

Original Article

# Chronic Ca<sup>2+</sup> influx through voltage-dependent Ca<sup>2+</sup> channels enhance delayed rectifier K<sup>+</sup> currents via activating Src family tyrosine kinase in rat hippocampal neurons

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**ABSTRACT** Excessive influx and the subsequent rapid cytosolic elevation of Ca<sup>2+</sup> in neurons is the major cause to induce hyperexcitability and irreversible cell damage although it is an essential ion for cellular signalings. Therefore, most neurons exhibit several cellular mechanisms to homeostatically regulate cytosolic Ca<sup>2+</sup> level in normal as well as pathological conditions. Delayed rectifier K<sup>+</sup> channels (I<sub>DR</sub> channels) play a role to suppress membrane excitability by inducing K<sup>+</sup> outflow in various conditions, indicating their potential role in preventing pathogenic conditions and cell damage under Ca<sup>2+</sup>-mediated excitotoxic conditions. In the present study, we electrophysiologically evaluated the response of I<sub>DR</sub> channels to hyperexcitable conditions induced by high Ca<sup>2+</sup> pretreatment (3.6 mM, for 24 hours) in cultured hippocampal neurons. In results, high Ca<sup>2+</sup>-treatment significantly increased the amplitude of I<sub>DR</sub> without changes of gating kinetics. Nimodipine but not APV blocked Ca<sup>2+</sup>-induced I<sub>DR</sub> enhancement, confirming that the change of I<sub>DR</sub> might be targeted by Ca<sup>2+</sup> influx through voltage-dependent Ca<sup>2+</sup> channels (VDCCs) rather than NMDA receptors (NMDARs). The VDCC-mediated I<sub>DR</sub> enhancement was not affected by either Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release (CICR) or small conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channels (SK channels). Furthermore, PP2 but not H89 completely abolished I<sub>DR</sub> enhancement under high Ca<sup>2+</sup> condition, indicating that the activation of Src family tyrosine kinases (SFKs) is required for Ca<sup>2+</sup>-mediated I<sub>DR</sub> enhancement. Thus, SFKs may be sensitive to excessive Ca<sup>2+</sup> influx through VDCCs and enhance I<sub>DR</sub> to activate a neuroprotective mechanism against Ca<sup>2+</sup>-mediated hyperexcitability in neurons.

## INTRODUCTION

Dynamic changes of cytosolic Ca<sup>2+</sup> level under physiological conditions are essential for cellular signalings of protein synthesis and signal transmission of neurons in central nervous system (CNS). However, due to its cytotoxic effects, excessive influx of Ca<sup>2+</sup> and subsequent rapid elevation of [Ca<sup>2+</sup>]<sub>i</sub> can induce irreversible damage of neurons or result in pathological dysfunctions such as epileptic seizure. Hence, mammalian

neurons exhibit several protective mechanisms to homeostatically regulate membrane excitability and cytosolic Ca<sup>2+</sup> level. Several types of voltage dependent ion channels are involved in these neuroprotective processes via regulating cation flow. In its original description based on neuronal excitability, intrinsic excitability can be defined as the ability to fire action potentials (APs), which are determined by voltage-dependent K<sup>+</sup> and Na<sup>+</sup> channels (K<sub>V</sub> and Na<sub>V</sub> channels) [1]. Therefore, cation channels especially sensitive to membrane potential are key factors to



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regulate excitability in most neurons even though they are inhibitory. In particular, several subtypes of Kv channels can determine resting membrane potential and effectively induce cation outflow to downregulate neuronal excitability in many conditions of membrane depolarization [2].

