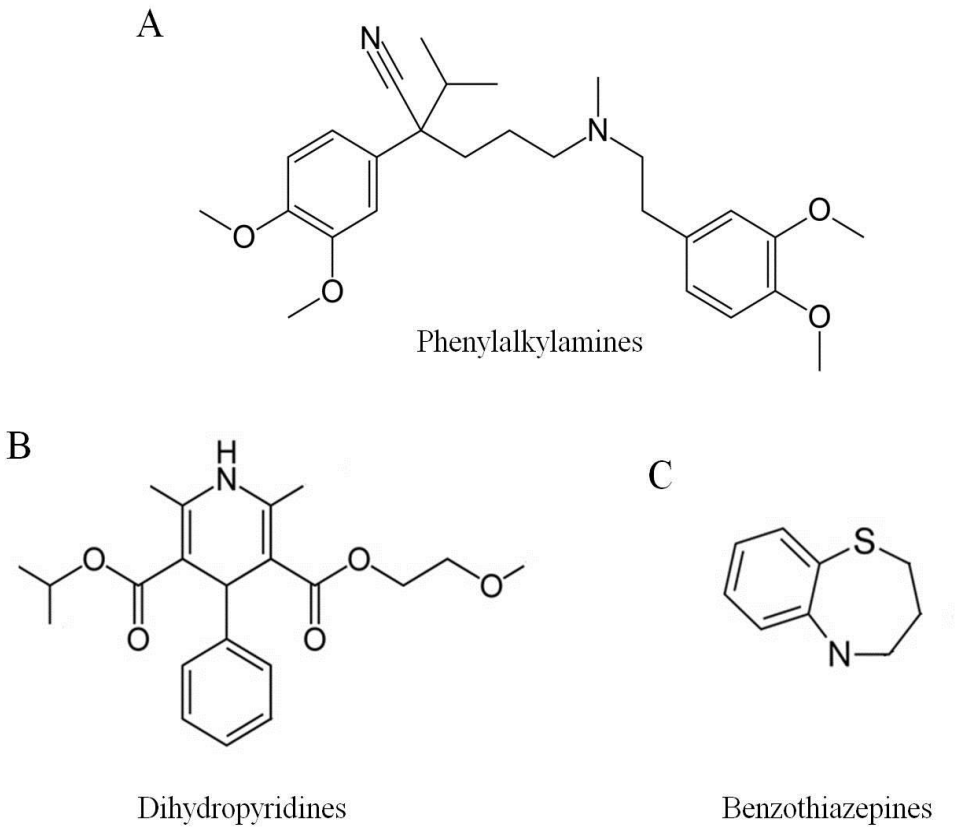

Figure 15. Chemical structures of VDCCs blockers. The VDCCs blockers are divided into three types. One is a phenylalkylamines containing verapamil and mybefradil, and another is dihydropyridines such as nifedipine and nimodipine and the other is venzothiazepines. **A-C** indicate the structures of them in order.

