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소뇌 대마제제 유발 단기약화 과정에 TRPC 이온통로 전류가 관여하는지에 대한 연구

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2012 년 7 월

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의학과 생리학 전공

장원석

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By

Wonseok Chang

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ABSTRACT

TRPC-mediated current is not involved in endocannabinoid-induced short-term depression in cerebellum

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It was found that large calcium influx caused by depolarization of neuron (Depolarization-induced suppression of inhibition/excitation, DSI/DSE) or strong activation of $G_{q/11}$-coupled receptors (receptor-driven endocannabinoid release, RER) can mediate endocannabinoid-induced retrograde signaling and induce transient depression of synaptic transmission. Besides, the endocannabinoids release also can be induced when a small Ca^{2+} increase and weak activation of $G_{q/11}$-coupled receptor occur

simultaneously (calcium-assisted RER, Ca-RER). In Ca-RER, calcium channels other than voltage-operated calcium channel or intra cellular calcium store can be important calcium sources for phospholipase C beta (PLCβ) activation because small amount of calcium can facilitate endocannabinoid release significantly when receptor activation is combined. Metabotropic glutamate receptor (mGluR) can induce not only activation of PLCβ, but also intracellular calcium increase. Two major different calcium signaling pathways have been known: (1) slow-kinetic evoked postsynaptic current (slow EPSC) carried by a non-selective cation channel, transient receptor potential canonical (TRPC) that is permeable to Ca^{2+} in cerebellar Purkinje cells (PCs); (2) IP_3-induced intracellular calcium release from intracellular calcium store. However, it is unclear that how much each calcium source contributes to Ca-RER. Here, we investigated whether calcium influx through mGluR-evoked TRPC channel activation contributes to Ca-RER in cerebellar PCs. We induced the transient depression by a burst stimulation of parallel fiber (PF) or combination of burst and postsynaptic depolarization and confirmed that those depressions are cannabinoid- and mGluR1-dependent. Furthermore, TRPC antagonist, SKF96365 blocked transient depression induced by endocannabinoid, and attenuated calcium transient during induction of the transient depression. However, an

alternative TRPC antagonist, BTP2 blocked neither the depression nor calcium increase though it blocked PF burst-induced slow inward current in both experiments with two different protocols. BTP2 inhibited the slow current mostly, so the data that Ca-RER was not impaired by BTP2 displays that Ca-RER can be induced without TRPC-mediated current, and the results from experiments with SKF96365 is interpreted in terms of nonspecificity of SKF96365. These findings suggest that TPRC-mediated inward current is overwhelmed by other calcium sources and does not have an important role in endocannabinoid signaling in cerebellar PF-PC synapse.

Keywords: TRPC, cerebellar Purkinje cell, metabotropic glutamate receptor, slow EPSC, endocannabinoid, synaptic plasticity, retrograde signaling, dendritic calcium

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LIST OF FIGURES

LIST OF ABBREVIATION

DSI/DSE: Depolarization-induced suppression of inhibition/excitation

RER: receptor-driven endocannabinoid release

Ca-RER: calcium-assisted RER

PLCβ: phospholipase C beta

mGluR: metabotropic glutamate receptor

EPSC/EPSP: evoked postsynaptic current/potential

TRPC: transient receptor potential canonical

PF: parallel fiber

CF: climbing fiber

PC: Purkinje cell

LTD/LTP: long-term depression/potentiation

VOCC: voltage-operated calcium channel

ER: endoplasmic reticulum

GPCR: G protein-coupled receptor

AEA: arachidonoyl ethanolamide

2-AG: 2-arachidonoyl glycerol

CB1R: cannabinoid receptor 1

CONTENTS

INTRODUCTION

Synaptic plasticity in cerebellum

Synapse is a structure that permits a neuron to transmit a signal to another neuron. An activation of presynaptic neuron is delivered to postsynaptic neuron with neurotransmitter in chemical synapse. So, the amplitude of response to given signal depends on the ability of the connection - synaptic strength. It is also well known that use and disuse or transmission over synapses can trigger modulation of synaptic function which is known as synaptic plasticity since Terje Lomo and Tim Bliss reported activity-dependent long-term potentiation (LTP) in early 70s (Bliss and Gardner-Medwin, 1973). Synaptic plasticity is suggested to be an important mechanism of neural development, storage of memory, and modulation of neural output and several pathologies like addiction, epilepsy or chronic neural pain. Especially, long-term synaptic plasticity is known as a molecular mechanism of memory storage in the central nervous system. In cerebellum, long-term depression (LTD) at parallel fiber-Purkinje cell (PF-PC) synapses have been widely accepted to underlie motor learning (Albus, 1971; Ito, 1972) while short-term depression is suggested to contribute to real-time modulation of output of neural circuit. Although both of cerebellar long-term

depression and short-term depression are thought to have different function, both of them have been reported to share the mechanisms: activation of mGluR and postsynaptic increase of calcium concentration.

Metabotropic glutamate receptors in cerebellar synaptic plasticity

Metabotropic glutamate receptor (mGluR) and calcium are well known as important molecules for many types of synaptic plasticity. mGluR is a G-protein coupled receptors that modulate neurotransmission and neuronal excitability throughout the CNS (Nakanishi, 1994; Conn and Pin, 1997). They are divided into three groups – Group I, Group II and Group III - based on sequence homology, agonist selectivity, signaling transduction pathways and have eight subtypes (mGluR1~mGluR8). In cerebellum, mGluR1, one of group I mGluR is expressed strongly at PCs, and located at perisynaptic region of the PC dendritic spine in PF-PC synapse. Single PF activation releases a moderate amount of glutamate that can not reach to mGluR1 at perisynaptic site. However, burst of PF stimuli releases glutamate enough to reach to mGluR1 and then activates mGluR1. PFs are axons of granule cell. Therefore mGluR1 can detect and deliver the signal of strong activation of granule cell. In other words, we can mimic strong activation of granule cell with a burst of PF stimuli and activate mGluR1. Activation of mGluR induces several cellular events in cerebellar PCs such as activation of PLCβ-

IP_3 signal cascade (Finch and Augustine, 1998; Takechi et al., 1998) or slow excitatory synaptic currents (Tempia et al., 1998; Kim et al., 2003). Contribution of mGluR1 to cerebellar LTD has been clear by pharmacologic study (Crepel et al., 1991; Linden and Connor, 1991), or mGluR1 antibody (Shigemoto et al., 1994), furthermore, mGluR1 null mutant mouse showed impairments of cerebellar function such as ataxia and motor coordination (Aiba et al., 1994; Conquet et al., 1994).

Calcium in cerebellar synaptic plasticity

Intracellular calcium is also well known to be involved in many kinds of synaptic plasticity, activating enzymes like various kinases, phophotases or adenylyl cyclase, which contribute to modulation of phosphorylation of channels or receptors, control of synaptic release machinery or regulation of gene expression. In cerebellum, voltage-operated calcium channels or intracellular calcium store are known as important calcium sources for various cellular events which need increases of calcium concentration. Cerebellar PCs spontaneously and repetitively fire when there is no synaptic input (Llinas and Sugimori, 1980). PC have two major excitatory inputs – PF and Climbing fiber (CF). Especially, activation of CF induces huge inward current that induces strong depolarization of PC (Konnerth et al., 1990) by activation of voltage-operated calcium channel. Activation of climbing fiber

already have been clear to be involved in cerebellar LTD (Sakurai, 1990), LTP (Salin et al., 1996), and endocannabinoid signaling (Stanton and Sejnowski, 1989). In addition, activation of mGluR causes release of calcium from IP_3-sensitive calcium store in PC dendrites and spines (Finch and Augustine, 1998; Takechi et al., 1998) and calcium release from intracellular stores by IP_3Rs is required for LTD (Inoue et al., 1998). However, contribution of calcium sources other than voltage-operated calcium channel (VOCC) or endoplasmic reticulum (ER) calcium store to synaptic plasticity in cerebellum is still unknown.

TRPC-mediated slow current and calcium influx

Slow currents are known to be mediated by transient receptor potential canonical (TRPC) channel which is a subfamily of TRP channels when mGluR is activated (Kim et al., 2003). Slow currents are non-selective cation currents include Na^+ and Ca^{2+} influxes (Chuang et al., 2000) so it can elevate intracellular calcium concentration in cerebellar PC. Furthermore, in TRPC3 knockout mice slow EPSCs by the burst stimuli of PF and walking behavior are impaired (Hartmann et al., 2008), however, it has not been reported whether TRPC-mediated calcium influx underlies synaptic plasticity such as cerebellar LTD or endocannabinoid release.

Mechanisms of Endocannabinoid-induced short-term depression

Endocannabinoids are metabolites of membrane lipid that activate cannabinoid receptor, the G protein-coupled receptor (GPCR) known to be targets for the active ingredient, Δ9-tetrahydrocannabinol, in *Cannabis savita*. Two major endocannabinoids are arachidonoyl ethanolamide (AEA or anadamide) and 2-arachidonoyl glycerol (2-AG) and their actions are mediated through activation of the cannabinoid1 receptor (CB1R), which express in whole CNS region, while CB2 receptors express in immune system mainly. Endocannabinoids function as a retrograde messenger that contributes to long-term and short-term plasticity in the CNS. (Yoshida et al., 2002; Maejima et al., 2005; Safo and Regehr, 2008). In early 1990s, it was first reported that endocannabinoids are synthesized from membrane lipids and released by large elevation of calcium concentration in postsynaptic neuron that is induced by strong depolarization of postsynaptic neuron (depolarization-induced suppression of inhibition/excitation, DSI/DSE) in hippocampus and cerebellum (Pitler and Alger, 1994; Kreitzer and Regehr, 2001). Then, endocannabinoid-induced retrograde modulation of synapse driven by strong activation of G protein-coupled receptor (receptor-driven endocannabinoid release, RER) was found in the cerebellum (Maejima et al., 2001). The activation of mGluR1 in PCs induced endocannabinoid-induced short-term depression and the depression did not require increase of calcium

concentration but PLCβ signaling cascade, which contributes to converting diacylglycerol to 2-AG (Ohno-Shosaku et al., 2005). PLCβ can act by itself, but the action of PLCβ is facilitated by calcium, therefore, if activation of mGluR1 and increase of intracellular calcium concentration is combined the endocannabinoid release is largely facilitated (Ca^{2+}-assisted RER, Ca-RER) (Hashimotodani et al., 2005). Therefore, even small elevation of calcium concentration that can only help the action of PLCβ may contribute to endocannabinoid release. Then endocannabinoid acts presynaptically to suppress neurotransmitter release and induce presynaptic depression of synaptic transmission transiently. As already described, unlike DSE/DSI, Ca-RER can be induced by weak elevation of calcium, which can be mediated by calcium sources other than VOCC such as ER calcium store or TRPC can contribute to induction of Ca-RER. It was reported that ER calcium release does not have a role in induction of Ca-RER (Brenowitz and Regehr, 2005), that means it is more worthwhile to investigate role of TRPC in endocannabinoid release.