Among the many types of  $K^+$  channels expressed in CNS neurons, Kv2.1 is a major component of delayed rectifier  $K^+$  channel ( $I_{DR}$  channel), exhibiting sustained outward  $K^+$  currents [3-5]. This subtype plays a direct role in lowering membrane potential thus inhibiting AP initiation as well as limiting repetitive APs firing. Thus, the hyperpolarization of membrane potential and blockade of depolarization by Kv2.1 can prevent hyperexcitability that activates cytotoxic cascades and induces neuronal damage [6,7]. Previous studies showed that Kv2.1 channels are sensitive to changes in cytosolic  $Ca^{2+}$  level. For example, cytosolic  $Ca^{2+}$  increase and subsequent calcineurin activation affects the gating kinetics of  $I_{DR}$  channels via dephosphorylating Kv2.1 channels, since calcineurin-dependent dephosphorylation of Kv2.1 decreases the threshold for  $I_{DR}$  channel opening and disrupts channel clustering, resulting in changes of activation kinetics [1,8]. Kv2.1 channels have many serine, threonine, and tyrosine phosphorylation sites. These participate in the regulation of  $I_{DR}$  channel kinetics; for e.g., PKA-mediated phosphorylation in serine/threonine sites induces the changes of gating kinetics but not membrane expression of Kv2.1 channels [9,10]. Dynamic changes of gating kinetics by de- or phosphorylation of Kv2.1 channels seem to be minor but significant for regulating  $K^+$  outflow and membrane excitability under physiological conditions. However, other studies have recently demonstrated that under several pathological conditions such as epileptic seizures and ischemic damages, acute increment of cytosolic  $Ca^{2+}$  rapidly potentiates  $I_{DR}$ , and thus suppresses hyperexcitability [11-13]. These results suggest that rapid changes of cytosolic  $Ca^{2+}$  may stimulate more effective signaling pathways by which  $I_{DR}$  channels directly regulate membrane excitability. However, whether excessive  $Ca^{2+}$  influx rapidly and potentially enhances  $I_{DR}$  remains unclear.

In the present study, we confirmed that dissociated hippocampal neurons of rats showed significant increase of  $I_{DR}$  after high  $Ca^{2+}$  treatment, which could enhance synaptic activities and membrane excitability. The  $Ca^{2+}$ -mediated  $I_{DR}$  upregulation was sensitive to activity of voltage-dependent  $Ca^{2+}$  channels (VDCCs) but not NMDA receptors (NMDARs), suggesting a neuroprotective signaling pathway that is not targeted by postsynaptic  $Ca^{2+}$  influx via NMDARs. In the additional experiment, we confirmed that the contribution of Src family tyrosine kinase (SFK) was necessary for upregulating  $I_{DR}$  under high  $Ca^{2+}$  condition, suggesting that the increased expression of  $I_{DR}$  channels via SFK activation is a protective mechanism against excitotoxicity and pathogenic processes in neurons.

## METHODS

### Animal preparation and hippocampal primary culture

Hippocampal primary cultures were prepared from embryonic 20-day SD rats. After pregnant rats were deeply anesthetized, the embryos were removed and 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. The pH level was adjusted to 7.4 with NaOH. Isolated hippocampi from embryonic rat brains were transferred to ice-cold minimal essential medium (MEM) containing Earle's salts and glutamine with 10% fetal bovine serum, 0.45% glucose, 1 mM sodium pyruvate, 25  $\mu$ M glutamate and antibiotics, and the hippocampi were triturated. The cells were counted and seeded on glass coverslips coated with poly-L-lysine (Sigma-Aldrich) at a density of  $9 \times 10^4$  cells/ml and transferred to an incubator gassed with 95%  $O_2$  and 5%  $CO_2$  at 37°C. After 7 hours, the whole plating medium was exchanged to Neurobasal medium (Sigma-Aldrich) containing B-27 (Invitrogen), and a half of the medium was replaced with new media once at DIV 5. Detail protocols for cell preparation and recording techniques were as previously reported [14,15]. All experiments and procedures with animals were approved by the Animal Care and Use Committee of Jeju National University.

### Drugs treatment

To electrophysiologically observe the changes of  $I_{DR}$  in cultured hippocampal neurons, high  $Ca^{2+}$  condition was induced by doubling  $Ca^{2+}$  concentration of culture media (normal 1.8 mM) up to 3.6 mM (with  $CaCl_2$ , Sigma-Aldrich) for 24 hours. APV (100 mM, Sigma-Aldrich) and nimodipine (10 mM, Sigma-Aldrich) were used with high  $Ca^{2+}$  treatment to block NMDARs and VDCCs, respectively, in some experiments. H89 was added to block the activation of PKA and ryanodine receptors and  $IP_3$  receptors of ER were blocked by Ryanodine and 2AP, respectively. Blockers of kinases and receptors were obtained from Sigma Aldrich. Caffeine or 4CMC (Sigma-Aldrich) were used to test the effects of CICR via ryanodine receptor activation. Details of drugs treatment are mentioned in results and legends of figures.

