

## TRPC-Mediated Current Is Not Involved in Endocannabinoid-Induced Short-Term Depression in Cerebellum

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It has been reported that activation of metabotropic glutamate receptor 1 (mGluR1) can mediate endocannabinoid-induced short-term depression of synaptic transmission in cerebellar parallel fiber (PF)-Purkinje cell (PC) synapse. mGluR1 has signaling pathways involved in intracellular calcium increase which may contribute to endocannabinoid release. Two major mGluR1-evoked calcium signaling pathways are known: (1) slow-kinetic inward current carried by transient receptor potential canonical (TRPC) channel which is permeable to  $\text{Ca}^{2+}$ ; (2)  $\text{IP}_3$ -induced calcium release from intracellular calcium store. However, it is unclear how much each calcium source contributes to endocannabinoid signaling. Here, we investigated whether calcium influx through mGluR1-evoked TRPC channel contributes to endocannabinoid signaling in cerebellar Purkinje cells. At first, we applied SKF96365 to inhibit TRPC, which blocked endocannabinoid-induced short-term depression completely. However, an alternative TRP channel inhibitor, BTP2 did not affect endocannabinoid-induced short-term depression although it blocked mGluR1-evoked TRPC currents. Endocannabinoid signaling occurred normally even though the TRPC current was mostly blocked by BTP2. Our data imply that TRPC current does not play an important role in endocannabinoid signaling. We also suggest precaution in applying SKF96365 to inhibit TRP channels and propose BTP2 as an alternative TRPC inhibitor.

**Key Words:** Endocannabinoid, TRPC, Short-term depression, Cerebellar Purkinje cell, BTP2

### INTRODUCTION

Metabotropic glutamate receptors (mGluRs) are a type of G-protein coupled receptor that modulate protein expression, neurotransmission and neuronal excitability throughout the CNS [1-4]. mGluR1 is a member of a Group I mGluR that is expressed in cerebellar Purkinje cells and in many other postsynaptic neurons. Activation of mGluR1 induces several cellular events in cerebellar Purkinje cells like the activation of PLC- $\beta$ - $\text{IP}_3$  signal cascade [5,6] or slow excitatory postsynaptic currents (EPSCs) [7,8]. Slow currents are known to be mediated by transient receptor potential canonical (TRPC) channels [8]. Slow currents are non-selective cation currents which include  $\text{Na}^+$  and  $\text{Ca}^{2+}$  influxes [9] so it may elevate intracellular  $\text{Ca}^{2+}$  concentration in cerebellar Purkinje cells. The function of such slow currents is still unknown, however.

Delta-9-tetrahydrocannabinol ( $\Delta^9$ -THC), the principal psychoactive constituent of marijuana affects brain function by activating the G-protein coupled receptor, cannabinoid receptor-1 (CB1R) [10,11], which is expressed throughout the brain [12]. Two major endogenous ligands of the receptor, 2-arachidonylglycerol (2-AG) and anandamide are known so far [13,14]. Endocannabinoids function as a retrograde messenger that contributes to long-term and short-term plasticity in the CNS [15-17]. It is reported that strong depolarization of postsynaptic neurons [18,19] or administration of mGluR agonists [20,21] can suppress presynaptic transmitter release and diminish EPSCs. It is found that the depression of EPSCs has an endocannabinoid-dependent mechanism and three different models for the mechanism of endocannabinoid release have been suggested. Endocannabinoids are released by large elevation of calcium concentration in the postsynaptic neuron that is usually induced by strong depolarization (depolarization-induced suppression of inhibition/excitation, DSI/DSE), strong activation of G protein-coupled receptors (receptor-driven endocannabinoid release, RER), or simultaneous small elevations of calcium concentration and weak

Received February 28, 2012, Revised March 25, 2012,  
Accepted March 25, 2012

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**ABBREVIATIONS:** PF, parallel fiber; PC, Purkinje cell; EPSC, excitatory postsynaptic current; TRPC channel, transient receptor potential canonical channel; CB1R, cannabinoid receptor-1; mGluR, metabotropic glutamate receptor; DSI/DSE, depolarization-induced suppression of inhibition/excitation; RER, receptor-driven endocannabinoid release; Ca-RER,  $\text{Ca}^{2+}$ -assisted RER; VOCC: voltage-operated calcium channel; ER, endoplasmic reticulum; CF, climbing fiber.

