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Miniature Synaptic Events Elicited by Presynaptic Ca^{2+} Rise Are Selectively Suppressed by Cannabinoid Receptor Activation in Cerebellar Purkinje Cells

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Activation of cannabinoid receptors suppresses neurotransmitter release in various brain regions. In cerebellar Purkinje cells (PCs), cannabinoid agonists suppress both EPSC and IPSC evoked by stimulating the corresponding inputs. However, cannabinoid agonists suppress miniature IPSC (mIPSC) but not miniature EPSC (mEPSC) at normal external Ca^{2+} concentration ($[Ca^{2+}]_o$). Therefore, cannabinoid agonists are thought to suppress release machinery for IPSCs but not that for EPSCs. Here we investigated the possible cause of this difference and found that cannabinoid agonists selectively suppressed Ca^{2+}-enhanced miniature events. A cannabinoid agonist, WIN55,212-2 (5 μM), did not affect mEPSC frequency with 2 mM extracellular Ca^{2+} ($Ca^{2+}{}_o$). However, WIN55,212-2 became effective when mEPSC frequency was enhanced by elevation of presynaptic Ca^{2+} level by perfusion with 5 mM $Ca^{2+}{}_o$ or bath application of A23187, a Ca^{2+} ionophore. In contrast, WIN55,212-2 suppressed mIPSC frequency with 2 mM $Ca^{2+}{}_o$, but it became ineffective when the presynaptic Ca^{2+} level was lowered by perfusion with a Ca^{2+}-free solution containing BAPTA-AM. Experiments with systematic $[Ca^{2+}]_o$ changes revealed that mIPSC but not mEPSC regularly involved events elicited by presynaptic Ca^{2+} rise with 2 mM $Ca^{2+}{}_o$. Importantly, Ca^{2+}-enhancement of mEPSC and mIPSC was not attributable to activation of voltage-dependent Ca^{2+} channels. Activation of $GABA_B$ receptor or group III metabotropic glutamate receptor, which couple to $G_{i/o}$-protein, also preferentially suppressed Ca^{2+}-enhanced miniature events in PCs. These results suggest that the occurrence of Ca^{2+}-enhanced miniature events at normal $[Ca^{2+}]_o$ determines the sensitivity to the presynaptic depression mediated by cannabinoid receptors and other $G_{i/o}$-coupled receptors in PCs.

Key words: **cannabinoid receptor; presynaptic modulation; mEPSC; mIPSC; Purkinje cell; cerebellum**

Introduction

Endogenous cannabinoids (endocannabinoids) are released from postsynaptic neurons, activate presynaptic cannabinoid receptors, and suppress transmitter release in various brain regions (Maejima et al., 2001a; Alger, 2002; Wilson and Nicoll, 2002; Piomelli, 2003; Kano et al., 2004). Although conditions required for endocannabinoid release have been studied intensively (Kreitzer and Regehr, 2001; Maejima et al., 2001b; Ohno-Shosaku et al., 2001; Varma et al., 2001; Wilson and Nicoll, 2001; Ohno-Shosaku et al., 2002; Hashimotodani et al., 2005; Maejima et al., 2005), precise mechanisms underlying the suppression of transmitter release remain to be determined. Cannabinoid agonists are reported to suppress the frequency of miniature events at some synapses (Llano et al., 1991; Misner and Sullivan, 1999; Sullivan, 1999; Takahashi and Linden, 2000; Morisset and Urban, 2001; Robbe et al., 2001; Diana et al., 2002; Trettel and Levine, 2002; Diana and Marty, 2003; Derbenev et al., 2004) but not at others (Pitler and Alger, 1994; Alger et al., 1996; Hajos et al., 2000; Takahashi and Linden, 2000; Huang et al., 2001; Katona et al., 2001; Gubellini et al., 2002). Changes in the miniature frequency are generally thought to reflect modulation of release machinery. Thus, the suppression of miniature events by cannabinoids has been taken as evidence that the release machinery is the target of cannabinoid-mediated suppression.

In the present study, we examined the cannabinoid sensitivity of miniature events in cerebellar Purkinje cells (PCs) to elucidate the mechanisms underlying the cannabinoid-mediated suppression. PCs receive excitatory inputs from parallel fibers (PFs) and climbing fibers (CFs) and inhibitory inputs from basket and stellate interneurons. Responses elicited by stimulating these fibers are all suppressed presynaptically by cannabinoids (Takahashi and Linden, 2000; Maejima et al., 2001b; Yoshida et al., 2002). However, the cannabinoid sensitivity of miniature events is different between excitatory and inhibitory inputs at normal external Ca^{2+} concentration ($[Ca^{2+}]_o$). The frequency of miniature IPSC (mIPSC) is consistently suppressed by the activation of cannabinoid receptors (Llano et al., 1991; Vincent and Marty, 1993; Glitsch et al., 1996; Takahashi and Linden, 2000; Glitsch and Jack, 2001; Diana et al., 2002; Diana and Marty, 2003), whereas that of miniature EPSC (mEPSC) is not (Takahashi and

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Linden, 2000) (but see Levenes et al., 1998). Thus, this system provides us a good model to study the cause of the difference in the cannabinoid sensitivity of miniature events.

Although miniature events can occur at the basal cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), their frequencies are greatly enhanced when the $[Ca^{2+}]_i$ is elevated. Here we show that, with 2 mM extracellular Ca^{2+} ($Ca^{2+}{}_o$), a significant fraction of mIPSCs is elicited by $[Ca^{2+}]_i$ rise (Ca^{2+}-enhanced mIPSCs), whereas mEPSCs do not involve Ca^{2+}-enhanced ones. Raising the $[Ca^{2+}]_o$ to 5 mM generates Ca^{2+}-enhanced mEPSCs, whereas preventing the $[Ca^{2+}]_i$ rise by BAPTA-AM loading and removal of $Ca^{2+}{}_o$ eliminates a major part of Ca^{2+}-enhanced mIPSCs. Importantly, cannabinoid receptor activation selectively suppresses Ca^{2+}-enhanced miniature events without affecting those present at the basal $[Ca^{2+}]_i$. Our results suggest that cannabinoid receptor activation regulates the processes of spontaneous transmitter release by acting on the downstream of Ca^{2+} entry.