### Electrophysiology

For patch-clamp recordings of dissociated hippocampal neurons, DIV 6~8 cultured cells 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 level was adjusted to 7.4 with NaOH, and bubbled with 95%  $O_2$  and 5%  $CO_2$ . TTX (0.5  $\mu$ M, Tocris) was added to the recording solution to block the Nav channels. The patch pipettes (4~6  $M\Omega$ ) 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. pH and osmolarity were adjusted to 7.2 and 280~300 mOsm, respectively. In all experiments, neurons showing 7~11 pF whole cell capacitance were used. Whole cell recording parameters were monitored throughout each experiment and recordings where series resistance (6~20 M $\Omega$ ) varied by more than 10% were rejected.

All recordings were performed at room temperature, low-pass filtered at 5 kHz, and digitized at 10 kHz by a Digidata 1322A convertor. Voltage-clamping whole cell patches, command pulse generation and data recording were performed using Axopatch 200B and pClamp 8 software. Thick-walled, filamented patch electrodes had tip resistance of 3~6 M $\Omega$ . For adjusting whole-cell parameters, membrane capacitance and series resistance were manually compensated in Axopatch 200B during applying command pulses. Series resistance varied between 8~30 M $\Omega$ , and recordings where series resistance varied by more than 10% were rejected. No electronic compensation for series resistance was employed during currents recording. Pulse commands to record sustained currents in voltage-clamping mode are described in detail in results and figures. Sustained  $K^+$  currents were digitally isolated using a prepulse protocol after subtracting leak currents. Peak currents were measured at +60 mV. All experimental data were additionally analyzed using IGOR pro software.

## Statistical analysis

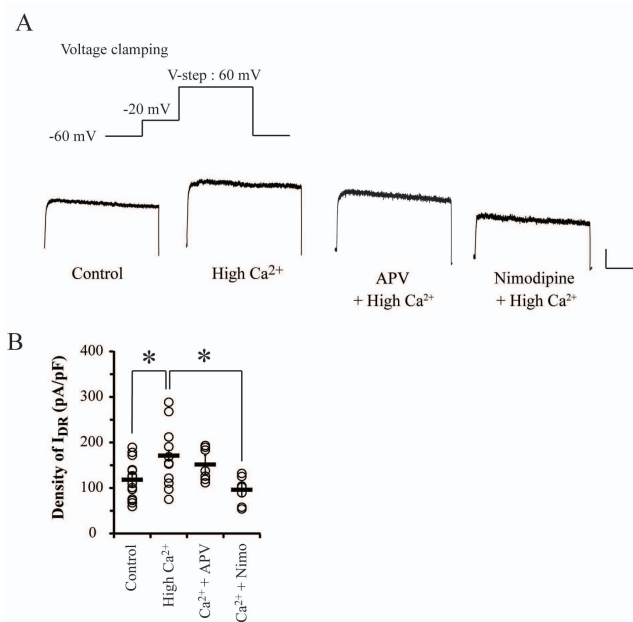
Further data and statistical analysis was performed using SPSS and Excel software and data were expressed as the mean value $\pm$ standard error (SEM). The Student's *t*-test was used, and statistical difference between groups was indicated for *p* values of <0.05 or 0.01.

## RESULTS

In physiological regulation of neuronal excitability, the dominant role of small but sustained  $K^+$  outflow through  $I_{DR}$  channels is to determine and stabilize sub- and suprathreshold membrane potentials. However, signaling-mediated changes of its kinetics suggest a possibility that  $I_{DR}$  channels may also be targeted by hyperexcitable conditions. In the present study, we tested  $K^+$  outflow through  $I_{DR}$  channels in response to hyperexcitable condition induced by high  $Ca^{2+}$  overnight treatment (3.6 mM  $CaCl_2$ , for 24 hours) in cultured hippocampal neurons. This experimental condition not only induces the downregulation of A-type  $K^+$  channels ( $I_A$  channels) but also enhances synaptic  $Ca^{2+}$  influx by remodeling NMDARs composition, resulting in the hyperexcitability of hippocampal neurons [14,16]. After treating high  $Ca^{2+}$  to culture media for 24 hours, neurons were transferred to a recording chamber and washed in normal ACSF solution

for electrophysiological measurement. In results, the current density of  $I_{DR}$  channels was significantly increased by high  $Ca^{2+}$  treatment, as shown in Fig. 1 (Control=112.62 $\pm$ 10.28 pA/pF, *n*=13; high  $Ca^{2+}$ =171.05 $\pm$ 18.30 pA/pF, *n*=12, *p*=0.02). This indicates that  $I_{DR}$  channels may be upregulated under hyperexcitable conditions.