The physiological roles of short-term depression in cerebellum

Synaptic plasticity is regarded a molecular mechanism of memory storage. However, short-term synaptic plasticity can not be a direct mechanism of

memory storage because it can not change a response for a given input permanently, unlike long-term synaptic plasticity. The physiological role of the short-term plasticity are still the subject of debate and investigation (Hampson and Deadwyler, 1998; Alger, 2002; Chevaleyre et al., 2006), but one of the most attractive possibility is regulation of the pattern and timing of neuronal activity. For example, if endocannabinoid is released by synaptic stimulation, depression would occur homosynapitcally, but if whole neuron is depolarized, every synapse – even synapse with inhibitory interneuron - of the neuron would be depressed by endocannabinoid. In this way, synaptic inputs can be modified and transformed in synapse-specific way or synapse-nonspecific way. In addition, GABAergic interneurons are known to synchronize activities of large groups of neurons, thus generating specific network rhythms that thought to be important in cognitive function. In this regard, depression of an interneuron by endocannabinoid can dissociate this synchrony, with likely important consequences on the cerebellar output. Furthermore, depression at a subset of synapses onto a given postsynaptic neuron might boost the relative efficacy, so short-term depression may help to equalize synaptic efficacy across extensive dendritic arbors (Rumsey and Abbott, 2006), so each synapse can yield more equal weighting in influencing action potential generation. In addition, short-term plasticity can not be a direct mechanism of memory storage, However, it can help to

induce long term change of synaptic efficacy. It is reported that disinhibition produced by endocannabinoid signaling at inhibitory synapse in hippocampus enhances neuronal excitability (Wagner and Alger, 1996), or induces LTP (Carlson et al., 2002; Chevaleyre and Castillo, 2003). Endocannabinoid-induced synaptic depression may also have a neuroprotective role. Intracellular calcium increases in response to excitatory synaptic transmission can trigger cellular toxicity during noxious events like brain injury or ischemia. Cannabinoid-induced depression in excitatory synapse may express neuroprotective role reducing glutamate release and postsynaptic calcium increase.

The Physiological roles of endocannabinoid

It is known that CB1R knock-out mice display neither gross anatomical defect nor apparent health problems. However, they showed hypoactivity, increased catalepsy, and hypoalgesia, increased mortality (Zimmer et al., 1999) and resistance to diet-induced obesity (Ravinet Trillou et al., 2004). Furthermore, diacylglycerol lipase knock-out mice, which can not synthesis 2-AG displayed reduced adult neurogenesis (Gao et al., 2010). The Mechanisms of these phenomenons are still remain unknown, but it is plausible that animals lack endocannabinoid signaling system show

impairments or altered behavioral manifestations because endocannabinoids contributed to various types of plasticity that can modulate learning and memory..

Here, we investigated whether endocannabinoid-induced retrograde signaling is regulated by TRPC-induced slow current. We could induce transient depression of synaptic transmission by PF burst stimulation in current clamp mode and associative stimulation of PF burst and PC depolarization in voltage clamp mode, however, any of them was affected by TRPC blocker. It suggests TRPC mediated slow current and calcium transient don’t play an important role in the endocannabinoid signaling and calcium source other than TRPC is needed for Ca-RER.

MATERIALS AND METHODS

Slice Preparation

Parasagittal slices of the cerebellar vermis (250 μm thick) were prepared from P15–P20 Sprague-Dawley rats using a vibrating tissue slicer (Microm HM650V, Germany) and ice-cold standard artificial cerebrospinal fluid (ACSF) containing 124 mM NaCl, 2.5 mM KCl, 1 mM Na_2HPO_4, 1.3 mM $MgCl_2$, 2.5 mM $CaCl_2$, 26.2 mM $NaHCO_3$, and 20 mM D-glucose, bubbled with 95% O_2 and 5% CO_2. After cutting, slices were kept for 30 min at 35℃ and then for up to 8 hours at 25℃ in ACSF.

Patch-Clamp Recordings

After a recovery period, the slices were placed in a submerged chamber that was perfused at a rate of 2 ml/min with ACSF supplemented with either 100 μM picrotoxin to block $GABA_A$ receptors. Somatic whole-cell recording were obtained by using a amplifier (HEKA Instruments Germany). The recording electrodes (resistance 2–4 MΩ) were filled with a solution containing 130 mM K-gluconate, 10 mM KCl, 10 mM HEPES, 0.2 EGTA, 10 mM Na_2-phosphocreatine, 3 mM Mg_2ATP, 0.3 mM Na_3GTP(pH 7.25) for current-clamp recording and 135 mM Cs-methanesulfonate, 10 mM CsCl, 10

mM HEPES, 4 mM Mg_2ATP, 0.4 mM Na_3GTP, and 0.2 mM EGTA (pH 7.25) for voltage-clamp recordings. 0.2mM EGTA was replaced to 0.2mM Fluo-5F (pH7.25) for calcium imaging. Currents were filtered at 1 kHz, digitized at 5 kHz, and acquired using Pulse software (HEKA Instruments, Germany). For PF stimulation, standard patch pipettes were used that were filled with external saline and placed in the middle third of the molecular layer. Synaptic responses were evoked every 30 s using 20–40 μA pulses (100 μs duration). When burst stimulation was employed, the interpulse interval was 10 ms. Recordings were performed at RT in ACSF.

Confocal fluorescence imaging and photolytic uncaging

A confocal laser-scanning head (Zeiss Pascal, U.S.A.) attached to an upright microscope (Zeiss Axioskop 2FSmot, 40X water-immersion objective, N.A. = 0.8, U.S.A.) was used to acquire fluorescence image in parallel to the whole-cell recordings. To measure postsynaptic calcium transients, EGTA was replaced to calcium indicator Fluo-5F (Invitrogen, U.S.A.). Calcium imaging was started at least 20 min after establishment of whole-cell configuration to allow for dendritic perfusion. Dendritic calcium transients were acquired at 0.1 Hz. Images were analyzed in Fluoview (Olympus, Japan).

Statistics and Drugs

All group data are shown as mean ± SEM. Comparisons were made using Student's t tests except for Fig. 4G (Anova-test). All drugs were purchased from Sigma (U.S.A) except for CPCCOEt, NBQX, AM251 (Tocris Cookson, U.S.A.).

RESULTS

PF burst-induced short-term depression is receptor-driven endocannabinoid release.

Several studies demonstrated that brief burst of PF can trigger retrograde inhibition of PF-PC synapse through cannabinoid receptor 1 (CB1R) and mGluR1-dependent pathway (Brenowitz and Regehr, 2005). This phenomenon is termed as receptor-driven endocannabinoid release (RER). To mimic Ca-RER, we made whole-cell patch clamp recordings from cerebellar PCs in cerebellar slices and recorded PF-PC excitatory postsynaptic potentials (EPSPs) every 2s, then applied short burst PF stimulation (100Hz×10) (Fig. 1A). Subsequent EPSCs were depressed transiently (Fig 1B, C, D; 52.05±10.57%, n=5), and this short-term depression was inhibited by 3 μM of cannabinoid receptor 1 blocker AM251 (Fig. 1B, C, D); -11.24±14.45 %, n=5). Furthermore, in another set of experiments, short-term depression (Fig 2; 49.73±25.25 %, n=5) was also inhibited by 100 μM of CPCCOEt, an mGluR inhibitor. (Fig 2; -12.37±17.65 %, n=5). This shows that depression induced by burst stimulation of PF is CB1R and mGluR1 dependent RER

RER is inhibited by SKF96365, a TRP channel blocker.

TRPC is known to mediate slow inward current activated by mGluR1 in cerebellar PC (Kim et al., 2003; Hartmann and Konnerth, 2008). TRPC is calcium-permeable non-selective cation channel. Thus it is possible that TRPC function as a calcium source at calcium dependent cellular events, but it has not been reported about TRPC-mediated calcium transient affecting any synaptic plasticity. To investigate whether TRPC-mediated calcium influx contribute to Ca-RER, we applied 30 μM of SKF96365, a TRP channel inhibitor. The short-term depression (Fig. 3; 38.42 ± 11.10 %, n=5) was inhibited by SKF96365 (Fig 3; -3.95 ± 20.30 %) significantly ($p<0.05$). Since Regehr and his colleagues reported associative stimulation can induce endocannabinoid signaling more effectively (Brenowitz and Regehr, 2005), They suggested that PF burst can make a massive release of glutamate, so that can activate mGluR1 on perisynaptic site of PC dendritic spines and concurrently CF can make global depolarization of PC so that may open VOCC, and increase intracellular calcium concentration of PC. To mimic this associative short-term depression and investigate a role of TRPC in the depression, we made whole-cell patch clamp recordings from cerebellar PCs in cerebellar slices and recorded PF-PC EPSCs every 4s, then applied associative stimulation consist of short burst PF stimulation (100Hz×10) followed by 50ms of depolarization of PC after 50ms (Fig. 4C). The

associative protocol induced short-term depression effectively (Fig. 4C, D; 45.68 ± 12.08 %, n=7, p=0.0000037, ANOVA test), while only depolarization of PC (Fig. 4A, D; 11.42 ± 9.64 %, n = 7) or only PF burst PC failed to induce depression (Fig. 4B, D; 10.34 ± 12.84 %, n = 7). This data is consistent with previous report that showed efficiencies of induction of PF burst, CF activation and association of both of them, although we replaced CF stimulation to depolarization of PC (Brenowitz and Regehr, 2005). In another data set of experiment, the associative protocol induced short-term depression effectively (Fig 5. B, C, D; 50.03 ± 14.84 %, n=7), and SKF96365 decreased this depression partially (Fig. 5 B, C, D; 14.69 ± 11.72 %, n=7) again.