Materials and Methods

Electrophysiology. Experiments were conducted according to the guidelines of the Animal Welfare Committee of Kanazawa University. Parasagittal cerebellar slices (250 μm in thickness) were prepared from C57BL6/J mice aged from 9 to 14 d postnatally as described previously (Edwards et al., 1989; Aiba et al., 1994; Kano et al., 1995, 1997). Whole-cell recordings were made from visually identified PCs using an upright microscope (Axioskop; Zeiss, Oberkochen, Germany). Patch pipettes had resistances of 2–4 MΩ when filled with an internal solution. For recording mEPSCs, the internal solution was composed of the following (in mM): 60 CsCl, 10 Cs D-gluconate, 20 tetraethylammonium-Cl, 20 BAPTA, 4 $MgCl_2$, 4 ATP, 0.4 GTP, and 30 HEPES, pH 7.3, adjusted with CsOH. For recording mIPSCs, Cs D-gluconate in the above solution was substituted by equimolar CsCl. The composition of the standard bathing solution was as follows (in mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 20 glucose, and 0.001 tetrodotoxin (TTX) (bubbled with 95% O_2 and 5% CO_2). To change the $[Ca^{2+}]_o$, Ca^{2+} in the bathing solution was replaced partially with equimolar Mg^{2+} to keep the total concentration of divalent cation constant. In 5 mM $Ca^{2+}{}_o$ solution, Mg^{2+} was omitted. Bicuculline (10 μM) or a mixture of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (10 μM)/3-((*R*)-2-carboxypiperazine-4-yl)-propyl-l-phosphonic acid (*R*-CPP) (10 μM) was added to the bathing solution for recording mEPSC or mIPSC, respectively. For recording quantal CF-EPSCs, external 2 mM Ca^{2+} and 1 mM Mg^{2+} were replaced with 1 mM Sr^{2+} and 2 mM Mg^{2+}. Ionic currents were recorded with a patch-clamp amplifier (EPC-9; HEKA Elektronik, Lambrecht/Pfalz, Germany). The signals were filtered at 2 kHz and digitized at 20 kHz. The holding potential was −70 mV. Series resistance and leak currents were monitored continuously, and recordings were terminated if these parameters changed significantly. Series resistance compensation was not used for minimizing the circuit-derived noise. On-line data acquisition was performed using PULSE software (HEKA Elektronik). All experiments were performed at room temperature.

Drugs. Drugs used in the present study included the following: bicuculline methochloride, CNQX, WIN55,212-2, 4-aminopyridine (4-AP), 2-amino-4-phosphonobutyrate (L-AP-4), (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl) glycine (DCG IV), *R*-CPP, and KB-R7943 (Tocris Cookson, Bristol, UK); EGTA-AM and BAPTA-AM (Invitrogen, Carlsbad, CA); SKF96365, A23187, and ryanodine mixture (Calbiochem, La Jolla, CA); α-latrotoxin (α-LTX) (Alomone Labs, Jerusalem, Israel); TTX (Wako Chemicals, Osaka, Japan); and SR141716A (generous gift from Sanofi, Libourne, France). All drugs were applied to the bath. For perfusion of solutions containing WIN55,212-2 and SR141716A, different tubes were used to avoid contamination. EGTA-AM or BAPTA-AM (100 μM), a membrane-permeable form of the calcium chelator, was bath applied for 25 min and then washed out. These two chelators freely entered the cells and then were deesterified to cell-impermeant EGTA or BAPTA (Cummings et al., 1996; Dittman and Regehr, 1998; Angleson and Betz, 2001). Given that ryanodine is an open channel blocker of ryanodine receptor, efficient blockade was achieved when the drug is applied at a high dose in combination with depolarization (Simkus and Stricker, 2002). Ryanodine (100 μM) was first applied for 15 min in a high K^+ solution ($[K^+]$, 15 mM) and for another 15 min in normal external solution ($[K^+]$, 2.5 mM). Then, WIN55,212-2 was applied in the continuous presence of ryanodine. For the control experiment, similar protocol without ryanodine was performed.

Data analysis. Miniature events were counted and analyzed off-line using Mini Analysis Program (version 5.6.4; Synaptosoft, Decatur, GA). The threshold for detection of an event was set at the level five times higher than the root-mean-square noise. By our detection parameter, the 10–90% rise times of detected miniature events did not exceed 10 ms. Miniature events were recorded continuously for 60 or 120 s in the control solution to calculate the baseline values of frequency and amplitude. After switching to a test solution, we waited until the effect of the applied solution reached the steady states. We then recorded miniature events continuously for 60 or 120 s to obtain the values in the test solution. The effect of the test solution was quantified as a test value represented in percentage of the control value. It usually took several tens of minutes for the effects of the test solutions to reach the steady states. To evaluate the steady states for such a prolonged period, we monitored the frequency and amplitude of miniature events for 500 ms in every 2 or 5 s. In the pilot experiments, we confirmed that mean frequency and amplitude analyzed from the recordings with these two sampling methods gave similar values. We constructed histograms for the time course of change as shown in Figures 1, 3, 4, and 6. Data are expressed as mean ± SEM throughout the text and figures. Differences between control and test data were examined using Student's *t* test. Statistical significance was assumed when $p < 0.05$.

Because PCs are innervated by two distinct excitatory inputs from CFs and PFs, mEPSCs recorded in PCs are the mixture of those originated from PFs and CFs. We judged that mEPSCs with 10–90% rise times longer than 1 ms mainly originated from PF terminals, whereas mEPSCs with rise times shorter than 1 ms originated from CF terminals, because of the following two reasons. First, ~90% of asynchronous CF mEPSCs elicited in Sr^{2+}-containing solution had 10–90% rise times shorter than 1 ms at room temperature (data not shown). Second, application of L-AP-4 (50 μM), a group III metabotropic glutamate receptor (mGluR) agonist, selectively suppressed the subpopulation of Ca^{2+}-enhanced mEPSCs with 10–90% rise times longer than 1 ms (see Fig. 8*B*). In contrast, Ca^{2+}-enhanced mEPSCs with 10–90% rise times shorter than 1 ms were suppressed preferentially by DCG IV (0.5 μM), a group II mGluR agonist (data not shown). As reported previously, PF terminals possess mGluR4 and are sensitive to L-AP-4 (Pekhletski et al., 1996; Neale et al., 2001), whereas CF terminals posses group II mGluRs and are sensitive to DCG IV but not to L-AP-4 (Maejima et al., 2001b). Therefore, mEPSCs with 10–90% rise times longer than 1 ms are thought to originate mainly from PF terminals and those with 10–90% rise times shorter than 1 ms from CF terminals. For the following analysis, we used the PF-originated mEPSCs (i.e., 10–90% rise times longer than 1 ms) because the occurrence of CF-originated mEPSCs was not stable over a prolonged recording period (1–2 h).