receptor activation ( $\text{Ca}^{2+}$ -assisted RER, Ca-RER) and acts presynaptically to suppress neurotransmitter release. Unlike DSE/DSI, Ca-RER can be induced by weak elevation of calcium, which can be mediated by calcium sources other than the voltage-operated calcium channel (VOCC) like the Endoplasmic reticulum (ER) calcium store or TRPC. It was reported that ER calcium release do not have a role in the induction of Ca-RER [22], indicating it is more worthwhile to investigate the role of TRPC in endocannabinoid release. Here, we investigated whether endocannabinoid-induced retrograde signaling is regulated by the TRPC-induced slow current. We induced transient depression of synaptic transmission by PF burst stimulation in current clamp mode and associative stimulation of parallel fiber (PF) burst and Purkinje cell (PC) depolarization in voltage clamp mode; however, neither was affected by TRPC blockers. This suggests TRPC mediated slow currents and calcium transients do not play an important role in the endocannabinoid signaling and that calcium sources other than TRPC is needed for Ca-RER.

## METHODS

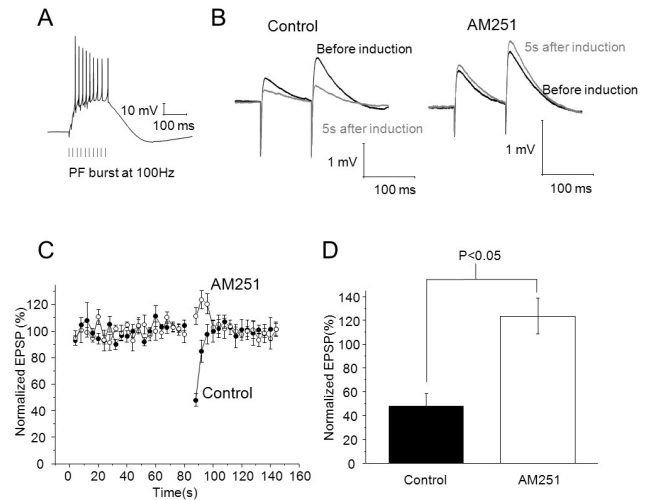
Parasagittal brain slices of the cerebellar vermis (250  $\mu\text{m}$  thick) were prepared from P15-P20 Sprague-Dawley rats using a vibrating tissue slicer and ice-cold standard artificial cerebrospinal fluid (ACSF) containing 124 mM NaCl, 2.5 mM KCl, 1 mM  $\text{Na}_2\text{HPO}_4$ , 1.3 mM  $\text{MgCl}_2$ , 2.5 mM  $\text{CaCl}_2$ , 26.2 mM  $\text{NaHCO}_3$ , and 20 mM D-glucose, bubbled with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ . After cutting, slices were kept for 30 min at 35°C and then for up to 8 hrs. at 25°C in ACSF.

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  $\mu\text{M}$  picrotoxin to block  $\text{GABA}_A$  receptors. Somatic whole-cell recording were obtained by using an EPC8 amplifier (HEKA Instruments Inc., Bellmore, NY). The recording electrodes (resistance 2~4 M $\Omega$ ) were filled with a solution containing 130 mM K-gluconate, 10 mM KCl, 10 mM HEPES, 0.2 EGTA, 10 mM  $\text{Na}_2$ -phosphocreatine, 3 mM  $\text{Mg}_2\text{ATP}$ , 0.3 mM  $\text{Na}_3\text{GTP}$  (pH 7.25) for current-clamp recording and 135 mM Cs-methanesulfonate, 10 mM CsCl, 10 mM HEPES, 4 mM  $\text{Mg}_2\text{ATP}$ , 0.4 mM  $\text{Na}_3\text{GTP}$ , and 0.2 mM EGTA (pH 7.25) for voltage-clamp recordings. Currents were filtered at 1 kHz, digitized at 5 kHz, and acquired using Pulse (HEKA Instruments). For parallel fiber 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  $\mu\text{A}$  pulses (100  $\mu\text{s}$  duration). When burst stimulation was employed, the interpulse interval was 10 ms. Recordings were performed at RT in ACSF.