SKF96365 decreased calcium transients during induction of short-term depression

To investigate an effect of TRPC to calcium influx during induction of short-term depression, we made whole-cell patch clamp and confocal calcium imaging. The PCs were filled with low affinity calcium indicator Fluo-5F to measure calcium concentration (Fig. 6A). Low affinity calcium indicator allowed detecting micromolar range of calcium with a high signal-to-noise ratio without overwhelming the calcium-binding buffer capacity of the PC. The calcium transients were recorded in the dendrite of the same PCs in

previous experiments (Fig. 5) during associative stimulation for induction of endocannabinoid-mediated short-term depression. The calcium transients were decreased by SKF96365, (Fig. 6 B, C; 9.12±2.23% to 6.40±2.05%, n=7). To make it clear that the effect of SKF96365 on calcium transient is not caused by a non-specific effect of SKF96365 on voltage operative calcium channels, we measured calcium fluorescence applying 50 ms of depolarization, which activates only VOCC. SKF96365 did not affect depolarization-evoked calcium signal (Fig. 6D). This means SKF96365 interfere calcium transient of PC during induction of the short-term depression, suggesting TRPC-mediated calcium influx contribute to endocannabinoid signaling.

TRPC specific blocker in concentration that block TRPC-mediated slow current did not affect RER

Although we used SKF96365 as TRP channel blocker, others reported that SKF96365 also has non-specific activity (Merritt et al., 1990; Singh et al., 2010). Therefore, to investigate whether TRPC-mediated calcium influx contribute to RER, we had to confirm that TRPC contributed to short-term plasticity. By using BTP2, an alternative TRPC inhibitor which is known to be more specific. One μM of BTP2 blocked TRPC-induced current mostly at cultured cells in previous studies (He et al., 2005), however, the

concentration of BTP2 can block TRPC effectively in slice hasn't been reported. To figure out optimum concentration of BTP2 to block slow current in brain slice, we induced TRPC-mediated slow current with PF burst (100Hz×10 times) applying NBQX (2.5 μM) to block AMPAR current (Fig. 7A), and examined concentration-dependent effect of BTP2 on amplitude of the slow current. Fifty μM of BTP2 blocked the slow current in slice, while 25 μM or lower concentration of BTP2 couldn't (Fig. 7B). Thus, we investigated whether 50μM of BTP2 could change the amplitude of transient depression of EPSC in cerebellar slice. In cells which showed the depression by 10 times of PF burst, 50μM of BTP2 didn't affect the amplitude of transient depression neither in the current clamp mode (Fig. 7 C, D), nor in the voltage clamp mode (Fig. 7 E, F) although it was enough to block TRPC-mediated slow current almost totally. We also performed calcium imaging using fluo-5F, but calcium transients during the induction protocol were not different between control and the BTP2-treated group significantly (Fig. 8). This data show TRPC-mediated slow current doesn't contribute to RER by associative protocol we used and calcium influx through TRPC is overwhelmed by calcium influx through VOCC or calcium release from intracellular calcium store.

RER was not inhibited by t-type calcium channel blocker

SKF96365 did block RER and diminish calcium transients, but data from more specific TRPC blocker BTP2 was not consistent with that. It is more likely that this inconsistence is from non-specificity of SKF96365 because BTP2 had no effect on RER although we confirmed BTP2 could block TRPC-mediated slow current mostly.

SKF96365 can inhibit voltage operated calcium channels. P/Q type calcium channel is the major high-voltage activated VOCC in PC, so it is less likely that SKF96365 inhibited P/Q type calcium channels because SKF96365 did not affect depolarization-induced calcium increase in PC. T-type calcium channels express in PCs (Isope et al., 2010; Gugger et al., 2012) and SKF96365 is known to inhibit T-type calcium channel (Singh et al., 2010). T-type calcium channel is low-voltage activated calcium channel, so there is a possibility that depolarization to 0mV didn't show contribution of t-type calcium channel effectively. To investigate this possibility, we induced short-term depression like we performed previously on current clamp mode and applied 20μM of mibefradil, a T-type calcium channel blocker. However, mibefradil didn't affect the short-term depression (Fig. 9), indicating T-type calcium channel doesn't contribute to RER and non-specific action of SKF96365 was not on T-type calcium channel.

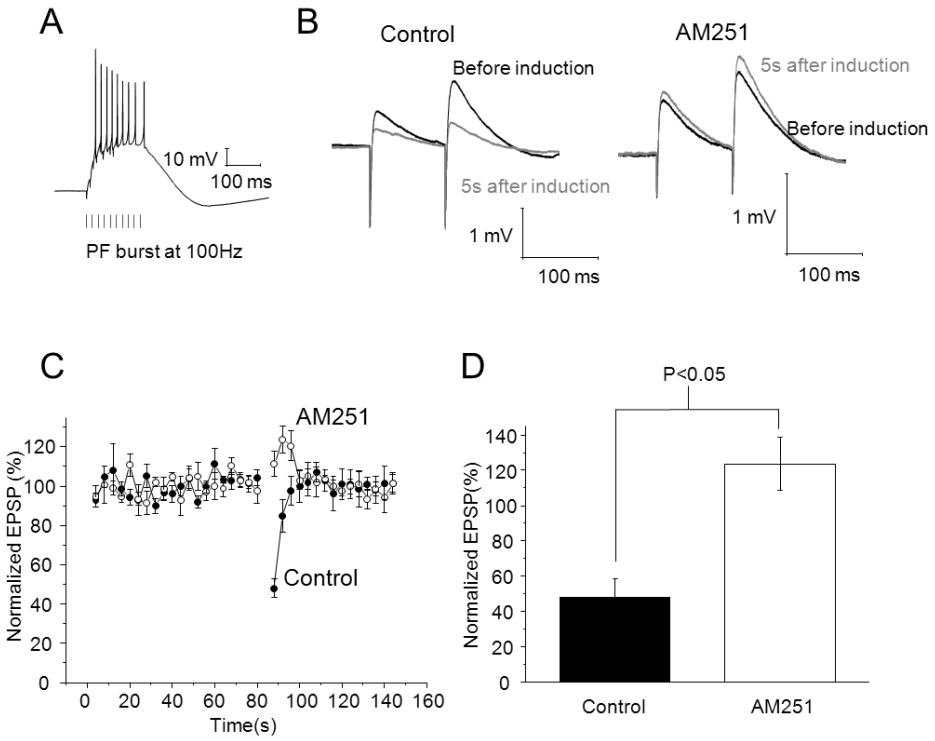

Fig. 1. Endocannabinoid-mediated short-term depression induced by PF burst in cerebellar Purkinje cell.

(A) The induction protocol for transient depression and trace in induction. Ten PF stimuli were applied at 100 Hz on the current clamp mode. (B) EPSPs before and 5 s after induction. (Left: control, Right: 3 μM of AM251 applied, Black: before induction, Grey: 5 s after induction) (C) EPSPs were evoked by single pulse of PF stimulus during a 80 seconds baseline and for 60 seconds and after induction of transient depression. After that, 3 μM of AM251 was applied for 10 minutes, and then recording of baseline and

induction of transient depression were established in the same cell. Amplitudes of EPSPs were normalized to baseline before and after application of AM251. (D) Summary bar graphs showed a decreased depression of EPSPs after application of AM251.

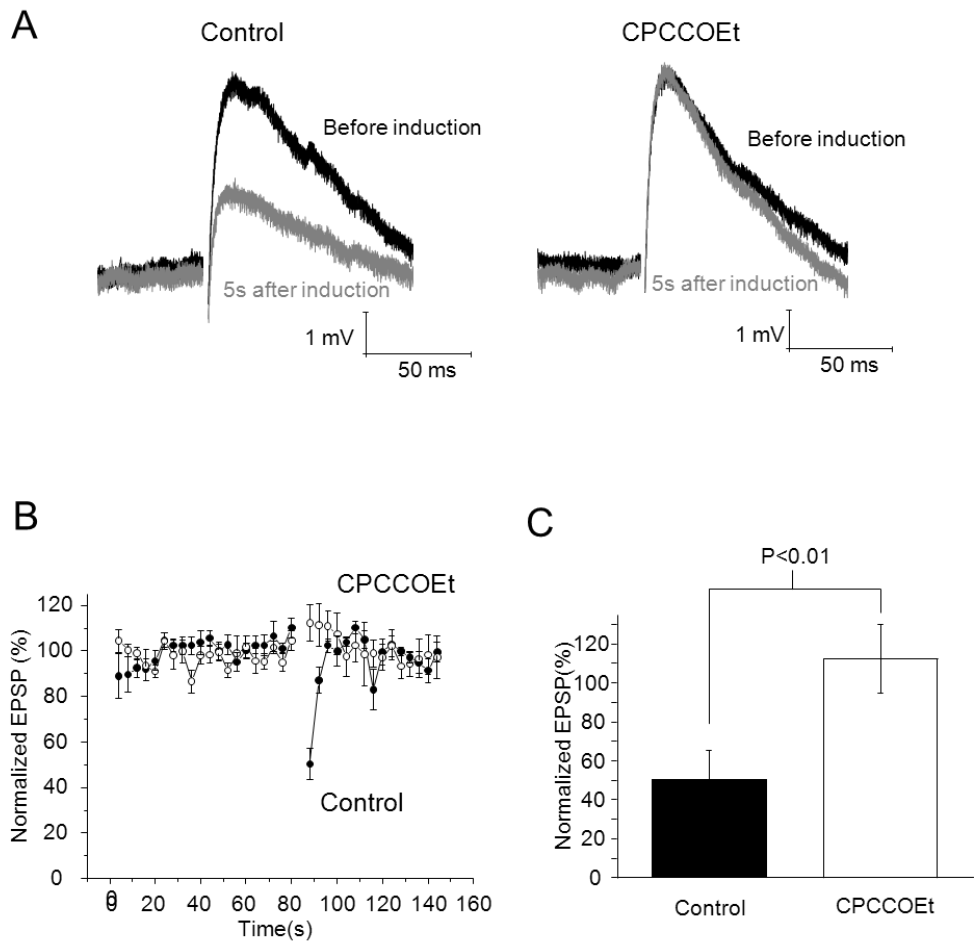

Fig. 2. PF burst-induced endocannabinoid-mediated short-term depression is mGluR-dependent.