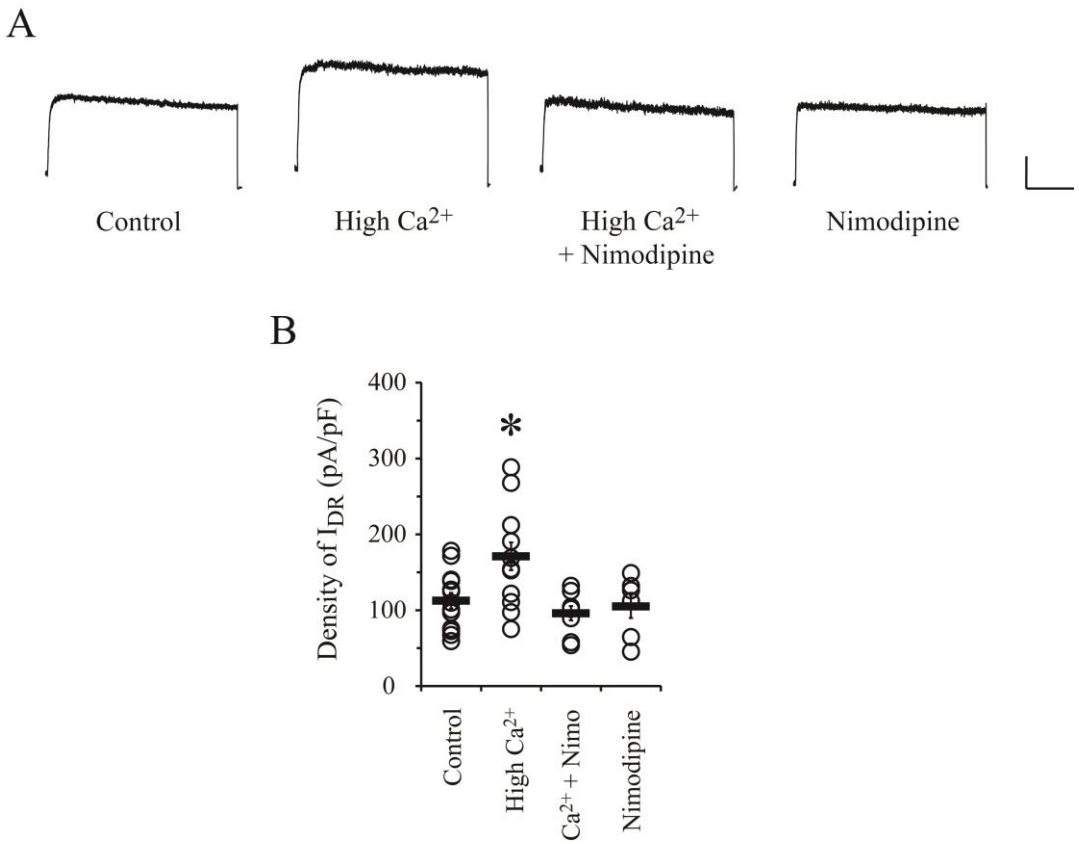

Figure 16. No effects of nimodipine alone on the density of I_{DR} channels were observed in hippocampal neurons. The density of I_{DR} was not changed by nimodipine alone treatment for 24 hrs compared with control. **A.** Example traces of I_{DR} after 10 μM nimodipine application. Scale bars 500 pA, 100 ms. **B.** Individuals (circles) and averaged (square bars) densities of I_{DR}. Error bars represent SEM. $p<0.05$.

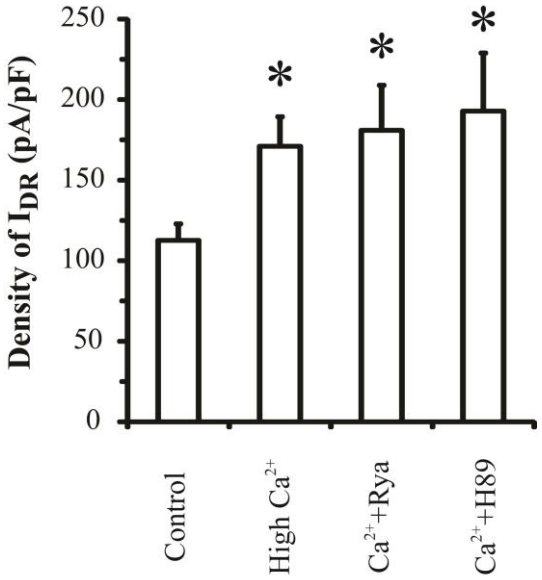

Figure 17. Activation of RyRs and PKA did not affect the upregulation of I_{DR} channels under the high Ca^{2+} condition. Ryanodine (10 μM) and H89 (10 μM) were treated with high Ca^{2+} for 24 hrs to inhibit the RyRs and PKA respectively. The induced amplitude of I_{DR} channels by high Ca^{2+} was not blocked by both Ryanodine and H89 application. Error bars represent SEM. $p<0.05$.

3.5. No involvement of small conductance Ca^{2+}-activated K^+ channels in increased sustained outward current under high Ca^{2+}condition.

Ledoux et al. (2006) reported that the enhancement of intracellular Ca^{2+} concentration induced the elevation of K^+ efflux through small conductance Ca^{2+} channels (SK channels). Therefore it is necessary to confirm whether the increased density of I_{DR} by high Ca^{2+} results from activation of SK channels. In this experiment, 100 nM apamin was treated for 24 hrs with high Ca^{2+} to block SK channels. Results showed that the increased density of I_{DR} by high Ca^{2+} treatment was not influenced by apamin, indicating that upregulating sustained K^+ currents for prevention of hyperexcitability does not require the contribution of SK channels (179.37 ± 26.56 pA/pF, p=0.04 and 0.81 compared with control and high Ca^{2+}, respectively).

3.6. High Ca^{2+} increases the density of I_{DR} through activation of SFKs in the dissociated hippocampal neurons.

The Figure 19 shows that Src family tyrosine kinases (SFKs) are crucial to regulation of I_{DR} channels under the high Ca^{2+} condition. 1 μM PP2 was treated with high Ca^{2+} to block SFKs and I_{DR} was recorded 24 hrs after treatment. PP2 significantly reduced density of I_{DR} channels as much as control levels in hyperexcitability condition induced by high Ca^{2+} (PP2= 99.01 ± 10.52 pA/pF, p= 0.43 and 0.02 compared with the control and high Ca^{2+}, respectively). PP2, however, did not affect I_{DR} channels in normal condition (123.46 ± 20.23 pA/pF, p= 0.63 compared with the control). These results demonstrate that Ca^{2+} influx via VDCCs regulate I_{DR} channels by activating SFKs to protect the neurons.