Results

Cannabinoid agonist suppresses mIPSC frequency but not mEPSC frequency with 2 mM $[Ca^{2+}]_o$

Previous reports have shown that mIPSCs recorded in PCs are consistently suppressed by cannabinoid agonists at a normal $[Ca^{2+}]_o$ (2 mM) (Takahashi and Linden, 2000; Diana et al., 2002). Moreover, the mIPSCs of a PC are suppressed by strong depolarization of the cell itself that triggers endocannabinoid-mediated retrograde suppression of inhibitory synaptic inputs known as depolarization-induced suppression of inhibition (DSI) (Llano et al., 1991; Vincent and Marty, 1993; Diana et al., 2002; Yoshida et al., 2002; Diana and Marty, 2003). In marked contrast, the mEPSCs of PCs are not affected by cannabinoid agonists at a normal $[Ca^{2+}]_o$ (Takahashi and Linden, 2000) (but see Levenes et al., 1998). We began by analyzing the effects of WIN55,212-2 (5

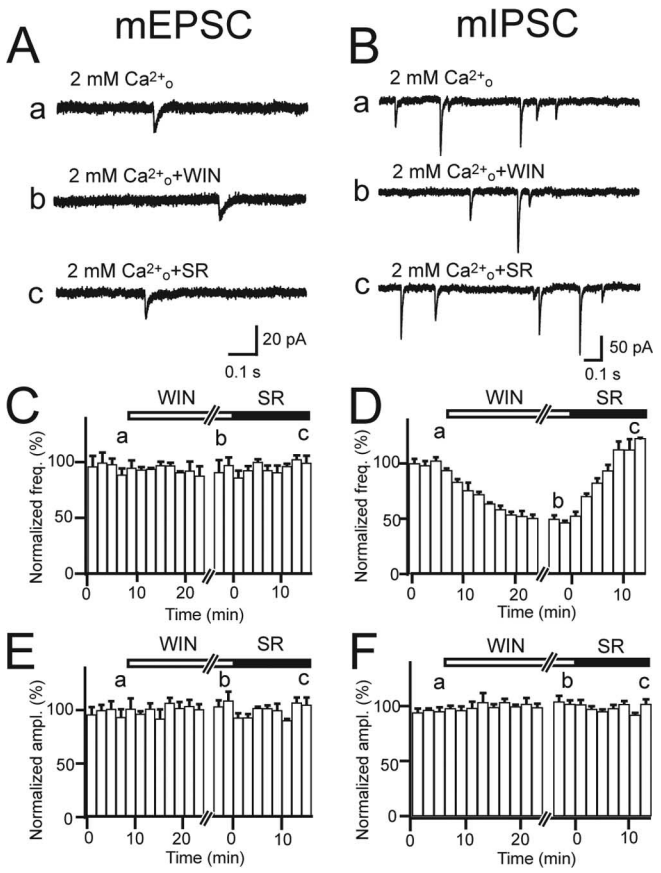

Figure 1. Effects of WIN55,212-2, a cannabinoid agonist, and SR141716A, a cannabinoid antagonist, on mEPSCs and mIPSCs at normal $[Ca^{2+}]_o$. ***A***, ***C***, ***E***, Representative data showing the effect of WIN55,212-2 on mEPSCs with 2 mM Ca^{2+}_o. ***A***, Sample traces taken from the indicated period in ***C*** and ***E*** from the same PC. ***C***, Average time plot of normalized mEPSC frequency ($n = 5$). WIN55,212-2 (5 μM) and SR141716A (2 μM) had no effect on mEPSC frequency. ***E***, Average time plot of normalized mEPSC amplitude ($n = 5$). WIN55,212-2 (5 μM) and SR141716A (2 μM) had no effect on mEPSC amplitude. ***B***, ***D***, ***F***, Representative data showing the effect of WIN55,212-2 on mIPSCs with 2 mM Ca^{2+}_o. ***B***, Sample traces were taken from periods labeled in ***D*** and ***F***. ***D***, Average time plot of normalized mIPSC frequency ($n = 4$). WIN55,212-2 (5 μM) reduced mIPSC frequency, and SR141716A (2 μM) reversed the reduction. ***F***, Average time plot of normalized mIPSC amplitude ($n = 4$). WIN55,212-2 (5 μM) and SR141716A (2 μM) had no effect on mIPSC amplitude. Error bars represent SEM.

μM), a cannabinoid agonist, on mEPSCs and mIPSCs recorded in the presence of TTX (1 μM). With 2 mM Ca^{2+}_o, WIN55,212-2 had no effect on the frequency and amplitude of mEPSCs (Fig. 1*A*,*C*,*E*). The mean control frequency and amplitude were 0.53 ± 0.14 Hz and 17.6 ± 1.0 pA, respectively ($n = 5$). WIN55,212-2 affected neither the frequency (89.1 ± 15.0% of the control) nor the amplitude (101.6 ± 1.5% of the control) of mEPSCs ($n = 5$; $p = 0.24$ and 0.35, respectively). In marked contrast, WIN55,212-2 reduced the mean mIPSC frequency (Fig. 1*B*,*D*,*F*). The mean baseline frequency was 4.5 ± 0.8 Hz ($n = 9$). The mIPSC frequency with WIN55,212-2 was 56.9 ± 5.2% of the control ($n = 9$; $p < 0.01$). This suppression was reversed to 119.7 ± 8.5% of the control ($n = 9$) by subsequent application of SR141716A (2 μM), a selective cannabinoid antagonist (Fig. 1*D*). Conversely, mean mIPSC amplitude did not change significantly (Fig. 1*F*). The mean baseline amplitude was 53.1 ± 9.5 pA and that with WIN55,212-2 was 101.4 ± 3.0% of the control ($n = 9$; $p = 0.88$). These results are consistent with a previous report that the mIPSCs of PCs are sensitive to cannabinoids at a normal $[Ca^{2+}]_o$ but the mEPSCs are not (Takahashi and Linden, 2000).