All group data are shown as mean $\pm$ SEM. Comparisons were made using Student's *t* tests. All drugs were purchased from Sigma (St. Louis, MO) except for CPCCOEt, NBQX, AM251, SKF96365 (Tocris Cookson, Ballwin, MO).

## RESULTS

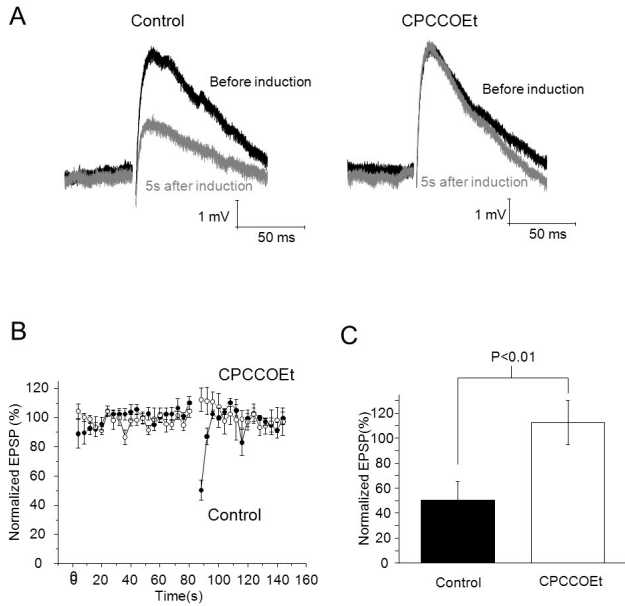
Several studies demonstrated that brief burst of parallel fiber can trigger retrograde inhibition of the PF-PC synapse [16,22]. This phenomenon is termed as Ca-RER and is



**Fig. 1.** PF burst induced Endocannabinoid-mediated short-term depression in cerebellar Purkinje cell. (A) The induction protocol for transient depression and trace in induction. 10 parallel fiber stimuli were applied at 100 Hz in current clamp mode. (B) EPSPs before induction and 5 s after induction. (Top: control, bottom: 3  $\mu\text{M}$  of AM251 applied, Black: before induction, Grey: 5 s after induction) (C) EPSPs were evoked by a single pulse of PF stimulus during an 80 seconds baseline and for 60 seconds after induction of transient depression. After that, 3  $\mu\text{M}$  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 showing change of depression of EPSPs after application of AM251.

mGluR-PLC $\beta$ 4 dependent. To mimic this Ca-RER, we made whole-cell patch clamp recordings from cerebellar Purkinje cells in cerebellar slices and recorded parallel fiber-Purkinje cell (PF-PC) excitatory postsynaptic potentials (EPSPs) every 2 s, then applied short burst PF stimulation (100 Hz $\times$ 10) (Fig. 1A). Subsequent EPSCs were depressed transiently (Fig. 1B, C, D;  $52.05\pm 10.57\%$ ,  $n=5$ ), and this short-term depression was inhibited by 3  $\mu\text{M}$  of cannabinoid receptor 1 blocker AM251 (Fig. 1B, C, D;  $-23.69\pm 15.20\%$ ,  $n=5$ ). Furthermore, in another set of experiments, short-term depression (Fig. 2;  $49.73\pm 25.25\%$ ,  $n=5$ ) was also inhibited by 100  $\mu\text{M}$  of CPCCOEt, an mGluR inhibitor (Fig. 2;  $-12.37\pm 17.65\%$ ,  $n=5$ ). This data shows that the depression induced by burst stimulation of PF is CB1R and mGluR1-dependent RER.