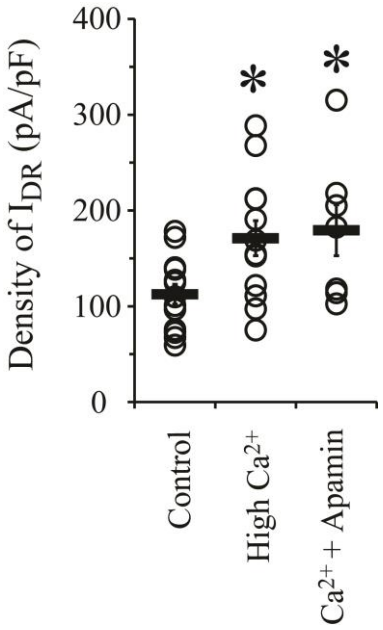

Figure 18. SK channels were not involved in the upregulation of sustained currents under high Ca^{2+}condition. Apamin **(**100 nM) was treated with high Ca^{2+} for 24 hrs to block SK channels. Averaged (square bars) densities of I_{DR} with individual values (open circles) were presented. Error bars represent SEM. $p < 0.05$

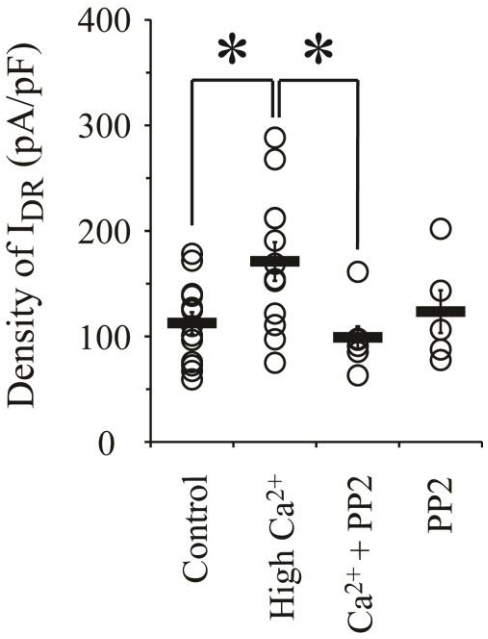

Figure 19. SFKs played important role in upregulation of I_{DR} channels under high Ca^{2+} condition in dissociated hippocampal neurons. PP2 **(**1 μM), specific SFKs inhibitor, was treated with high Ca^{2+} for 24 hrs and blocked upregulation of I_{DR} channels by high Ca^{2+}. Averaged (square bars) densities of I_{DR} with individual values (open circles) were presented. Error bars represent SEM. $p < 0.05$

4. Discussion

This study was conducted in oreder to confirm roles of I_{DR} channels regulating membrane excitability under neuronal hyperexcitability conditions. I demonstrate that Ca^{2+} influx through VDCCs increases I_{DR} without changes of activation kinetics (Figure 11-14) and it can actively prevent neuronal overexcitation by increasing K^+ efflux. The enhanced I_{DR} by high Ca^{2+} was not affected by APV, NMDARs antagonist, indicating independent modulation on the special functions of spatially restricted- Ca^{2+} around synaptic NMDARs during synaptic transmission. These results indicate that the enhancement of I_{DR} by high Ca^{2+} may compensate for downregulation of I_A channels, but it is surely possible that there are the separated signaling pathways for I_{DR} upregulation. Virtually, blocking RyRs and PKA participating in downregulation of I_A channels did not affect upregulation of I_{DR} (Figure 17). Some studies have reported on the effects of VDCC blockers such as verapamil and mybefradil in directly inhibitting the I_{DR} channels (Rampe et al., 1993; Perchenet and Clement-Chomienne, 2000; Choe et al., 2003). VDCCs blocker of phenylalkylamines type mainly acts by binding pores during channels opening. On the other hand, dihydropyridines block channels by binding the specific domain. Verapamil and Mybefradil are included in phenylalkylamines and nimodipine is a dihydropyridine. Nimodipine, used in the present study, did not alter I_{DR} (Figure 16).