Depolarization of membrane potential activates two major  $Ca^{2+}$  influx pathways: VDCC and NMDAR. High  $Ca^{2+}$ -enhanced neuronal excitability is reportedly dependent on  $Ca^{2+}$  influx via NMDARs, hence it was necessary to confirm if the increase of  $I_{DR}$  was dependent on NMDARs [16]. APV (100  $\mu$ M) or nimodipine (10  $\mu$ M) with high  $Ca^{2+}$  treatment was applied to culture media for 24 hours before recording. APV-induced NMDARs block showed no effects on the high  $Ca^{2+}$ -enhanced  $I_{DR}$  in this experiment; whereas, nimodipine, a VDCCs antagonist, significantly inhibited the effect of high  $Ca^{2+}$  treatment on  $I_{DR}$  (Fig. 1, APV=151.55 $\pm$ 12.56 pA/pF, Nimodipine=96.03 $\pm$ 9.34 pA/pF, *p*=0.48, 0.008 respectively, compared with high  $Ca^{2+}$ ). Thus,  $I_{DR}$  upregulation may be triggered by  $Ca^{2+}$  influx through VDCCs rather than NMDARs. Furthermore, because it is well known that  $Ca^{2+}$ -induced  $Ca^{2+}$  release (CICR) is responsive to  $Ca^{2+}$



**Fig. 1.  $I_{DR}$  is enhanced under high  $Ca^{2+}$  condition in cultured hippocampal neurons, showing the dependence on VDCCs but not NMDARs.** (A) The experimental protocol of voltage clamping to record  $I_{DR}$  in hippocampal neurons and example traces of  $I_{DR}$ . High  $Ca^{2+}$  condition was induced by doubling  $Ca^{2+}$  concentration ( $CaCl_2$ , 3.6 mM) of culture media for 24 hours (High  $Ca^{2+}$ ) and then neurons were washed in normal ACSF during recording. Either APV (100  $\mu$ M, APV+High  $Ca^{2+}$ ) or nimodipine (10  $\mu$ M, Nimodipine + High  $Ca^{2+}$ ) was added to culture media for 24 hours under high  $Ca^{2+}$  condition. Scale bars 500 pA, 100 ms. (B) Individuals (circles) and averaged (square bars) densities of  $I_{DR}$ . Currents were adjusted with whole cell capacitance for measuring current densities of each group. Error bars represent SEM. \**p*<0.05.

influx, we also tested if it might participate in  $I_{DR}$  upregulation resulted from  $Ca^{2+}$  influx via VDCCs. In Table 1, blocking of two major receptors of endoplasmic reticulum, ryanodine receptors (Ryanodine) and  $IP_3$  receptors (2APB), showed no effects on enhanced  $I_{DR}$  under high  $Ca^{2+}$  condition. Additionally, caffeine or 4CMC, agonists of ryanodine receptors, showed no enhancement effects on  $I_{DR}$ , unlike high  $Ca^{2+}$  treatment. These results strongly indicated that  $Ca^{2+}$  influx through VDCCs may directly regulate  $I_{DR}$  channels regardless of CICR activation.