(A) EPSPs before induction and 5 s after induction. (Left: control, Right: 100 μM of CPCCOEt applied, Black: before induction, Grey: 5 s after induction) (B) EPSPs were evoked by single pulse of PF stimulus during a 80 seconds baseline and for 60 seconds after induction of transient depression. After that, 100 μM of CPCCOEt was applied for 10 minutes, and then recording of

baseline and induction of transient depression were established in the same cell. Amplitude of EPSPs was normalized to baseline before and after application of CPCCOEt. (C) Summary bar graphs showed a decreased depression of EPSPs after application of CPCCOEt.

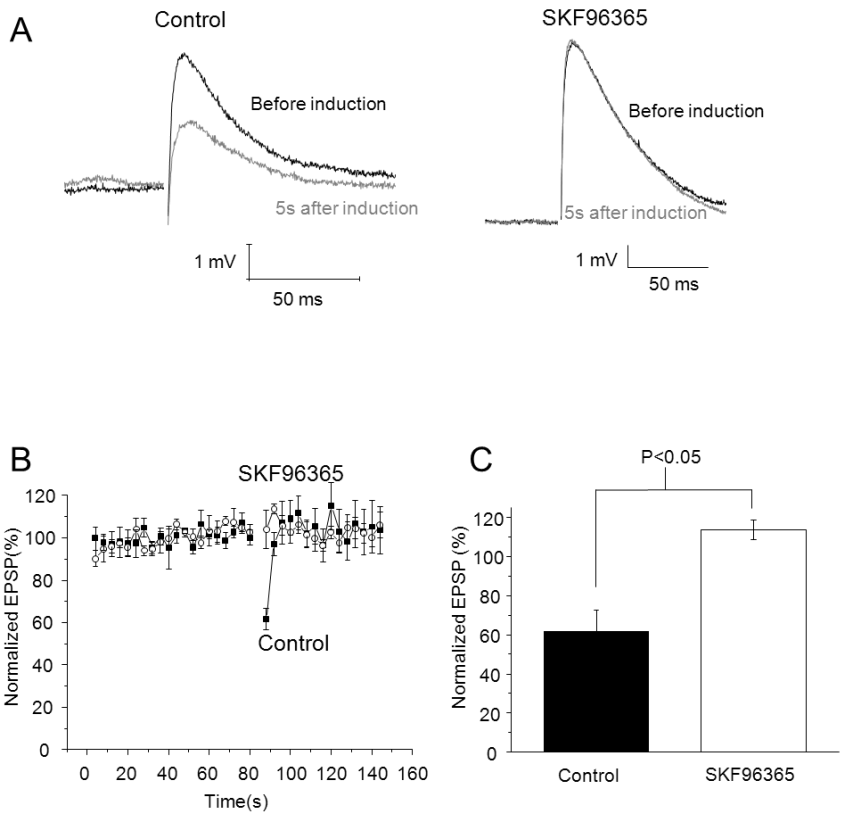

Fig. 3. A TRP channel blocker SKF96365 inhibited endocannabinoid-mediated short-term depression induced by PF burst.

(A) EPSPs before induction and 5 s after induction. (Left: control, Right: 30 μM of SKF96365 applied, Black: before induction, Grey: 5 s after induction) (B) EPSPs were evoked by single pulse of PF stimulus during a 80 seconds baseline and for 60 seconds after induction of transient depression. After that, 30 μM of SKF96365 was applied for 10 minutes, and then recording of baseline and induction of transient depression were established in the same

cell. Amplitude of EPSPs were normalized to baseline before and after application of SKF96365. (C) Summary bar graphs showed a decreased depression of EPSPs after application of SKF96365.

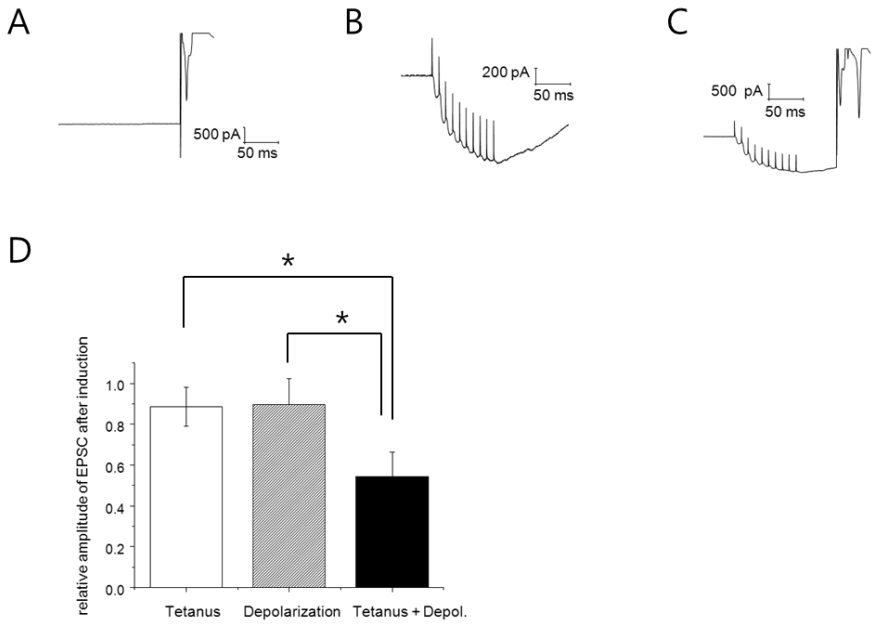

Fig. 4. Induction of Ca-RER with various protocols and the amount of depression in each protocol

(A) The induction protocol for transient depression and current trace during 50 ms of PC depolarization in the voltage clamp mode. (B) The induction protocol for transient depression and current trace with. Ten PF stimuli in the voltage clamp mode. (C) The induction protocol for transient depression and current trace with association of. Ten PF stimuli at 100 Hz and a 50ms of PC depolarization 50 ms after PF burst applied in the voltage clamp mode. (D) Summary bar graphs showed amount of depression with each protocol. *P<0.05

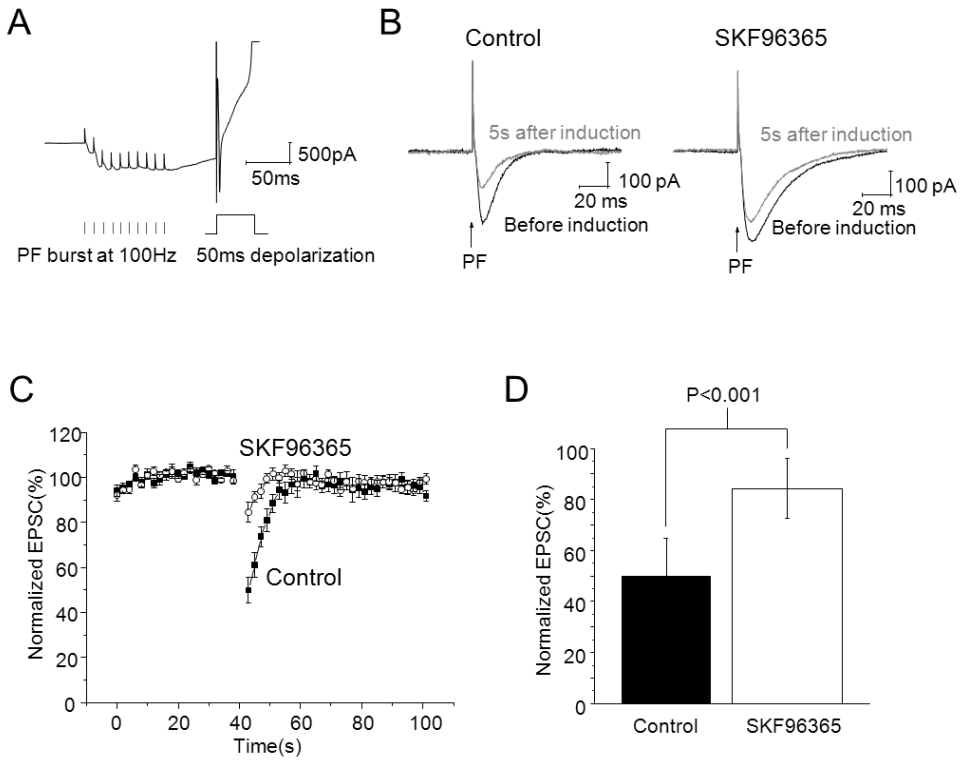

Fig. 5. A TRP channel blocker SKF96365 inhibited endocannabinoid-mediated short-term depression induced by association of PF burst and PC depolarization.