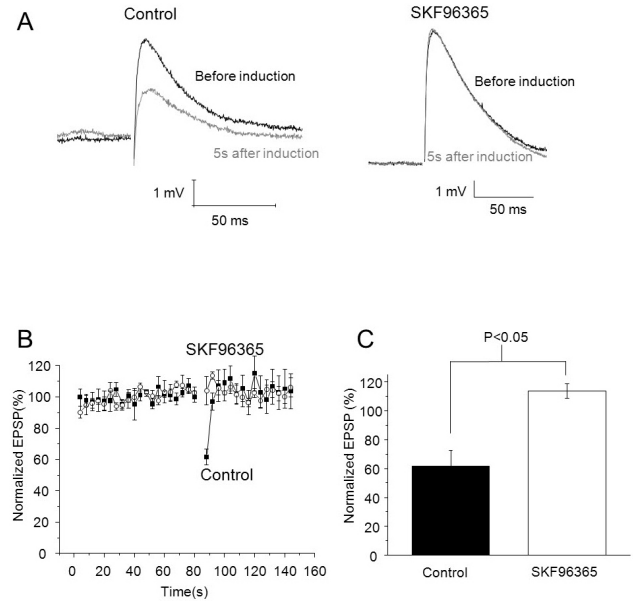
TRPC is known to mediate slow inward currents activated by mGluR1 in cerebellar Purkinje cells [8,23]. TRPC is a calcium-permeable non-selective cation channel, thus it is possible that TRPC functions as a calcium source in calcium-dependent cellular events. To investigate whether TRPC-mediated calcium influx contributes to Ca-RER, we applied SKF96365, a TRP channel inhibitor. The short-term depression (Fig. 3;  $38.42\pm 11.10\%$ ,  $n=5$ ) was inhibited by 30  $\mu\text{M}$  of SKF96365 (Fig. 3;  $-13.62\pm 4.99\%$ ) significantly. Regehr and his coworkers reported that associative stimulation can induce endocannabinoid signaling more effectively [22]. It is suggested that PF burst can cause a massive release of glutamate which can activate mGluR1 on the perisynaptic site of Purkinje cell dendritic spines, and climbing fiber (CF) can make global depolarization of Purkinje cell that may open VOCC, increasing the intra-



**Fig. 2.** PF burst-induced Endocannabinoid-mediated short-term depression is mGluR-dependent. (A) EPSPs before induction and 5s after induction (Top: control, bottom: 100  $\mu$ M of CPCCOEt applied, Black: before induction, Grey: 5 s after induction). (B) EPSPs were evoked by a single pulse of PF stimulus during an 80 seconds baseline and for 60 seconds after induction of transient depression. After that, 100  $\mu$ M of CPCCOEt 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 CPCCOEt. (C) Summary bar graphs showing change of depression of EPSPs after application of CPCCOEt.

cellular calcium concentration of the Purkinje cell. To mimic this associative short-term depression and investigate the role of TRPC in the depression, we made whole-cell patch clamp recordings from cerebellar Purkinje cells in cerebellar slices and recorded PF-PC EPSCs, then applied associative stimulation consisting of short burst PF stimulation (100 Hz $\times$ 10) followed by 50 ms of depolarization of PC after 50 ms (Fig. 4A). The associative protocol induced short-term depression effectively (Fig. 4B, C, D;  $50.03 \pm 14.84\%$ ,  $n=7$ ), and SKF96365 decreased this depression partially (Fig. 4B, C, D;  $14.69 \pm 11.72\%$ ,  $n=7$ ).

Although we observed the effect of a TRP channel blocker, we had to confirm that TRPC specifically contributed to short-term plasticity since SKF96365 is known for its non-specificity [24,25], especially to VOCCs. To investigate whether TRPC-mediated calcium influx contribute to RER, we applied BTP2, another TRPC inhibitor. 1  $\mu$ M of BTP2 blocked most TRPC-induced currents in cultured cells in previous studies [26], however, the concentration of BTP2 to block TRPC effectively in slices has not been reported. To find out the optimum concentration of BTP2 to block slow currents in brain slices, we induced TRPC-mediated slow current with parallel fiber burst (100 Hz $\times$ 10 times) while applying NBQX (2.5  $\mu$ M) to block the overlaying AMPAR current (Fig. 5A), and examined the concentration-dependent effect of BTP2 on the amplitude of the slow current. 50  $\mu$ M of BTP2 blocked the slow current in slice, while 25  $\mu$ M or lower concentrations of BTP2 could