Although the current density of  $I_{DR}$  was increased, the gating kinetics of  $I_{DR}$  channels was not affected under high  $Ca^{2+}$  condition. In Fig. 2, the increase rate of  $I_{DR}$  positively reflected the increase rates of command pulse potentials in all experimental groups. At +80 mV injection, significant differences of  $I_{DR}$  amplitudes were observed between control and high  $Ca^{2+}$  treated neurons (Fig. 2A and B, Control=139.82±15.62 pA/pF; High  $Ca^{2+}$ =226.79±17.34 pA/pF,  $p=0.02$ ). The current density of  $I_{DR}$  under high  $Ca^{2+}$  condition was enhanced up to 60% in each injection range from -40 to 80 mV compared with the control group. However, in the analysis of conductance compensated by each membrane potential, activation properties of  $I_{DR}$  channels were consistent in all groups, indicating that the gating kinetics of  $I_{DR}$  channels may not be subjected to high  $Ca^{2+}$  condition (Fig. 2C and D,  $V_h$  of activation: Control=12.38±2.62 mV; High  $Ca^{2+}$ =22.21±4.4 mV,  $p=0.1$ ). This result suggests that the upregulation of  $I_{DR}$  channels under high  $Ca^{2+}$  condition may not be dependent on their dephosphorylation by calcineurin activated by  $Ca^{2+}$  signaling. Additionally, both antagonists, APV and nimodipine, showed no effects on the activation kinetics of  $I_{DR}$  channels ( $V_h$  of activation; APV group=10.11±1.91; Nimodipine group=14.92±1.63,  $p=0.55$  and  $0.45$ , respectively).

Previously, Ledoux et al. [17] reported that the enhancement of intracellular  $Ca^{2+}$  concentration induced the elevation of  $K^+$  efflux through small conductance  $Ca^{2+}$  channels (SK channels). Because of possible change in intracellular  $Ca^{2+}$  concentration under the experimental condition, it was necessary to confirm whether the enhanced density of  $I_{DR}$  by high  $Ca^{2+}$  was contaminated by the flow of SK channels even though the two channels exhibit totally different gating mechanisms. In Fig. 3, the effect of 100

nM apamin with high  $Ca^{2+}$  was tested by adding to culture media for 24 hours to block SK channels. The addition of apamin did not influence the increased density of  $I_{DR}$  by high  $Ca^{2+}$  treatment, indicating that upregulating  $I_{DR}$  is not via the activation of SK channels (179.37±26.56 pA/pF,  $p=0.04$  and  $0.81$ , as compared with control and high  $Ca^{2+}$ , respectively).

$Ca^{2+}$  signaling cascades activated by  $Ca^{2+}$  influx through synaptic NMDARs induce the phosphorylation of protein kinase A (PKA) resulting in the downregulation of  $I_A$  in hippocampal neurons [15,18]. Although it is still under argument, it is possible that PKA regulates the turnover rate of proteins from plasma membrane and currents through various channels. In this study, PKA was not involved in the cellular processing to enhance  $I_{DR}$  under high  $Ca^{2+}$  condition (Table 1, +H89). Rather, we confirmed that SFKs have a potentially important role in  $I_{DR}$  upregulation under high  $Ca^{2+}$  condition (Fig. 3). Adding SFK blocker, PP2 (1  $\mu$ M), to culture media with high  $Ca^{2+}$  showed no enhancement of  $I_{DR}$ , with similar levels as in control neurons that were not treated with any drugs ( $Ca^{2+}$ +PP2=99.01±10.52 pA/pF,  $p=0.43$  and  $0.02$  compared with the control and high  $Ca^{2+}$ , respectively). Furthermore, PP2 alone showed no effect on the density of  $I_{DR}$ , indicating that high  $Ca^{2+}$  condition possibly activate SFKs to prevent neuronal excitotoxicity or damage (123.46±20.23 pA/pF,  $p=0.63$  compared with the control).