(A) The induction protocol for transient depression and current trace with. Ten PF stimuli at 100 Hz and a 50 ms of PC depolarization 50 ms after PF burst were applied in the voltage clamp mode. (B) EPSCs before induction and 5 s after induction. (Left: control, right: 30 μM of SKF96365 applied, Black: before induction, Grey: 5s after induction) (C) EPSCs were evoked by paired pulse of PF stimulus during a 40 seconds baseline and for 60 seconds after induction of transient depression. After that, 30 μM of SKF96365 was

applied for 10 minutes, and then recording of baseline and induction of transient depression were established in the same cell. Amplitude of EPSCs were normalized to baseline before and after application of SKF96365. (D) Summary bar graphs showed change of depression of EPSCs after application of SKF96365.

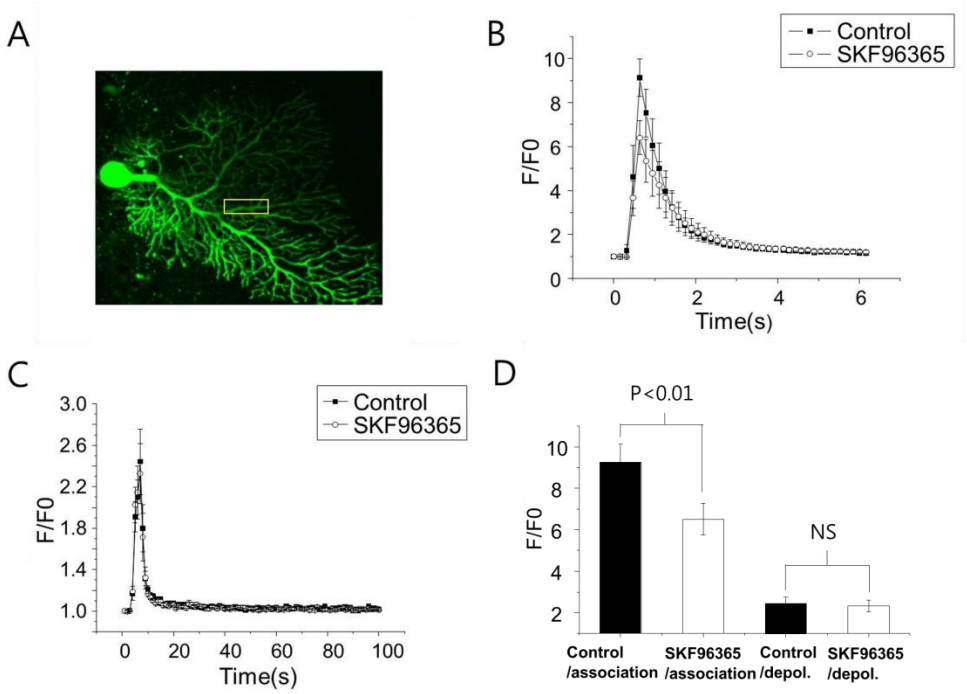

Fig. 6. Calcium transient of Purkinje cell dendrite during induction of short-term depression was decreased by SKF96365

(A) The PC was filled with Fluo-5F through a patch pipette. The fluorescence near the stimulating electrode for PF (square) was estimated and analyzed, and the background was substracted. (B) Increase of the fluoroscence during the induction of short-term-depression before and after application of SKF96365. (C) Increase of the fluorescence during 50ms of PC depolarization. (D) Summary bar graphs showed change of fluoroscence after application of SKF96365.

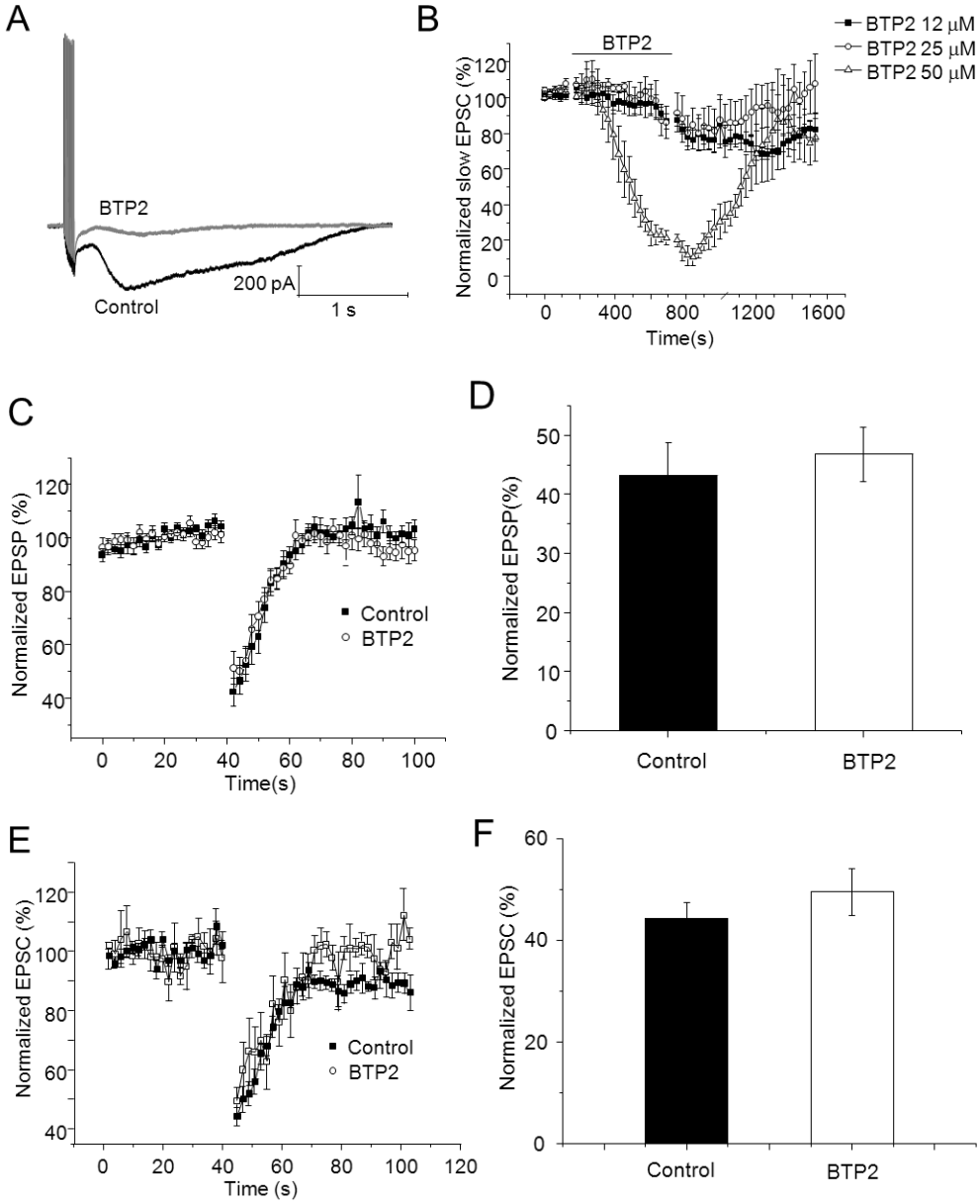
A
BTP2
200 pA
Control
1 s
B
BTP2
BTP2 12 μM
BTP2 25 μM
BTP2 50 μM
Normalized slow EPSC (%)
Time(s)
C
Normalized EPSP (%)
Control
BTP2
Time(s)
D
Normalized EPSP(%)
Control
BTP2
E
Normalized EPSC (%)
Control
BTP2
Time (s)
F
Normalized EPSC (%)
Control
BTP2

Fig. 7. Endocannabinoid-mediated short-term depression in cerebellar Purkinje cell was not inhibited by BTP2.

(A) Slow current traces evoked by 10 PF stimuli at 100 Hz during 5 μM of NBQX. (Black: Control, Grey: 50 μM of BTP2. Slow current was blocked mostly by 50 μM of BTP2. (B) Slow EPSCs were evoked during a 200 seconds baseline and various concentrations of BTP2 were applied for 10 minutes, and then BTP2 was washed out for 15 minutes. (C) EPSPs were evoked by paired pulse of PF stimulus during a 40 seconds baseline and for 60 seconds after induction of transient depression. After that, 50 μM of BTP2 was applied for 10 minutes, and then recording of baseline and induction of transient depression were established in the same cell. Amplitudes of EPSCs were normalized to baseline before and after application of BTP2. (D) Summary bar graphs showing change of depression of EPSPs after application of BTP2. (E) EPSPs were evoked by paired pulse of PF stimulus during a 40 seconds baseline and for 60 seconds after induction of transient depression. After that, 50 μM of BTP2 was applied for 10 minutes, and then recording of baseline and induction of transient depression were established in the same cell. Amplitudes of EPSCs were normalized to baseline before and after application of BTP2. (F) Summary bar graphs showing change of depression of EPSCs after application of BTP2

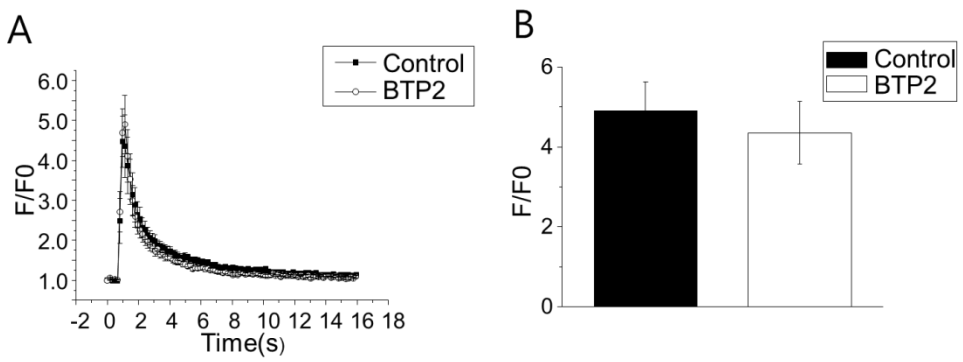

Fig. 8. Calcium transient of Purkinje cell dendrite during induction of short-term depression was not decreased by BTP2.

(A) Increase of the calcium fluoroscence during induction of short-term-depression before and after application of BTP2. (B) Summary bar graphs showed change of fluoroscence after application of BTP2.