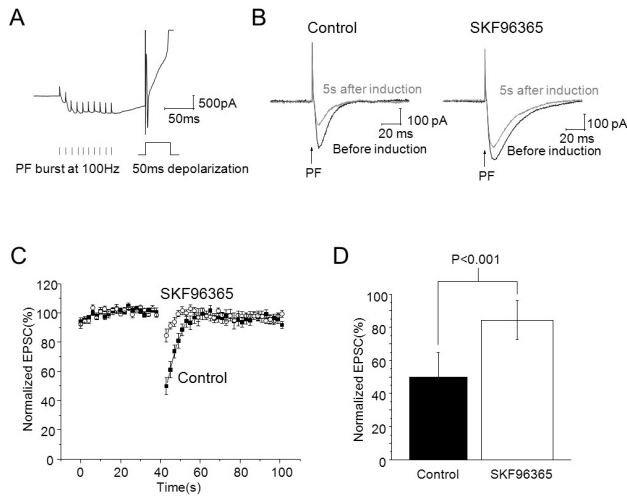


**Fig. 3.** TRP blocker SKF96365 inhibited PF burst-induced Endocannabinoid-mediated short-term depression. (A) EPSPs before induction and 5 s after induction (Top: control, bottom: 30  $\mu$ M of SKF96365 applied, Black: before induction, Grey: 5 s after induction). (B) EPSPs were evoked by a single pulse of PF stimulus during an 80 seconds baseline and for 60 seconds after induction of transient depression. After that, 30  $\mu$ M of SKF96365 was applied for 10 minutes, 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 SKF96365. (C) Summary bar graphs showing change of depression of EPSPs after application of SKF96365.

not (Fig. 5B). Thus, we investigated whether 50  $\mu$ M of BTP2 changed the amplitude of transient depression of EPSC in cerebellar slices. In cells which showed the depression by 10 times of parallel fiber burst on current clamp mode, 50  $\mu$ M of BTP2 did not affect the amplitude of transient depression (Fig. 5C, D), although it was enough to block TRPC-mediated slow current almost completely. Furthermore, BTP2 did not affect short-term depression induced by the associative protocol in the voltage clamp mode (Fig. 5E, F). This data indicates that TRPC-mediated slow current does not contribute to RER by the associative protocol we used and that the calcium influx through TRPC is overwhelmed by the calcium influx through VOCC or calcium release from intracellular calcium stores.

## DISCUSSION

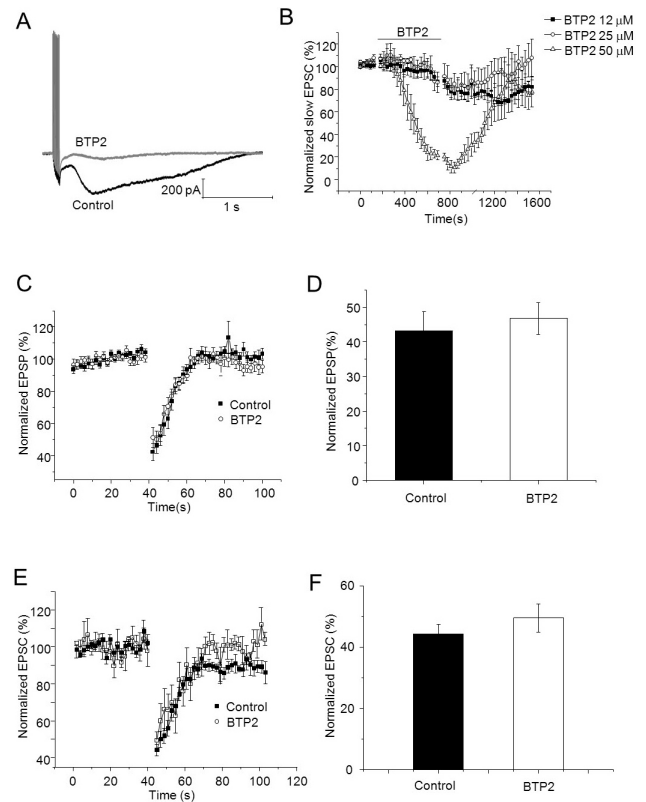
SKF96365 has been reported to elevate intracellular calcium ion levels [27] and inhibit VOCCs [24,28] – especially T-type calcium channels in cerebellar Purkinje cells [25], potassium channels [29,30], cytochrome P450 [31,32], and calcium ATPase [28,33]. SKF96365 blocked the endocannabinoid-induced depression effectively; we assumed the SKF96365 inhibited endocannabinoid release by reducing postsynaptic calcium increase through TRPC. However, BTP2, another blocker of TRP channel did not block RER, even though same concentration of BTP2 could block the