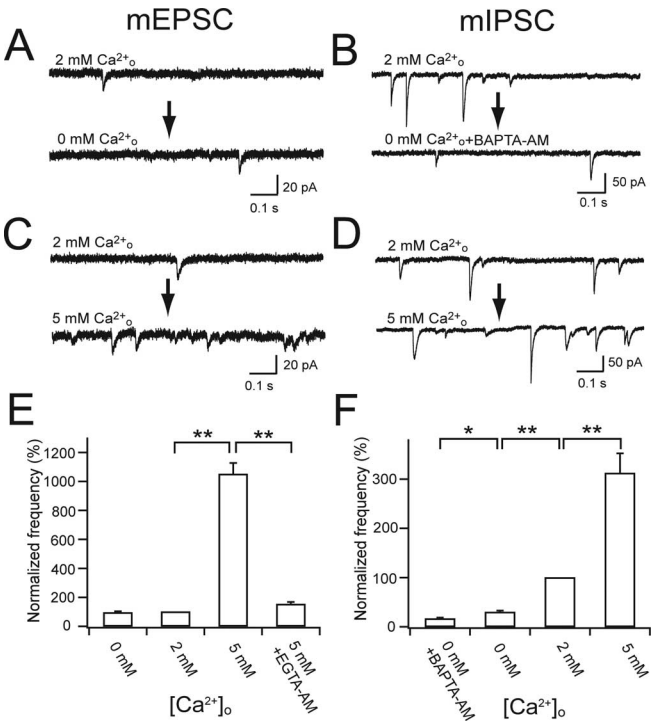

Figure 2. Ca^{2+}_o dependency of mEPSC and mIPSC. ***A***, ***C***, ***E***, Effect of changing $[Ca^{2+}]_o$ on mEPSC frequency. ***A***, Sample mEPSCs recorded with 2 mM Ca^{2+}_o and 0 mM Ca^{2+}_o. ***C***, Sample mEPSCs recorded with 2 mM Ca^{2+}_o and 5 mM Ca^{2+}_o. ***E***, Summary bar graph for the effects of changing $[Ca^{2+}]_o$ on mEPSC frequency. Data with 0 mM Ca^{2+}_o ($n = 12$), with 5 mM Ca^{2+}_o ($n = 52$), and with 5 mM Ca^{2+}_o solution preincubated with EGTA-AM (100 μM) ($n = 11$) are normalized to those with 2 mM Ca^{2+}_o. ***B***, ***D***, ***F***, Effect of changing $[Ca^{2+}]_o$ on mIPSC frequency. ***B***, Sample mIPSCs recorded with 2 mM Ca^{2+}_o and with 0 mM Ca^{2+}_o EGTA-containing solution preincubated with BAPTA-AM. ***D***, Sample mIPSCs recorded with 2 mM Ca^{2+}_o and with 5 mM Ca^{2+}_o. ***F***, Summary bar graph for the effect of changing $[Ca^{2+}]_o$ on the mIPSC frequency. Data with EGTA-containing 0 mM Ca^{2+}_o solution preincubated with BAPTA-AM ($n = 6$), with EGTA-containing 0 mM Ca^{2+}_o solution ($n = 6$), and with 5 mM Ca^{2+}_o ($n = 7$) are normalized to those with 2 mM Ca^{2+}_o. *$p < 0.05$, **$p < 0.01$, *t* test. Error bars represent SEM.

Effect of changing $[Ca^{2+}]_o$ on mEPSC and mIPSC

To elucidate the cause of the apparent difference in the cannabinoid sensitivity between mEPSC and mIPSC, we recorded miniature responses at various $[Ca^{2+}]_o$. The mean frequency of mIPSC was decreased as the $[Ca^{2+}]_o$ was lowered from 2 to 0 mM (Fig. 2*B*,*F*). This is consistent with the previous report by Llano et al. (2000) that mIPSCs recorded in PCs with 2 mM Ca^{2+}_o include those elicited by spontaneous Ca^{2+} release from intracellular Ca^{2+} stores (Llano et al., 2000). These Ca^{2+}-enhanced mIPSCs seemed to remain even after the slices were perfused with Ca^{2+}-free solution containing 200 μM EGTA for >30 min, because the mIPSC frequency was further suppressed when application of EGTA-containing, Ca^{2+}-free solution was combined with presynaptic infusion of BAPTA-AM (100 μM; see Materials and Methods) (Fig. 2*F*). Incubation with BAPTA-AM in 2 mM Ca^{2+}_o solution reduced mIPSC frequency (normalized mIPSC frequency was 67.2 ± 8.5%; $n = 6$; $p < 0.01$). However, subsequent perfusion with EGTA-containing, Ca^{2+}-free solution further reduced mIPSC frequency to 16.4 ± 2.3% of the control, and this was 28.0 ± 4.2% of the value with BAPTA-AM in 2 mM Ca^{2+}_o solution ($n = 6$; $p < 0.01$). As reported previously, mIPSC with 2 mM Ca^{2+}_o was not affected by Cd^{2+} (100 μM), a nonselective blocker of voltage-dependent Ca^{2+} channels (VDCCs) (Llano et al., 2000; Harvey and Stephens, 2004), indicating that VDCCs were not involved in such spontaneous Ca^{2+} rise (the

mean frequency and amplitude were 4.0 ± 0.6 Hz and 61.4 ± 10.6 pA with 100 μM Cd^{2+} compared with 3.9 ± 0.7 Hz and 61.2 ± 10.2 pA in control; $n = 5$; $p = 0.87$ and 0.98, respectively). In marked contrast, mean mEPSC frequency did not change after the $[Ca^{2+}]_o$ was switched from 2 to 0 mM (Fig. 2*A*,*E*). Consistent with a previous report (Chen and Regehr, 1997), mEPSC was also not affected by Cd^{2+} (100 μM) (the mean baseline frequency and amplitude was 0.78 ± 0.35 Hz and 17.2 ± 0.7 pA compared with 0.83 ± 0.24 Hz and 17.6 ± 0.7 pA in control; $n = 4$; $p = 0.84$ and 0.87, respectively). These results suggest that, with 2 mM Ca^{2+}_o, mEPSCs mainly consist of miniature events independent of spontaneous or persistent presynaptic $[Ca^{2+}]_i$ rise (we hereafter term such mEPSCs "basal mEPSCs") and do not include miniature events elicited by presynaptic $[Ca^{2+}]_i$ rise (we hereafter term such mEPSCs "Ca^{2+}-enhanced mEPSCs"). In contrast, mIPSCs consist of both "basal mIPSCs" and "Ca^{2+}-enhanced mIPSCs" with 2 mM Ca^{2+}_o.

If cannabinoid agonists preferentially suppress Ca^{2+}-enhanced miniature events, this could account for the differential sensitivities of mEPSC and mIPSC to cannabinoid agonists with 2 mM Ca^{2+}_o. In the subsequent sections, we tested this hypothesis by addressing the following two questions. First, are Ca^{2+}-enhanced mEPSCs suppressed by cannabinoid agonists? Second, even after Ca^{2+}-enhanced mIPSCs are eliminated, are the remaining mIPSCs (i.e., basal mIPSCs) suppressed by cannabinoid agonists?