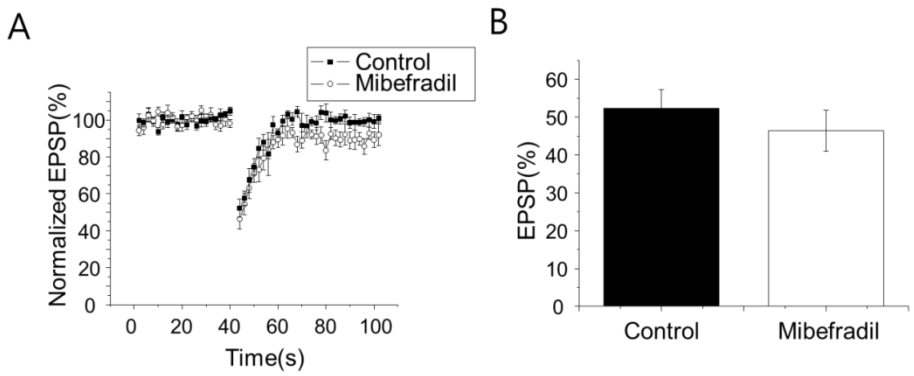

Fig. 9. Endocannabinoid-mediated short-term depression in cerebellar Purkinje cell was not affected by 20 μM of Mibefradil.

(A) EPSPs were evoked by single pulse of PF stimulus during a 40 seconds baseline and for 60 seconds and after induction of transient depression. After that, 20 μM of Mibefradil was applied for 10 minutes, and then recording of baseline and induction of transient depression were established in the same cell. Amplitude of EPSPs were normalized to baseline before and after application of mibefradil. (B) Summary bar graphs showed change of depression of EPSPs after application of mibefradil.

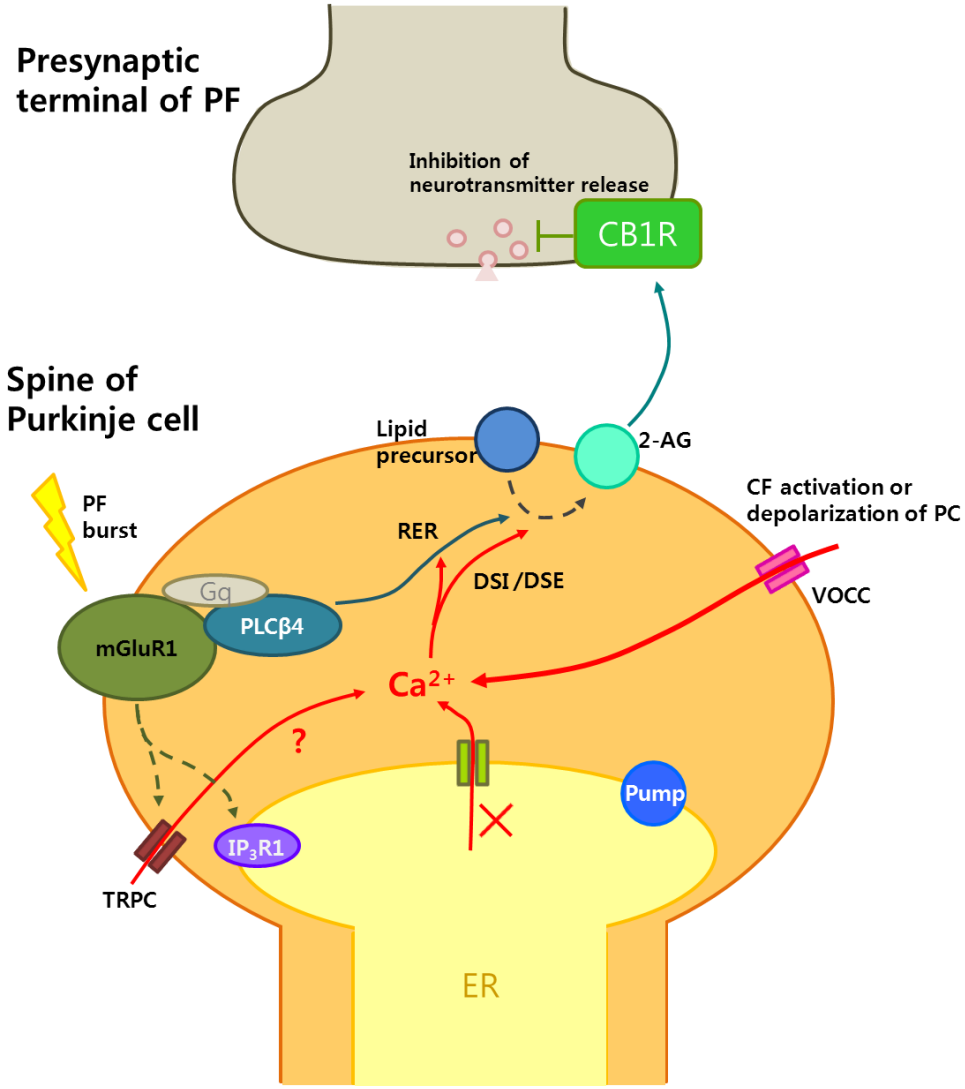

Fig. 10. Summary schematic diagram for role of TRPC-mediated calcium influx in the endocannabinoid release.

A burst of PF induces glutamate spillover, so it can activate mGluR1 on the perisynaptic site of PC. Then PLCβ4 induces RER, which can be assisted by calcium increase in PC. Activation of mGluR can induce TRPC-mediated

calcium influx and IP3-induced calcium release from store contribute to calcium increase, while depolarization of PC activates VOCC, which mediate a very strong calcium influx. It is reported that calcium release from ER calcium store does not contribute to endocannabinoid release, and our data suggest that neither does TRPC.

DISCUSSION

This study investigated whether mGluR1-evoked calcium influx through TRPC channels underlies cerebellar Ca-RER in PF-PC synapses. Pairing brief burst of PF with PC depolarization in voltage clamp mode or brief burst of PF in current clamp mode induced calcium transient and cannabinoid-dependent transient depression. However, this calcium transient and synaptic depression was not inhibited by TRPC channel specific antagonist. These finding suggest that mGluR1-evoked calcium influx does not underlie Ca-RER in cerebellum.

mGluR1-evoked calcium transients and slow EPSCs through parallel fiber burst stimuli

mGluR1 is expressed in cerebellar PCs, and coupled to Gq protein which activate Phospholipase C (PLC)-dependent signaling cascades. Activation of PLC produces IP_3 which binds to IP_3R at ER calcium store and leads to calcium release from the calcium store. Also, activation of mGluR evoked slow currents and calcium influx through TRPC channel. Calcium release from intracellular calcium store is required for coupling mGluR activation to

the opening of TRP channel in other cell, however, mGluR-evoked calcium transients and slow EPSCs were not blocked in the PC even if intracellular calcium stores were depleted by CPA (SJ Ahn, 2012). Furthermore, in TRPC3 knock-out mice, slow EPSCs and calcium transients through the brief burst of PF stimuli are impaired although the stores are unchanged (Hartmann et al., 2008). These suggest PF burst stimuli we applied induced TRPC-mediated slow EPSCs and calcium transient.

TRPC is not an important calcium source for endocannabinoid release

The role of voltage operated calcium channel or ER calcium store as calcium source for endocannabinoid release is well known already, while that of calcium influx through TRPC is unclear (Fig. 10). Calcium influx through VOCC is large enough to make an endocannabinoid release by itself or with activation of mGluR, but when VOCC is not activated enough to induce DSI/DSE or Ca-RER – when only PFs burst or climbing fiber is associated weakly with PF burst – TRPC can be an important calcium source for endocannabinoid release. Moreover, the brief burst of PF and climbing fiber stimuli trigger supralinear calcium increases in the PC and we observed that this supralinear calcium increases is diminished by SKF96365, TRP channel blocker. This finding support the possibility that TRPCs act a role as an co-incidence detector increasing calcium concentration supralinearly when PF

and CF activate simultaneously, in spite of relatively low calcium conductance of TRPC. We investigated a possibility that TRPC plays a role as a novel calcium source other than VOCC or ER calcium store in Ca-RER. However, TRPC specific blocker BTP2 did not affect endocannabinoid release. It was also revealed previously that ER calcium store has little effect on elevation of calcium concentration and endocannabinoid release (Brenowitz and Regehr, 2005). After all of these findings, there is quite a possibility that Voltage operated calcium channel act as a critical and sole calcium source. We could not induce transient depression by only PF burst on voltage clamp mode (-70 mV), unlike associative stimulation or PF burst on current clamp mode, which VOCC can activate by; This data is consistent with the idea that VOCC is most important calcium source for endocannabinoid release.

The role of TRPC for cerebellar LTD

We revealed that TRPC has an important role in induction of cerebellar LTD (SJ Ahn, 2012) recently. We observed that activation of TRPC contributes to induction of cerebellar LTD, increasing intracellular calcium concentration supralinearly. Induction of cerebellar LTD and Ca-RER is quite similar – both of them need activation of mGluR by PF burst and activation of VOCC by postsynaptic depolarization while TRPC has an important role for only

cerebellar LTD. Though TRPC is not involved in Ca-RER, it might suggest TRPC has a partial role for distinction between a strong input which is supposed to induce a long-term change and a weak input that is not enough to make a long-term change.

Non-specific action of SKF96365

SKF93635 is well known for TRP channel blocker, but several nonspecific actions have been reported. SKF96365 is known to elevate intracellular calcium ion level, (Harper and Daly, 2000) and inhibit voltage dependent calcium channels (Ciardo and Meldolesi, 1990; Merritt et al., 1990) – especially T type calcium channel in cerebellar PC (Singh et al., 2010), potassium channel (Alvarez et al., 1992; Schwarz et al., 1994), cytochrome P450 (Rodrigues et al., 1987; Capdevila et al., 1988), and calcium ATPase (Ciardo and Meldolesi, 1990; Mason et al., 1993). We observed that SKF96365 attenuated calcium increase during induction of RER, but not during just 50ms depolarization, so we presumed the SKF96365 inhibited endocannabinoid release, reducing postsynaptic calcium increase. However, BTP2, specific blocker of TRP channel did not affect RER, thus the action of SKF96365 seemed to be through some nonspecific effect.