**Fig. 4.** TRP 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 in induction. 10 parallel fiber stimuli at 100 Hz and 50 ms of PC depolarization 50 ms after PF burst were applied in voltage clamp mode. (B) EPSCs before induction and 5 s after induction (Left: control, right: 30  $\mu$ M of SKF96365 applied, Black: before induction, Grey: 5 s after induction). (C) EPSCs were evoked by a paired pulse of PF stimulus during a 40 seconds baseline and for 60 seconds after induction of transient depression. After that, 30  $\mu$ M of SKF96365 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 SKF96365. (D) Summary bar graphs showing change of depression of EPSCs after application of SKF96365.

most of the slow current. This means the action of SKF96365 was a nonspecific effect, although the mechanism by which SKF96365 inhibited RER is unknown. Drugs like SKF96365 or ruthenium red have been used as TRP channel blockers so far, but now they are known to have nonspecific effects on Endoplasmic reticulum (ER) calcium stores or channels other than TRPC. BTP2 has been known to function as a store-operated  $Ca^{2+}$  channel/ $Ca^{2+}$  release-activated  $Ca^{2+}$  channel blocker [34,35], and inhibit overexpressed TRPC channels in HEK293 cells. BTP2 almost fully blocks TRPC at about 1  $\mu$ M, and had little effect on ER,  $K^+$  channel or VOCC in cultured single cell. However, a concentration of BTP2 that blocks TRPC effectively in brain slices has not been reported yet. We found that 50  $\mu$ M of BTP2 is needed to block the slow current; it is much larger than the concentration that blocks TRPC in cultured single cell, and specificity of BTP2 to TRPC at this concentration is not clear yet. However, based on the fact that BTP2 did not affect endocannabinoid release, we conclude that TRPC does not contribute to endocannabinoid release although 50  $\mu$ M of BTP2 may be nonspecific. The results described above suggest that BTP2 can be an alternative or additional option as a pharmacologic blocker of TRP channels.

The role of VOCC or ER calcium stores as a calcium source is well known, while that of calcium influx through TRPC is unclear. 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 did not



**Fig. 5.** 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  $\mu$ M of NBQX (Black: Control, Grey: 50  $\mu$ M of BTP2). (B) Slow EPSCs were evoked during a 200 seconds baseline and for 500 seconds after induction of transient depression application. After that, 50  $\mu$ M of BTP2 was applied for 10 minutes, and then recording of baseline and induction of transient depression were established in the same cell. (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  $\mu$ M 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  $\mu$ M 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.

affect endocannabinoid release. It was also revealed previously that ER calcium store has little effect on the elevation of calcium concentration and endocannabinoid release [22]. Based on these findings, there is a large possibility that VOCC acts as the critical and sole calcium source. We could not induce transient depression by only PF burst on voltage clamp mode at  $-70$  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 the most important calcium source for endocannabinoid release.

Ca-RER can be induced by 1  $\mu$ M or higher concentration of calcium [22]. Therefore, if calcium increase induced by VOCC was more than 1  $\mu$ M and hence sufficient to mediate endocannabinoid release, TRPC-mediated calcium increase could be overwhelmed by the activation of VOCC. Thus, a possibility that TRPC can contribute to endocannabinoid release in the condition that VOCC activates to a lesser degree still remains. However, the amplitude of endocannabinoid-induced depression increases in a calcium dependent manner to almost 80% of control, up to calcium concentrations of 5  $\mu$ M [22]. The amplitude of attenuation of EPSC we observed was about 50%, which is not maximal degree of endocannabinoid-mediated depression. If TRPC contributes to endocannabinoid release, BTP2 should inhibit the depression of EPSC at least partially in spite of VOCC. Therefore, our data suggest TRPC is not involved in endocannabinoid release.

## ACKNOWLEDGEMENTS

This study was supported by grants of Korea Research Foundation funded by the ministry of Education, Science and Technology (No. 2009-0080939), the National Research Foundation of Korea (NRF) grant funded by the Korea government (MESF) (No. 2011-0030737), and Research Program 2011 funded by Seoul National University College of Medicine Research Foundation and Su Foundation.

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