Next, the relationship between increased sustained currents and SK channels was investigated. These channels are known to be activated by increased intracellular calcium through VDCCs (Herrera and Nelson, 2002; Yamada et al., 2004; Jones and Stuart, 2013). The increased sustained current by high Ca^{2+} was not affected by SK channels antagonist apamin (Figure 18), suggesting that upregulation of sustained currents by high Ca^{2+} does not involve the contribution of SK channels.

How can I_{DR} channels be upregulated by neuronal hyperexcitability? I_{DR} channels in

neurons are predominantly regulated by calcineurin-dependent dephosphorylation, which is accompanied by changes of both threshold and activation kinetics (Misonou et al., 2005a; Mohapatra and Trimmer, 2006). In the current study, however, upregulation of I_{DR} channels apears to be due to the increase of I_{DR} channel expression into plasma membrane without alterations of gating kinetics. According to previous reports confirming regulatory mechanisms of $K_V2.1$ channel, these channels possess a large number of kinase binding sites such as serine, threonine, and tyrosine, and are usually affected by those kinases except threonine (Jonas and Kaczmarek, 1996; Murakoshi et al., 1997). Tyrosine kinases, such as Src and Fyn, regulate the expression of $K_V2.1$ channels while the gating kinetics of I_{DR} channels are targeted by serine kinases (Sobko et al., 1998; Peretz et al., 2000; Tiran et al., 2003; Song et al., 2011). It is quietly reasonable that the upregulation of I_{DR} by high Ca^{2+} shown in the present study may originate from the modulatory functions of tyrosine kinases as no significant changes in gating kinetics were observed. Accordingly, SFKs inhibitor PP2 was used and the results demonstrate that upregulation of I_{DR} under the high Ca^{2+} condition require the SFKs activation induced by Ca^{2+} influx via VDCC (Figure 19).

In conclusion, overexcitable condition induced by high Ca^{2+} increased Ca^{2+} influx via VDCC and then VDCC-mediated Ca^{2+} signaling can prevent neuronal hyperexcitability by upregulating the I_{DR} channels through SFKs activation independently of I_A channels regulation. Furthermore, this cascade is not influenced by SK channels.

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국문 초록

해마 신경세포에서 신경연접의 활성에 의해서 조절되는 내재적 흥분성은 정보처리, 인지와 같은 신경세포의 기능을 결정하는데 중요하지만 과도한 흥분성은 세포사멸을 유도할 수 있다. 따라서 막전도도와 막전위를 조절함으로써 신경세포의 흥분성에 관여하는 전압 의존적 칼륨 통로의 조절 기전을 확인하는 것은 신경세포의 기능을 이해하기 위해 꼭 필요할 것이라 생각하였다. 전압 의존적 칼륨 통로는 빠르게 비활성화되는 A-type K^+ channel(I_A channel)과 느리게 비활성화되거나 비활성화 되지 않는 Delayed rectifier K^+ channel(I_{DR} channel)로 나눌 수 있다. 본 연구에서는 part 1과 2로 나누어 초대 배양한 흰쥐 해마 신경세포에서 I_A channel과 I_{DR} channel의 칼슘 매개 기전을 확인하고자 하였다.

신경연접에 존재하는 NMDA 수용체를 통한 칼슘유입에 의해 세포체의 I_A channel의 세포막 발현정도가 감소된다는 것이 알려졌다. 그러나 신경연접의 활성화에 의해 세포체에 존재하는 I_A channel이 어떻게 조절되는지는 거의 연구되지 않았다. 신경연접의 활성화를 유도하기 위해 고농도의 칼슘(3.6 mM $CaCl_2$)과 글라이신(200 μM)을 처리했을 때 I_A 전류가 감소하였다. NMDA 수용체와 전압 의존적 칼슘 통로(Voltage-dependent Ca^{2+} channel, VDCC) 길항제를 처리했을 때 고농도의 칼슘에 의한 I_A 전류의 감소는 나타나지 않았으며, 이는 고농도의 칼슘에 의한 I_A 전류 감소가 신경연접의 활성화에 의해 나타난다는 것을 의미한다. 신경연접과 세포체 사이의 공간적 제약을 해결할 수 있는 인자를 찾기 위해 소포체에 존재하는 두 개의 칼슘방출 수용체인 Ryanodine 수용체와 IP_3 수용

체의 관련성을 확인했다. 고동노의 칼슘과 같이 2APB(100 μM)를 처리하여 IP_3 수용체를 막았을 때에도 여전히 I_A 전류가 감소한 반면, Ryanodine 수용체의 길항제인 Ryanodine(10 μM) 처리 시에는 신경연접의 활성화에 의해 나타나는 I_A 전류의 감소가 나타나지 않았다. 또한, Ryanodine은 칼슘에 의해서 증가되는 PKA의 활성을 감소시켰다. 이러한 결과들은 세포체에 존재하는 I_A channel을 조절하기 위해 소포체와 PKA를 포함하는 일련의 신호전달과정이 필요하다는 것을 시사한다. 이상의 결과를 통해 Ryanodine 수용체가 세포체의 I_A channel 감소기전을 통해 신경세포의 흥분성 조절에 관여한다는 것을 확인할 수 있었다.