Effective concentration and specificity of BTP2 in brain slice

SKF96365 have been used as TRP channel blocker so far, but now they are known to have nonspecific effect on ER calcium store or channels other than TRPC. BTP2 has been known to function as a SOC/CRAC channel blocker, (Ishikawa et al., 2003; Zitt et al., 2004) and inhibit TRPC channel in HEK293 cells specifically. BTP2 block TRPC at about 1μM almost fully, and had little effect on ER, K+ channel or VOCC in cultured single cell. However, a concentration of BTP2 that block TRPC effectively in brain slice has not been reported yet. We found that 50 μM of BTP2 is needed to block the slow current from data we obtained; it is much larger than the concentration that block TRPC in cultured single cell, and specificity of BTP2 to TRPC at this concentration is not clear yet. However, based on the facts that BTP2 didn't affect to endocannabinoid release, we conclude that TRPC doesn't contribute to endocannabinoid release although 50μM of BTP2 may be nonspecific. But a specificity of BTP2 may be a important issue for further investigation about TRPC hence more examination about the specificity is needed.

A possibility of Calcium saturation by VOCC

Ca-RER can be induced by 1μM or more concentration of calcium. Therefore, if receptor is activated and calcium influx through VOCC is more

than 1μM, Ca-RER can be induced without any other calcium source. During our induction protocol in voltage clamp mode, we applied brief depolarization of PC so that VOCC can be activated. We applied only PF burst stimuli in another induction protocol in current clamp mode, but PCs were depolarized or fired since we did not clamp voltage and VOCC could be activated in this experiment, too. Moreover, burst stimuli of PF activate mGluR-evoked calcium influx and ER calcium release. Therefore, calcium transients that we measured during our induction protocol contain calcium influx through VOCC, TRPC and calcium release from ER. Previously Brenowitz and Regehr (2005) reported that calcium release from ER is not involved in endocannabinoid release, however, TRPC-mediated calcium increase could be overwhelmed by VOCC if our induction protocol activated VOCC enough. Thus, a possibility that TRPC can contribute to endocannabinoid release in the condition that VOCC activate lesser still remains. However, amplitude of endocannabinoid-induced depression increase calcium dependently to almost 80 %, until calcium concentration attain 5 μM (Brenowitz and Regehr, 2005). The extent of attenuation of EPSC we observed was about 50 %, which is not the maximal range of endocannabinoid-mediated depression. It means that calcium increase by VOCC was not saturated for Ca-RER. If TRPC contribute to endocannabinoid release partially, BTP2 should inhibit the depression of

EPSC in spite of VOCC. Therefore, our data suggest TRPC is not involved in endocannabinoid release.

REFERENCES

Aiba, A., M. Kano, C. Chen, M. E. Stanton, G. D. Fox, K. Herrup, T. A. Zwingman and S. Tonegawa (1994). "Deficient cerebellar long-term depression and impaired motor learning in mGluR1 mutant mice." Cell **79**(2): 377-388.

Albus, J. (1971). "A Theory of Cerebellar Function." Math Biosci **10**: 25-61.

Alger, B. E. (2002). "Retrograde signaling in the regulation of synaptic transmission: focus on endocannabinoids." Prog Neurobiol **68**(4): 247-286.

Alvarez, J., M. Montero and J. Garcia-Sancho (1992). "High affinity inhibition of Ca(2+)-dependent K+ channels by cytochrome P-450 inhibitors." J Biol Chem **267**(17): 11789-11793.

Bliss, T. V. and A. R. Gardner-Medwin (1973). "Long-lasting potentiation of synaptic transmission in the dentate area of the unanaestetized rabbit following stimulation of the perforant path." J Physiol **232**(2): 357-374.

Brenowitz, S. D. and W. G. Regehr (2005). "Associative short-term synaptic plasticity mediated by endocannabinoids." Neuron **45**(3): 419-431.

Capdevila, J., L. Gil, M. Orellana, L. J. Marnett, J. I. Mason, P. Yadagiri and

J. R. Falck (1988). "Inhibitors of cytochrome P-450-dependent arachidonic acid metabolism." Arch Biochem Biophys **261**(2): 257-263.

Carlson, G., Y. Wang and B. E. Alger (2002). "Endocannabinoids facilitate the induction of LTP in the hippocampus." Nat Neurosci **5**(8): 723-724.

Chevaleyre, V. and P. E. Castillo (2003). "Heterosynaptic LTD of hippocampal GABAergic synapses: a novel role of endocannabinoids in regulating excitability." Neuron **38**(3): 461-472.

Chevaleyre, V., K. A. Takahashi and P. E. Castillo (2006). "Endocannabinoid-mediated synaptic plasticity in the CNS." Annu Rev Neurosci **29**: 37-76.

Chuang, S. C., R. Bianchi and R. K. Wong (2000). "Group I mGluR activation turns on a voltage-gated inward current in hippocampal pyramidal cells." J Neurophysiol **83**(5): 2844-2853.

Ciardo, A. and J. Meldolesi (1990). "Multiple actions of SC 38249: the blocker of both voltage-operated and second messenger-operated Ca2+ channels also inhibits Ca2+ extrusion." Eur J Pharmacol **188**(6): 417-421.

Conn, P. J. and J. P. Pin (1997). "Pharmacology and functions of metabotropic glutamate receptors." Annu Rev Pharmacol Toxicol **37**:

205-237.

Conquet, F., Z. I. Bashir, C. H. Davies, H. Daniel, F. Ferraguti, F. Bordi, K. Franz-Bacon, A. Reggiani, V. Matarese, F. Conde and et al. (1994). "Motor deficit and impairment of synaptic plasticity in mice lacking mGluR1." Nature **372**(6503): 237-243.

Crepel, F., H. Daniel, N. Hemart and D. Jaillard (1991). "Effects of ACPD and AP3 on parallel-fibre-mediated EPSPs of Purkinje cells in cerebellar slices in vitro." Exp Brain Res **86**(2): 402-406.

Finch, E. A. and G. J. Augustine (1998). "Local calcium signalling by inositol-1,4,5-trisphosphate in Purkinje cell dendrites." Nature **396**(6713): 753-756.

Gao, Y., D. V. Vasilyev, M. B. Goncalves, F. V. Howell, C. Hobbs, M. Reisenberg, R. Shen, M. Y. Zhang, B. W. Strassle, P. Lu, L. Mark, M. J. Piesla, K. Deng, E. V. Kouranova, R. H. Ring, G. T. Whiteside, B. Bates, F. S. Walsh, G. Williams, M. N. Pangalos, T. A. Samad and P. Doherty (2010). "Loss of retrograde endocannabinoid signaling and reduced adult neurogenesis in diacylglycerol lipase knock-out mice." J Neurosci **30**(6): 2017-2024.

Gugger, O. S., J. Hartmann, L. Birnbaumer and J. P. Kapfhammer (2012). "P/Q-type and T-type calcium channels, but not type 3 transient receptor potential cation channels, are involved in inhibition of

dendritic growth after chronic metabotropic glutamate receptor type 1 and protein kinase C activation in cerebellar Purkinje cells." Eur J Neurosci **35**(1): 20-33.

Hampson, R. E. and S. A. Deadwyler (1998). "Role of cannabinoid receptors in memory storage." Neurobiol Dis **5**(6 Pt B): 474-482.

Harper, J. L. and J. W. Daly (2000). "Effect of calmidazolium analogs on calcium influx in HL-60 cells." Biochem Pharmacol **60**(3): 317-324.

Hartmann, J., E. Dragicevic, H. Adelsberger, H. A. Henning, M. Sumser, J. Abramowitz, R. Blum, A. Dietrich, M. Freichel, V. Flockerzi, L. Birnbaumer and A. Konnerth (2008). "TRPC3 channels are required for synaptic transmission and motor coordination." Neuron **59**(3): 392-398.

Hartmann, J. and A. Konnerth (2008). "Mechanisms of metabotropic glutamate receptor-mediated synaptic signaling in cerebellar Purkinje cells." Acta Physiol (Oxf).

Hashimotodani, Y., T. Ohno-Shosaku, H. Tsubokawa, H. Ogata, K. Emoto, T. Maejima, K. Araishi, H. S. Shin and M. Kano (2005). "Phospholipase Cbeta serves as a coincidence detector through its Ca2+ dependency for triggering retrograde endocannabinoid signal." Neuron **45**(2): 257-268.

He, L. P., T. Hewavitharana, J. Soboloff, M. A. Spassova and D. L. Gill

(2005). "A functional link between store-operated and TRPC channels revealed by the 3,5-bis(trifluoromethyl)pyrazole derivative, BTP2." J Biol Chem **280**(12): 10997-11006.

Inoue, T., K. Kato, K. Kohda and K. Mikoshiba (1998). "Type 1 inositol 1,4,5-trisphosphate receptor is required for induction of long-term depression in cerebellar Purkinje neurons." J Neurosci **18**(14): 5366-5373.

Ishikawa, J., K. Ohga, T. Yoshino, R. Takezawa, A. Ichikawa, H. Kubota and T. Yamada (2003). "A pyrazole derivative, YM-58483, potently inhibits store-operated sustained Ca2+ influx and IL-2 production in T lymphocytes." J Immunol **170**(9): 4441-4449.

Isope, P., M. E. Hildebrand and T. P. Snutch (2010). "Contributions of T-Type Voltage-Gated Calcium Channels to Postsynaptic Calcium Signaling within Purkinje Neurons." Cerebellum.

Ito, M. (1972). "Neural design of the cerebellar motor control system." Brain Res **40**(1): 81-84.