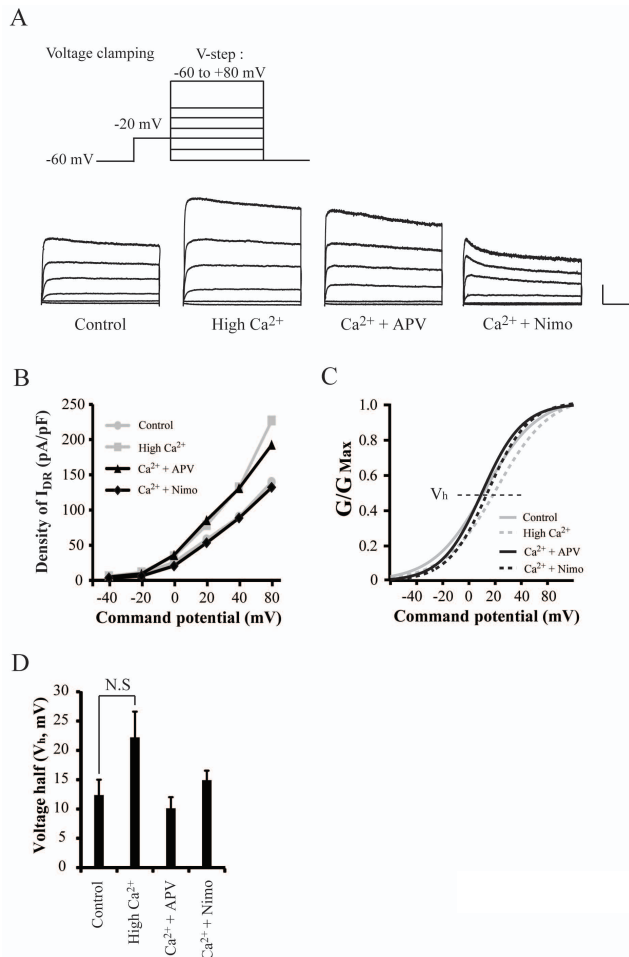
## DISCUSSION

Neurons in CNS exhibit dynamic regulatory signalings to protect from hyperexcitable conditions that result in neuronal damage. Herein, we reported upregulation of  $I_{DR}$  channels, as a neuroprotective regulator, which increased  $K^+$  outflow under hyperexcitable condition induced by high  $Ca^{2+}$  overnight treatment in cultured hippocampal neurons. The high  $Ca^{2+}$ -induced enhancement of  $I_{DR}$  was dependent on  $Ca^{2+}$  influx through VDCCs but not NMDARs and was not affected by CICRs that synergistically increase intracellular  $Ca^{2+}$  level (Fig. 1 and Table 1). These results indicate that hippocampal neurons may homeostatically modulate  $I_{DR}$  channels in response

**Table 1. The densities of  $I_{DR}$  recorded in hippocampal neurons under each condition**

	$I_{DR}$ density (pA/pF) average	n	p value, compared with	
			Control	high $Ca^{2+}$
Control	118.11±10.91	14	-	-
High $Ca^{2+}$	171.05±18.30	12	0.021*	-
High $Ca^{2+}$ +Ryanodine	180.93±27.97	11	0.039*	0.776
High $Ca^{2+}$ +2APB	157.54±22.42	8	0.106	0.663
Caffeine	99.88±11.91	11	0.292	0.006**
4CMC	118.24±10.47	8	0.993	0.049*
High $Ca^{2+}$ +H89	192.84±36.08	7	0.027*	0.579

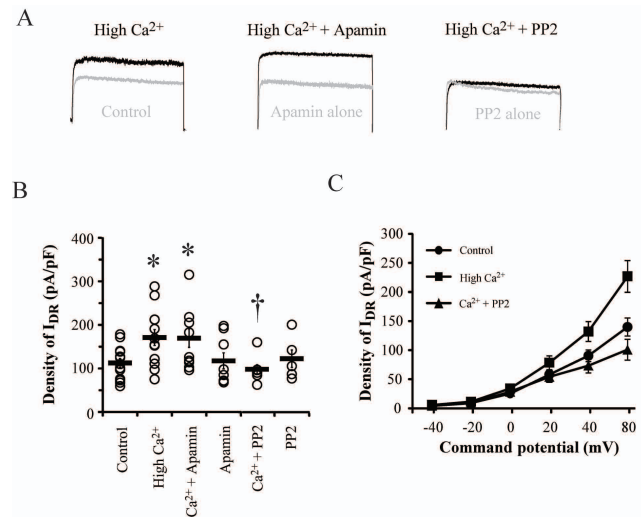
\* $p<0.05$ , \*\* $p<0.01$ .



**Fig. 2. The enhancement of  $I_{DR}$  under high  $Ca^{2+}$  condition does not involve the significant alteration of activation properties of  $I_{DR}$  channels.** The activation property was measured at  $-60$  to  $80$  mV with 20 steps after prepulse injection ( $-20$  mV, 200 ms duration). (A) The experimental protocol of voltage clamping to measure the activation kinetics of  $I_{DR}$  channels and example traces of  $I_{DR}$ . The amplitude of  $I_{DR}$  is increased by steps of voltage clamping. However, the constant rate of increment indicates no changes of  $I_{DR}$  activating kinetics. Scale bars 500 pA, 100 ms. (B) Averaged densities of  $I_{DR}$  at each clamping voltage ( $-40$  to  $80$  mV). (C) and (D) Boltzmann fitted curves of gating kinetics of  $I_{DR}$  activation and averaged values of voltage half ( $V_h$ , dotted line in C). The significant change of activation curves was not observed. Error bars represent SEM.