Emergence of Ca^{2+}-enhanced mEPSC by raising $[Ca^{2+}]_i$

As reported by Llano et al. (2000), changing the $[Ca^{2+}]_o$ from 2 to 5 mM resulted in a marked increase in the frequency (15.4 ± 2.7 Hz with 5 mM Ca^{2+}_o compared with 4.9 ± 0.7 Hz with 2 mM Ca^{2+}_o; $n = 7$; $p < 0.01$) (Fig. 2*D*,*F*) but not in the amplitude of Ca^{2+}-enhanced mIPSCs (mean amplitude, 60.2 ± 8.4 pA with 5 mM Ca^{2+}_o compared with 53.5 ± 6.5 pA with 2 mM Ca^{2+}_o; $n = 7$; $p = 0.15$). We first examined whether Ca^{2+}-enhanced mEPSC could be also elicited by raising the $[Ca^{2+}]_o$. As expected, mEPSC frequency markedly increased after changing the $[Ca^{2+}]_o$ from 2 to 5 mM (0.59 ± 0.14 Hz with 2 mM Ca^{2+}_o and 6.2 ± 0.5 Hz with 5 mM Ca^{2+}_o; $n = 52$; $p < 0.01$) (Fig. 2*C*,*E*) and rapidly recovered to the initial value during reperfusion with 2 mM Ca^{2+}_o solution. There was no significant change in the mean amplitude (17.5 ± 0.4 pA with 2 mM Ca^{2+}_o and 17.9 ± 0.4 pA with 5 mM Ca^{2+}_o; $n = 52$; $p = 0.85$). The increase in mEPSC frequency was blocked by preincubation of the slices with EGTA-AM (100 μM) (1.0 ± 0.2 Hz; $n = 11$; see Materials and Methods) (Fig. 2*E*), indicating that it required Ca^{2+} rise in the presynaptic terminals. The increased component of mEPSC frequency was thought to be attributable to Ca^{2+}-enhanced events, and, therefore, we used this manipulation to elicit Ca^{2+}-enhanced mEPSCs in the following experiments. The increase in mEPSC frequency with 5 mM Ca^{2+}_o was not affected by Cd^{2+} (100 μM) or by SKF96365 (100 μM), a broad-spectrum blocker of nonselective cation channels (data not shown). Another possible mechanism to produce this $[Ca^{2+}]_i$ increase is contribution of Na^+/Ca^{2+} exchanger (NCX). NCX is known to exist on the plasma membrane of nerve terminals and may cause net Ca^{2+} influx at the nerve terminal in certain conditions (Mulkey and Zucker, 1992; Blaustein and Lederer, 1999). To test the possible contribution of NCX, we applied an NCX blocker, KB-R7943 (50 μM) or bepridil (50 μM), before changing the $[Ca^{2+}]_o$ from 2 to 5 mM. However, neither of these blockers inhibited the increase in mEPSC frequency (data not shown). Therefore, the $[Ca^{2+}]_i$ elevation after changing the $[Ca^{2+}]_o$ is mediated by mechanisms other than the above tested Ca^{2+} entry pathways.

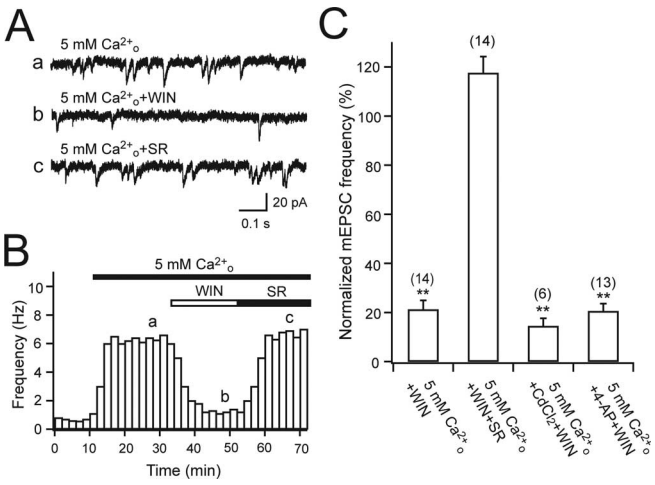

Figure 3. Effect of WIN55,212-2 on mEPSCs with 5 mM Ca^{2+}_o. ***A***, Sample traces of mEPSCs recorded at the time points labeled in ***B***. ***B***, Miniature EPSCs elicited by raising $[Ca^{2+}]_o$ from 2 to 5 mM were suppressed by WIN55,212-2, and the WIN55,212-2-induced suppression was reversed by SR141716A (2 μM). ***C***, Summary bar graph for the effect of WIN55,212-2 on mEPSC frequency in various experimental conditions. Data are obtained from the indicated number of experiments and normalized to the baseline frequency with 5 mM Ca^{2+}_o. **$p < 0.01$, *t* test. Error bars represent SEM.

WIN55,212-2 suppresses Ca^{2+}-enhanced but VDCC-independent mEPSCs

We examined whether activation of cannabinoid receptors could suppress Ca^{2+}-enhanced but VDCC-independent mEPSCs elicited with 5 mM Ca^{2+}_o. As exemplified in Figure 3, *A* and *B*, bath application of WIN55,212-2 effectively suppressed mEPSC frequency with 5 mM Ca^{2+}_o. The mean baseline frequency with 5 mM Ca^{2+}_o was 6.2 ± 0.8 Hz ($n = 14$). WIN55,212-2 reduced the frequency to 21.1 ± 3.8% of the baseline ($n = 14$; $p < 0.01$). This suppression was completely reversed by subsequent application of SR141716A (117.5 ± 6.7% of the level before WIN55,212-2 application; $n = 14$) (Fig. 3*A*–*C*). These results indicate that Ca^{2+}-enhanced mEPSCs elicited with 5 mM Ca^{2+}_o are sensitive to cannabinoids.

Because Ca^{2+} influx through VDCCs is not the primary factor of eliciting Ca^{2+}-enhanced mEPSCs with 5 mM Ca^{2+}_o, the suppression of mEPSC by WIN55,212-2 is not likely to result from cannabinoid-induced inhibition of VDCCs. This notion was confirmed by our observation that Cd^{2+} (100 μM) had no effect on suppression of mEPSC frequency by WIN55,212-2. In the continuous presence of Cd^{2+}, WIN55,212-2 decreased mEPSC frequency to 14.4 ± 3.2% of the baseline ($n = 6$; $p < 0.01$) (Fig. 3*C*). We also examined the possible contribution of 4-AP-sensitive K^+ channels because their activation is reported to suppress PF–PC synaptic transmission (Daniel and Crepel, 2001). Application of 4-AP (1 mM) with 5 mM Ca^{2+}_o had no effect on mEPSC frequency (data not shown). Furthermore, an application of WIN55,212-2 in the continuous presence of 4-AP decreased mEPSC frequency to 20.4 ± 3.1% of the baseline ($n = 13$; $p < 0.01$) (Fig. 3*C*). These results suggest that neither VDCCs nor 4-AP-sensitive K^+ channels contribute to the cannabinoid-mediated suppression of Ca^{2+}-enhanced mEPSC.