Kim, S. J., Y. S. Kim, J. P. Yuan, R. S. Petralia, P. F. Worley and D. J. Linden (2003). "Activation of the TRPC1 cation channel by metabotropic glutamate receptor mGluR1." Nature **426**(6964): 285-291.

Konnerth, A., I. Llano and C. M. Armstrong (1990). "Synaptic currents in cerebellar Purkinje cells." Proc Natl Acad Sci U S A **87**(7): 2662-

2665.

Kreitzer, A. C. and W. G. Regehr (2001). "Retrograde inhibition of presynaptic calcium influx by endogenous cannabinoids at excitatory synapses onto Purkinje cells." Neuron **29**(3): 717-727.

Linden, D. J. and J. A. Connor (1991). "Participation of postsynaptic PKC in cerebellar long-term depression in culture." Science **254**(5038): 1656-1659.

Llinas, R. and M. Sugimori (1980). "Electrophysiological properties of in vitro Purkinje cell somata in mammalian cerebellar slices." J Physiol **305**: 171-195.

Maejima, T., K. Hashimoto, T. Yoshida, A. Aiba and M. Kano (2001). "Presynaptic inhibition caused by retrograde signal from metabotropic glutamate to cannabinoid receptors." Neuron **31**(3): 463-475.

Maejima, T., S. Oka, Y. Hashimotodani, T. Ohno-Shosaku, A. Aiba, D. Wu, K. Waku, T. Sugiura and M. Kano (2005). "Synaptically driven endocannabinoid release requires Ca2+-assisted metabotropic glutamate receptor subtype 1 to phospholipase Cbeta4 signaling cascade in the cerebellum." J Neurosci **25**(29): 6826-6835.

Mason, M. J., B. Mayer and L. J. Hymel (1993). "Inhibition of Ca2+ transport pathways in thymic lymphocytes by econazole, miconazole,

and SKF 96365." Am J Physiol **264**(3 Pt 1): C654-662.

Merritt, J. E., W. P. Armstrong, C. D. Benham, T. J. Hallam, R. Jacob, A. Jaxa-Chamiec, B. K. Leigh, S. A. McCarthy, K. E. Moores and T. J. Rink (1990). "SK&F 96365, a novel inhibitor of receptor-mediated calcium entry." Biochem J **271**(2): 515-522.

Nakanishi, S. (1994). "Metabotropic glutamate receptors: synaptic transmission, modulation, and plasticity." Neuron **13**(5): 1031-1037.

Ohno-Shosaku, T., Y. Hashimotodani, T. Maejima and M. Kano (2005). "Calcium signaling and synaptic modulation: regulation of endocannabinoid-mediated synaptic modulation by calcium." Cell Calcium **38**(3-4): 369-374.

Pitler, T. A. and B. E. Alger (1994). "Depolarization-induced suppression of GABAergic inhibition in rat hippocampal pyramidal cells: G protein involvement in a presynaptic mechanism." Neuron **13**(6): 1447-1455.

Ravinet Trillou, C., C. Delgorge, C. Menet, M. Arnone and P. Soubrie (2004). "CB1 cannabinoid receptor knockout in mice leads to leanness, resistance to diet-induced obesity and enhanced leptin sensitivity." Int J Obes Relat Metab Disord **28**(4): 640-648.

Rodrigues, A. D., G. G. Gibson, C. Ioannides and D. V. Parke (1987). "Interactions of imidazole antifungal agents with purified cytochrome P-450 proteins." Biochem Pharmacol **36**(24): 4277-4281.

Rumsey, C. C. and L. F. Abbott (2006). "Synaptic democracy in active dendrites." J Neurophysiol **96**(5): 2307-2318.

Safo, P. and W. G. Regehr (2008). "Timing dependence of the induction of cerebellar LTD." Neuropharmacology **54**(1): 213-218.

Sakurai, M. (1990). "Calcium is an intracellular mediator of the climbing fiber in induction of cerebellar long-term depression." Proc Natl Acad Sci U S A **87**(9): 3383-3385.

Salin, P. A., R. C. Malenka and R. A. Nicoll (1996). "Cyclic AMP mediates a presynaptic form of LTP at cerebellar parallel fiber synapses." Neuron **16**(4): 797-803.

Schwarz, G., G. Droogmans and B. Nilius (1994). "Multiple effects of SK&F 96365 on ionic currents and intracellular calcium in human endothelial cells." Cell Calcium **15**(1): 45-54.

Shigemoto, R., T. Abe, S. Nomura, S. Nakanishi and T. Hirano (1994). "Antibodies inactivating mGluR1 metabotropic glutamate receptor block long-term depression in cultured Purkinje cells." Neuron **12**(6): 1245-1255.

Singh, A., M. E. Hildebrand, E. Garcia and T. P. Snutch (2010). "The transient receptor potential channel antagonist SKF96365 is a potent blocker of low-voltage-activated T-type calcium channels." Br J Pharmacol **160**(6): 1464-1475.

SJ Ahn, H. C., YH Hong, WS Chang, J Kim, SJ Kim (2012). "TRPCs regulate the induction of cerebellar long-term depression." Journal of Neuroscience **Accepted in June**.

Stanton, P. K. and T. J. Sejnowski (1989). "Associative long-term depression in the hippocampus induced by hebbian covariance." Nature **339**(6221): 215-218.

Takechi, H., J. Eilers and A. Konnerth (1998). "A new class of synaptic response involving calcium release in dendritic spines." Nature **396**(6713): 757-760.

Tempia, F., M. C. Miniaci, D. Anchisi and P. Strata (1998). "Postsynaptic current mediated by metabotropic glutamate receptors in cerebellar Purkinje cells." J Neurophysiol **80**(2): 520-528.

Wagner, J. J. and B. E. Alger (1996). "Increased neuronal excitability during depolarization-induced suppression of inhibition in rat hippocampus." J Physiol **495 (Pt 1)**: 107-112.

Yoshida, T., K. Hashimoto, A. Zimmer, T. Maejima, K. Araishi and M. Kano (2002). "The cannabinoid CB1 receptor mediates retrograde signals for depolarization-induced suppression of inhibition in cerebellar Purkinje cells." J Neurosci **22**(5): 1690-1697.

Zimmer, A., A. M. Zimmer, A. G. Hohmann, M. Herkenham and T. I. Bonner (1999). "Increased mortality, hypoactivity, and hypoalgesia in

cannabinoid CB1 receptor knockout mice." Proc Natl Acad Sci U S A **96**(10): 5780-5785.

Zitt, C., B. Strauss, E. C. Schwarz, N. Spaeth, G. Rast, A. Hatzelmann and M. Hoth (2004). "Potent inhibition of Ca2+ release-activated Ca2+ channels and T-lymphocyte activation by the pyrazole derivative BTP2." J Biol Chem **279**(13): 12427-12437.

국문 초록

소뇌 대마제제 유발 단기약화 과정에 TRPC 이온통로 전류가 관여하는지에 대한 연구

장원석

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의학과 생리학 전공

신경세포의 저분극에 의한 대량의 칼슘 이온 유입 (DSI/DSI)이나 G 단백질 결합수용체의 활성화 (RER)에 의하여 내인성 대마제제가 시냅스 후 신경세포에서 방출되어 시냅스 전 신경세포의 수용체에 역행성으로 작용함으로써 시냅스 전도의 일시적 저하를 유발하는 현상이 보고된바 있다. 그 외에도, 내인성 대마제제는 소량의 칼슘 농도 상승과 G 단백질 결합수용체의 약한 활성화가 동반되어 일어날 때 상승적 작용에 의해 방출되기도 한다 (Ca-RER). Ca-RER은 그리 높은 농도의 칼슘이온을 필요로 하지 않으므로, 전압 의존성 칼슘통로 외에도 인지질 분해효소 C (PLC)의 활성화를 유도함으로써 결과적으로 내인성 대마제제를 방출할 수 있는 칼슘 농도상승의 경로의 존재가 가능하다. 내인성 글루타메이트 수용체의 활성화는 PLC의 활성화뿐만이 아니라 그 자체가 세포내 칼슘 농도를 상승시키는 것으로 알려져 있는데, 이러한 칼슘 농도의 상승 기전으로 크

게 두 가지가 알려져 있다. 비선택적 양이온 통로인 TRPC를 통한 내향성 완만 전류, 그리고 이노시톨-트리스인산 (IP_3)의 유리에 의한 세포 내 칼슘 저장고에서의 칼슘의 방출이 그것이다. 하지만 어떠한 경로의 칼슘이 얼마나 Ca-RER에 기여하는지는 확실치 않으며, 이에 TRPC 이온통로를 통한 칼슘의 유입이 Ca-RER에 영향을 미치는지를 조사하였다. 이를 위해 소뇌 평행섬유의 강축성 자극 및 퍼킨지세포의 저분극 자극을 하여 평행섬유-퍼킨지세포 시냅스의 일시적 저하를 유발하였고, 이 시냅스 저하현상이 대마수용체 및 대사성 글루타메이트 수용체에 의존적임을 관찰하였다. 뿐만 아니라, TRPC 이온통로의 저해제인 SKF96365를 투여하였을 때, 시냅스 저하 및 시냅스 저하 유도 시의 세포내 칼슘 농도 상승이 억제되는 현상을 볼 수 있었다. 그러나, 또 다른 TRPC 이온통로의 선택적 억제제인 BTP2를 투여하였을 때는 시냅스 저하 및 칼슘농도의 상승 모두 변화하지 않았으며, 앞서의 실험에서 SKF96365가 보였던 효과는 SKF96365의 비선택적 작용에 의한 것으로 풀이된다. 따라서 본 연구는 TRPC 이온통로에 의한 칼슘 유입은 소뇌 대마수용체 유발 단기약화과정에 기여하는 역할이 미미함을 시사한다.

주제어: TRPC, 퍼킨지 세포, 대사성 글루타메이트 수용체, 완만 전류, 내인성 대마제제, 시냅스 가소성, 역행성 신호 전달, 수지상 칼슘

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