고농도의 칼슘을 처리했을 때, I_A 전류의 감소와 더불어 I_{DR} 전류의 증가도 나타났다. 그러나 I_{DR} 전류의 증가가 어떤 경로에 의해 나타나는지는 아직 많이 연구되지 않았다. I_{DR} channel은 간질 발작과 같은 병적인 상황에서 신경세포의 흥분성을 조절함으로써 항상성 유지에 관여한다. Part 2에서는 고농도의 칼슘 하에서 신경세포의 과흥분성을 조절할 수 있는 I_{DR} channel의 조절 기전을 확인했다. 고농도의 칼슘에 의해 유도된 I_{DR} 전류의 증가는 어떠한 통로 단백질 기질의 변화도 동반하지 않았으며, VDCC의 길항제인 Nimodipine(10 μM)에 의해 증가가 차단되지만 NMDA 수용체의 길항제인 APV(100 μM)에 의해서는 영향을 받지 않았다. 이는 I_A channel과는 독립적으로, VDCC를 통해 유입된 칼슘에 의해 I_{DR} channel이 증가할 수 있다는 것을 의미한다. 세포내의 칼슘 증가에 따른 SK channel을 통한 칼륨의 외부전류 증가로 인해 I_{DR} 전류가 증가된 것처럼 보였는지 확인해 본 결과, SK channel은 I_{DR} 전류 증가에 영향을 미치지 않았다. 본 실험에서 VDCC를

통한 칼슘 유입이 어떤 경로를 통해서 I_{DR} 전류를 증가시켰는지 확인하기 위해 Src-familiy 타이로신 인산화효소의 억제제인 PP2를 사용하여 I_{DR} channel의 변화를 확인했다. 그 결과 PP2(1 μM)는 고농도의 칼슘에 의한 I_{DR} 전류의 증가를 차단했다. 이러한 결과들을 통해 고농도의 칼슘에 의해 유도된 신경세포의 과흥분성 상황에서 VDCC를 통한 칼슘 유입에 의해 활성화된 Src-familiy 타이로신 인산화효소가 I_{DR} channel의 증가를 유도함으로써 신경세포의 흥분성을 낮춘다는 것을 확인할 수 있었다.

결론적으로, 본 연구는 다양한 생리적, 병리적 상황에서 I_A channel과 I_{DR} channel같은 칼륨 통로들을 통해 신경세포의 흥분성을 능동적으로 조절함으로써 신경세포의 기능과 항상성을 적절히 유지할 수 있음을 실험적으로 증명한다고 할 수 있다.

감사의 글

생물학과 생리학을 공부한지 어느덧 10년이 지났습니다. 10년 동안 정말 많은 분들의 도움과 격려로 지금 이 자리까지 올 수 있었습니다. 이 글을 빌어 많은 분들께 감사의 마음을 전합니다.

지난 6년간 부족한 저에게 많은 가르침을 주시고 지도해주신 정성철 교수님께 진심으로 감사드리며 존경한다는 말을 전하고 싶습니다. 그리고 연구와 강의로 바쁘신 가운데에도 불구하고 많은 조언을 해주신 은수용 교수님과 박주민 교수님, 그리고 저의 박사학위 논문 심사를 맡아주시고 조언을 아끼지 않으신 윤상필 교수님, 서영호 교수님께도 진심으로 감사 드립니다. 또한 학부와 석사과정 동안 지도해주신 김세재 교수님께도 깊은 감사의 말씀을 드립니다.

이 논문이 완성되기까지 애정과 관심을 보여주신 강희경 교수님, 유은숙 교수님, 고영상 교수님, 조문제 교수님, 현진원 교수님, 박덕배 교수님, 이영기 교수님, 김진우 교수님께 진심으로 감사 드립니다.

의대에 들어와 자리잡을 수 있게 많이 도와준 김선희 선생님, 최연희 박사님, 오금희 박사님, 문석오빠, 그리고 잘 따라주고 많이 도와준 지연이, 지형언니, 승혜, 유리, 홀란과 근로장학생을 하며 고생하고 있는 진우에게도 감사의 마음을 전합니다.

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모두 건강하시고 원하시는 일 모두 잘 되시길 바랍니다.