Ca^{2+} ionophore-induced mEPSCs are suppressed by WIN55,212-2, whereas α-latrotoxin-induced ones are not

It could be possible that cannabinoid agonists block unknown Ca^{2+} entry pathways and thereby decrease the $[Ca^{2+}]_i$ and reduce mEPSC frequency. To test this possibility, we directly raised Ca^{2+} concentration in the presynaptic terminals by using A23187, a Ca^{2+} ionophore. Bath application of A23187 (5 μM) with 2 mM Ca^{2+}_o markedly enhanced mEPSC frequency (773.2 ± 58.1% of the baseline; average frequency, 7.9 ± 0.8 Hz; $n = 6$; $p < 0.01$). If a cannabinoid agonist acts on the Ca^{2+} entry pathways, it should not affect mEPSCs elicited by A23187. However, subsequent application of WIN55,212-2 effectively suppressed mEPSC frequency to the baseline level before A23187 application (Fig. 4*A*,*B*). The suppression induced by WIN55,212-2 was completely reversed by subsequent application of SR141716A (Fig. 4*A*,*B*). These results indicate that cannabinoid agonists suppress the Ca^{2}-enhanced mEPSCs by acting on processes downstream of Ca^{2+} entry.

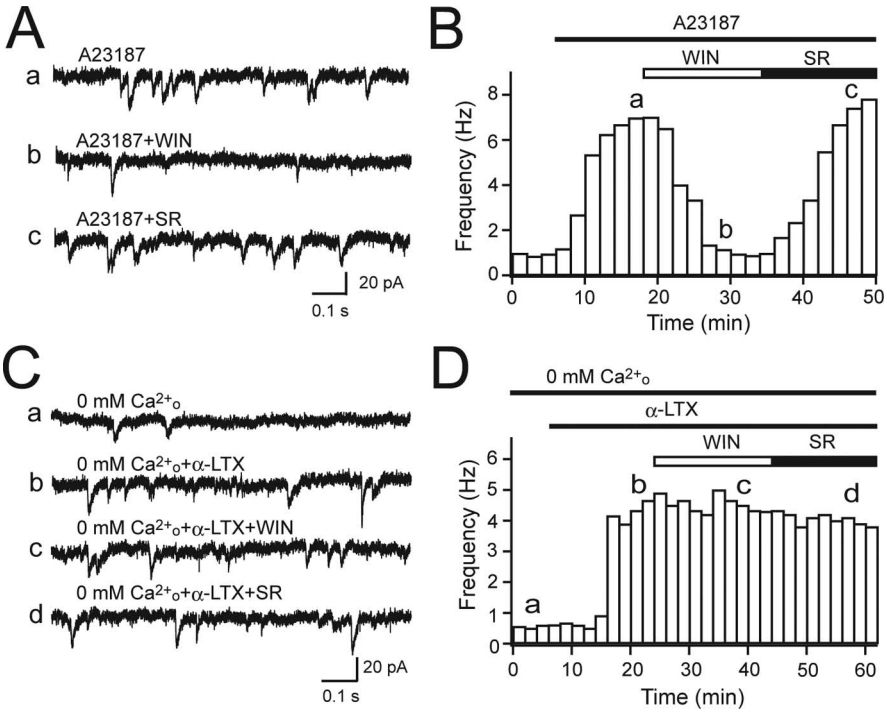

Figure 4. Effects of WIN55,212-2 on mEPSCs elicited by application of Ca^{2+} ionophore and α-LTX. ***A***, ***B***, Representative data showing the effect of WIN55,212-2 on mEPSC frequency enhanced by application of a Ca^{2+} ionophore, A23187. ***A***, Sample traces of mEPSCs recorded at the time points labeled in ***B***. ***B***, Miniature EPSCs elicited by A23187 (5 μM) were suppressed by WIN55,212-2 (5 μM), and this suppression was reversed by SR141716A (2 μM). ***C***, ***D***, Representative data showing the effect of WIN55,212-2 on mEPSC frequency enhanced by application of α-LTX. ***C***, Sample traces of mEPSCs recorded at the time points labeled in ***D***. ***D***, WIN55,212-2 (5 μM) and SR141716A (2 μM) had no effect on mEPSC frequency elicited by application of α-LTX.

We then examined whether cannabinoid agonists affect the mEPSCs enhanced independently of $[Ca^{2+}]_i$ rise. α-LTX is the major active component of a black widow spider toxin and is known to elicit a high rate of spontaneous transmitter release from nerve terminals (Capogna et al., 1996; Zhou et al., 2000; Harvey and Stephens, 2004). We examined whether cannabinoid agonist interfered with the release machinery activated by α-LTX. Because α-LTX facilitates vesicle release via both Ca^{2+}-dependent and Ca^{2+}-independent mechanisms (Sudhof, 2001), we used this toxin in the absence of Ca^{2+}_o to minimize Ca^{2+} mobilization. Bath application of α-LTX (0.3 nM) produced a marked increase in mEPSC frequency (935.9 ± 103.0% of the baseline; 5.1 ± 0.6 Hz; $n = 4$). WIN55,212-2 did not affect mEPSC frequency in the presence of α-LTX (106.9 ± 5.6% of the baseline; $n = 4$; $p = 0.82$) (Fig. 4*C*,*D*). These results indicate that the Ca^{2+}-independent release machinery activated by α-LTX is not a target of cannabinoid receptor-mediated signaling.

Figure 5 illustrates the relationship between the effects of WIN55,212-2 on mEPSC frequency and its baseline values in various experimental conditions. WIN55,212-2 markedly inhibited mEPSC frequency in the presence of 5 mM Ca^{2+}_o or A23187, when the predominant component of mEPSC was a Ca^{2+}-enhanced one. In contrast, WIN55,212-2 did not suppress mEPSCs with 0 or 2 mM Ca^{2+}_o, when the Ca^{2+}-enhanced mEPSCs were merely occurring. Furthermore, at all concentrations examined (0.01, 0.05, 0.5, and 5 μM), WIN55,212-2 selectively suppressed Ca^{2+}-enhanced mEPSCs (supplemental Fig. 1*A*, available at www.jneurosci.org as supplemental material). The inhibitory effect by WIN55,212-2 is not simply correlated with the frequency of mEPSC because the α-LTX-elicited mEPSCs occurred in high frequency are not affected by WIN55,212-2. Together, these results suggest that activation of cannabinoid receptors selectively suppresses mEPSCs elicited by elevation of the $[Ca^{2+}]_i$, which is produced by a mechanism other than VDCCs (Ca^{2+}-enhanced but VDCC-independent mEPSCs).