to  $Ca^{2+}$  influx through VDCCs. Thus, neuronal excitability is consequently downregulated by the enhancement of  $K^+$  outflow via  $I_{DR}$  channels. Furthermore,  $I_{DR}$  enhancement was dependent on tyrosine kinases of Src family, indicating the possibility of a unique signaling cascade for the downregulation of excitability to prevent neuronal damages under hyperexcitable or pathogenic conditions.

High  $Ca^{2+}$  treatment for 24 hours used in this study (i.e. doubling  $Ca^{2+}$  concentration in culture media), is reported to mediate changes in the subunit composition of synaptic NMDARs,  $I_A$  density and  $Ca^{2+}$ -calmodulin dependent kinase



**Fig. 3. SFKs are participated in  $I_{DR}$  enhancement under high  $Ca^{2+}$  condition.** (A) Example traces of  $I_{DR}$  in each group. To confirm the contribution of SK channels, apamin (100 nM,  $Ca^{2+}$ +Apamin) was added to culture media. PP2 (1  $\mu$ M) abolished the  $Ca^{2+}$ -induced enhancement of  $I_{DR}$ , indicating the involvement of SFK in  $I_{DR}$  upregulation. (B) Individual (open circles) and averaged values (square bars) of  $I_{DR}$  densities. Error bars represent SEM. \* $p < 0.05$  compared with control and  $^{\dagger}p < 0.05$  compared with High  $Ca^{2+}$ . (C) Averaged densities of  $I_{DR}$  at each clamping voltage ( $-40$  to  $80$  mV). Error bars represent SEM.

activity [14,16,19]. Increased  $Ca^{2+}$  influx through NMDARs and subsequent  $I_A$  downregulation critically increases neuronal excitability due to changes in membrane resistance, firing rates of APs and after-hyperpolarizing potentials resulting from direct effects of the cationic flow through channels or receptors [16,19]. These effects of high  $Ca^{2+}$  condition possibly result in irreversible damage to hippocampal neurons. However, hippocampal neurons showing  $Ca^{2+}$ -induced downregulation of Kv4.2 channels had no effect on the resting membrane potential even though suprathreshold properties of membrane excitability were significantly changed [19]. This phenomenon is consistent with previous results in hippocampal neurons pretreated with high  $Ca^{2+}$ , indicating that membrane potentials may also be targeted by a regulatory mechanism to prevent excitotoxicity [14]. In this study, the enhancement of  $I_{DR}$  strongly suggested a neuroprotective mechanism related with  $I_{DR}$  channels and  $Ca^{2+}$  influx through VDCCs (Fig. 1).  $I_{DR}$  channels contribute to the restoration of depolarized membrane potential to resting membrane potential in cardiac cells, determining refractory period as well as heart rate. In nervous system, these channels seem to contribute to the regulation of somatodendritic excitability. Kv2 family mediated with  $I_{DR}$  acts as a dominant regulatory factor for membrane excitability in hippocampal and cortical pyramidal neurons [20–22]. Experimental evidence indicates that the genetic manipulation of Kv2.1 directly affects intrinsic excitability, showing epileptiform activity, and that Kv2.1 knock-out mice exhibit defects in spatial learning [23].

These pathogenic outcomes are likely due to the hyperexcitability of neurons during development, as  $I_{DR}$  channels are targeted by cellular signaling pathways of various growth factors in young brain [24]. Therefore, enhanced  $I_{DR}$  under high  $Ca^{2+}$  condition observed in this study may be a rapid but effective neuroprotective modulation to prevent entry into pathogenic stages.