Basal mIPSCs are not suppressed by WIN55,212-2

We then examined whether basal mIPSCs were sensitive to cannabinoid agonists or not. A previous report shows that prolonged removal of external Ca^{2+} depletes intracellular Ca^{2+} stores and results in reduction of the frequency and amplitude of Ca^{2+}-enhanced mIPSCs (Llano et al., 2000). When slices were perfused with a Ca^{2+}-free solution containing 200 μM EGTA for >30 min, mIPSC frequency and amplitude markedly decreased. Under this condition, the magnitude of suppression induced by WIN55,212-2 became smaller than that with 2 mM Ca^{2+}_o (73.0 ± 4.3% of the baseline; $n = 6$), although it was still significant ($p < 0.05$). This is presumably because Ca^{2+}-enhanced mIPSC are not completely eliminated even in the absence of Ca^{2+}_o (Fig. 2*F*). Therefore, we performed a similar experiment in the condition in which removal of Ca^{2+}_o was combined with loading BAPTA-AM to slices to minimize an elevation in the $[Ca^{2+}]_i$ in the presynaptic terminals. Under this condition, WIN55,212-2 did not reduced mIPSC frequency (102.1 ± 6.8% of the baseline; $n = 6$; $p = 0.91$) (Fig. 6*A*,*B*).

It is reported that ryanodine-sensitive Ca^{2+} stores contribute to the generation of Ca^{2+}-enhanced mIPSCs (Llano et al., 2000). Therefore, we tried to eliminate Ca^{2+}-enhanced mIPSCs by interfering with ryanodine-sensitive Ca^{2+} stores. After mIPSCs were recorded in normal solution containing 2 mM Ca^{2+}_o, the slice was perfused for 15 min with a high-K^+ solution (15 mM) supplemented with 100 μM ryanodine. Then the slice was perfused for another 15 min with normal external solution (2.5 mM K^+) supplemented with ryanodine. After this treatment, both

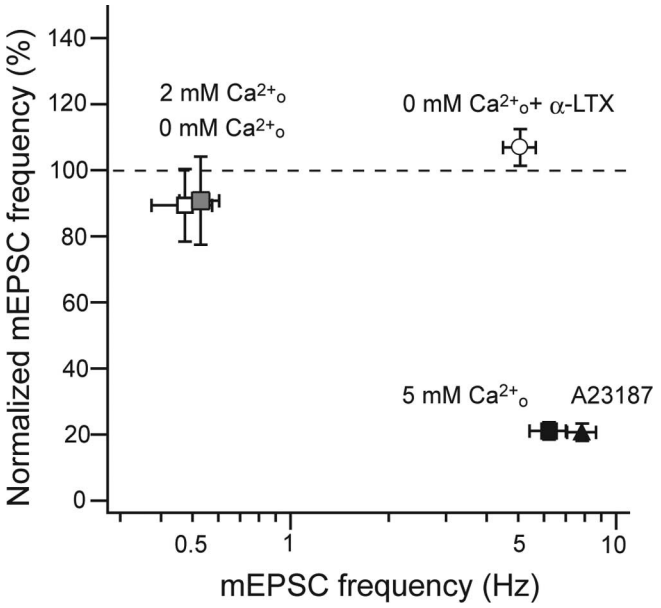

Figure 5. Summary plots for the effects of WIN55,212-2 on the mEPSC frequency in various experimental conditions. Values on the ordinate are expressed relative to the baseline mEPSC frequency before application of WIN55,212-2 (5 μM). Each symbol represents mean ± SEM. White rectangle, 0 mM Ca^{2+}_o ($n = 8$); light gray rectangle, 2 mM Ca^{2+}_o ($n = 5$); black rectangle, 5 mM Ca^{2+}_o ($n = 14$); black triangle, A23187 ($n = 6$); white circle, 0 mM Ca^{2+}_o plus α-LTX ($n = 4$).

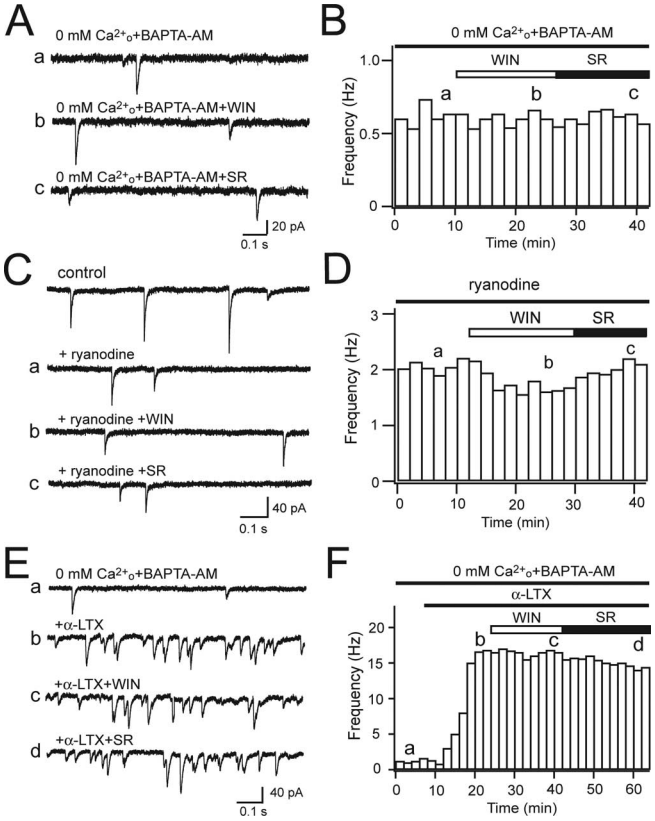

Figure 6. Effects of WIN55,212-2 on basal and α-LTX-induced mIPSCs. ***A***, ***B***, Representative data showing the effect of WIN55,212-2 on mIPSC frequency with EGTA-containing 0 mM Ca^{2+}_o solution preincubated with BAPTA-AM. ***A***, Sample traces of mIPSCs at the time points labeled in ***B***. ***B***, WIN55,212-2 (5 μM) and SR141716A (2 μM) had no effect on mIPSC frequency. ***C***, ***D***, Representative data showing the effect of WIN55,212-2 when Ca^{2+}-enhanced mIPSC is suppressed by 100 μM ryanodine. ***C***, Sample traces of mIPSCs at the time points labeled in ***D***. ***D***, The depressant effect of WIN55,212-2 (5 μM) is not prominent compared with the example shown in Figure 1*D*. ***E***, ***F***, Representative data showing the effect of WIN55,212-2 on the frequency of α-LTX-induced mIPSCs. To eliminate Ca^{2+}-enhanced mIPSC, application of 0 mM Ca^{2+}_o solution containing EGTA (200 μM) was combined with presynaptic loading of BAPTA-AM. ***E***, Sample traces of mIPSCs at the time points labeled in ***F***. ***F***, WIN55,212-2 (5 μM) and SR141716A (2 μM) had no effect on mIPSC frequency enhanced by application of α-LTX.