As a critical regulator of neuronal excitability,  $I_{DR}$  channels are dominantly targeted by  $Ca^{2+}$ -mediated signaling in the manner of activity- and use-dependence. Even though  $I_{DR}$  channels are highly phosphorylated in neurons, the expression and gating kinetics of these channels are bidirectionally subjected to activity-dependent dephosphorylation, which is mediated by glutamatergic transmission [11,13,25]. The dephosphorylation of these channels can rapidly and homeostatically suppress neuronal excitability under pathological conditions via the enhancement of  $K^+$  outflow. Dephosphorylation of  $I_{DR}$  channels under anoxic stress is dependent on  $Ca^{2+}$  influx through NMDARs that are activated by excess glutamate release in synapses [25]. Thus, overexcitability of glutamatergic synapses in various pathological conditions possibly activates neuroprotective signalings to dephosphorylate and upregulate  $I_{DR}$  channels. However, we observed that enhanced  $I_{DR}$  under high  $Ca^{2+}$  condition was clearly dependent on  $Ca^{2+}$  influx through VDCCs but not NMDARs. This is evidenced by the effect of nimodipine to abolish the enhancement of  $I_{DR}$  (Fig. 1). Despite previous reports on the effects of VDCC blockers to inhibit  $I_{DR}$ , nimodipine, one of the dihydropyridines, showed no direct inhibition of  $I_{DR}$  in this study (data not shown) [26-28]. Because  $I_A$  downregulation is dependent on NMDARs under high  $Ca^{2+}$  condition,  $I_{DR}$  enhancement may be a possible mechanism of compensatory regulation in hippocampal neurons that are chronically exposed to high  $Ca^{2+}$  [14].  $I_{DR}$  channels are predominantly regulated by calcineurin-dependent dephosphorylation, which is accompanied by changes of threshold as well as activation kinetics [1,8]. In this study, however, we confirmed that high  $Ca^{2+}$ -induced upregulation of  $I_{DR}$  involved only the increase of  $I_{DR}$  peak but not the alteration of gating kinetics, suggesting the possibility of another signaling mechanism distinctive to calcineurin-dependent dephosphorylation. Moreover, the bidirectional and homeostatic upregulation of  $I_{DR}$  observed in neurons is due to the overexcitation of glutamatergic synapses, hence, the enhancement of  $I_{DR}$  peak without a kinetic change may be originated from  $Ca^{2+}$  influx via VDCCs rather than NMDARs [11,13,25].

The upregulation of  $I_{DR}$  peak without changes of gating kinetics suggests the involvement of specific modulatory signalings of kinases, which can be activated under high  $Ca^{2+}$  condition (Fig. 1 and 2). Previous reports focusing on regulatory mechanisms of KV2.1 channel indicate that these channels possess a large number of kinase binding sites such as serine, threonine, and tyrosine, and are usually affected by all except threonine [9,10]. In particular, tyrosine kinases, such as Src and Fyn, seem to play an important role in the regulation of KV2.1 channel expression.

Although, tyrosine 124 phosphorylated by src regulates channels activities, tyrosine 810 phosphorylation influences the intracellular trafficking, regulating Kv2.1 channels expression [29-31]. Furthermore, growth differentiation factor 15 (GDF15)-induced upregulation of Kv2.1 channels expression was also abolished by the inhibition of Src-mediated phosphorylation of TGF $\beta$  receptors [24]. These previous reports indicate that SFKs are dominantly mediated with the cellular regulation of Kv2.1 channel expression, while the gating kinetics of  $I_{DR}$  channels is specifically targeted by serine kinases. Thus, the upregulation of  $I_{DR}$  by high  $Ca^{2+}$  treatment observed in this study may be originated from the modulatory function of tyrosine kinases. Fig. 2 clearly shows that PP2 blocked the enhancement of  $I_{DR}$  under high  $Ca^{2+}$  condition, indicative of SFKs' roles in  $I_{DR}$  channels for preventing hyperexcitability.

Because excessive  $Ca^{2+}$  influx is sufficient to cause hyperexcitability and irreversible damage to hippocampal neurons, resulting in pathological conditions, a number of subcellular signalings in neurons target the expression and gating kinetics of Kv channels which actively regulate membrane excitability. The current study confirmed that a neuroprotective regulatory mechanism involving  $I_{DR}$  channels in hippocampal neurons possibly plays a role to suppress neuronal excitability under various pathogenic conditions. Although further study is needed to clarify the relationship(s) between  $Ca^{2+}$  influx via VDCCs and SFK signaling in  $I_{DR}$  upregulation, VDCC-mediated upregulation of  $I_{DR}$  suggests another signaling pathway to actively regulate membrane excitability of hippocampal neurons.

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## CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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