mIPSC frequency and mean amplitude were markedly reduced from those before ryanodine application (2.1 ± 0.5 Hz and 45.7 ± 8.0 pA compared with 4.0 ± 0.4 Hz and 59.0 ± 12.3 pA in control period; $n = 6$; $p < 0.01$). These changes were not observed after the challenge of high-K^+ solution (15 mM) without ryanodine (data not shown). We then compared the depressant effect of WIN55,212-2 in these two groups. WIN55,212-2 reduced mIPSC frequency to 45.2 ± 8.9% after the control manipulation without ryanodine, whereas the effect was significantly smaller after the manipulation with ryanodine (69.5 ± 4.2%; $n = 6$; $p < 0.01$) (Fig. 6*C*,*D*).

Together, the results from the two sets of experiments strongly suggest that mIPSCs become insensitive to cannabinoid agonists when generation of Ca^{2+}-enhanced mIPSCs is suppressed.

WIN55,212-2 does not interfere with the α-LTX-activated release process

We then examined whether cannabinoid agonists affect Ca^{2+}-independent enhancement of mIPSCs by α-LTX. To minimize Ca^{2+}-enhanced mIPSCs, application of EGTA-containing Ca^{2+}-free solution was combined with presynaptic loading of BAPTA-AM, and then α-LTX was applied with Ca^{2+}-free solution. α-LTX significantly increased mIPSC frequency (1672.3 ± 198.8% of the control; 15.4 ± 1.9 Hz; $n = 4$), but the α-LTX-induced mIPSCs were not suppressed by WIN55,212-2 (96.4 ± 3.5% of the baseline; $n = 4$; $p = 0.71$) (Fig. 6*E*,*F*). These results indicate that Ca^{2+}-independent mIPSCs activated by α-LTX are insensitive to cannabinoid agonists.

As shown in Figure 7, in the experiments changing the $[Ca^{2+}]_o$, the magnitude of mIPSC suppression by WIN55,212-2 was correlated to the baseline mIPSC frequency that reflected the proportion of Ca^{2+}-enhanced mIPSC. In contrast, WIN55,212-2 did not affect Ca^{2+}-independent mIPSCs elicited by α-LTX. We also examined the depressant effect of WIN55,212-2 at 0.01, 0.05, 0.5, and 5 μM. At all concentrations tested, WIN55,212-2 selectively suppressed Ca^{2+}-enhanced mIPSCs (supplemental Fig. 1*B*, available at www.jneurosci.org as supplemental material).

These results strongly suggest that the mIPSCs independent of spontaneous $[Ca^{2+}]_i$ rise are not suppressed by cannabinoid agonists.

Baclofen and L-AP-4 selectively suppress Ca^{2+}-enhanced miniature events

Finally, we tested whether selective suppression of miniature events was commonly seen for other $G_{i/o}$-coupled receptor-mediated presynaptic inhibition. PF to PC excitatory synaptic transmission is known to be suppressed presynaptically by activating $GABA_B$ receptors or group III mGluRs (Dittman and Regehr, 1996; Pekhletski et al., 1996). In the presence of 2 mM Ca^{2+}_o, the mEPSC frequency was not affected by baclofen (5 μM), a $GABA_B$ receptor agonist, or L-AP-4 (50 μM), a group III mGluR agonist (normalized mEPSC frequency was 96.2 ± 3.0% with baclofen, $n = 12$, $p = 0.61$; 106.7 ± 7.1% with L-AP-4, $n = 6$, $p = 0.72$) (Fig. 8*A*,*B*). In contrast, in the presence of 5 mM Ca^{2+}_o, the mEPSC frequency was markedly suppressed by baclofen and L-AP-4 (normalized mEPSC frequency was 16.3 ± 4.0% with baclofen, $n = 6$, $p < 0.01$; 19.1 ± 2.1% with L-AP-4,

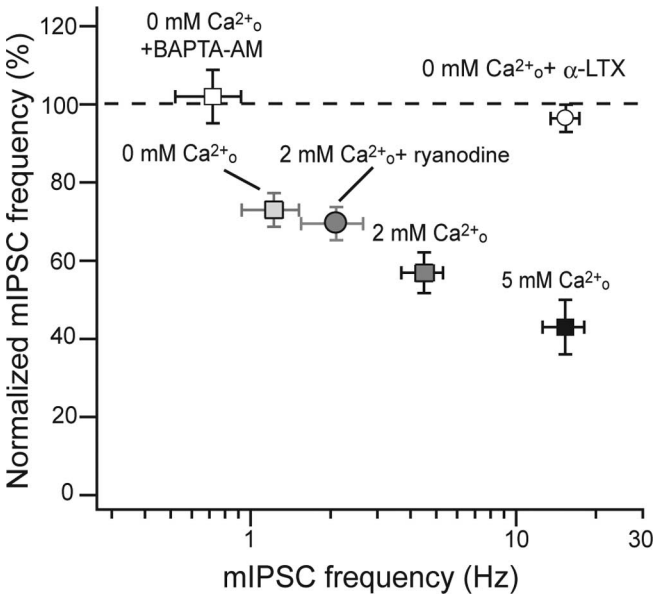

Figure 7. Summary plots for the effects of WIN55,212-2 on mIPSC frequency in various experimental conditions. Values on the ordinate are expressed relative to the baseline mIPSC frequency before application of WIN55,212-2 (5 μM). Each symbol represents mean ± SEM. White rectangle, 0 mM $Ca^{2+}{}_o$ plus BAPTA-AM ($n = 6$); light gray rectangle, 0 mM $Ca^{2+}{}_o$ ($n = 6$); gray circle, 2 mM $Ca^{2+}{}_o$ plus 100 μM ryanodine ($n = 6$); gray rectangle, 2 mM $Ca^{2+}{}_o$ ($n = 9$); black rectangle, 5 mM $Ca^{2+}{}_o$ ($n = 6$); white circle, 0 mM $Ca^{2+}{}_o$ plus BAPTA-AM plus α-LTX ($n = 4$).

$n = 18$, $p < 0.01$) (Fig. 8*A*,*B*). These results indicate that activation of presynaptic $GABA_B$ receptor or group III mGluR preferentially suppresses Ca^{2+}-enhanced mEPSCs.

Previous studies show that baclofen effectively suppresses mIPSC frequency in the presence of 2 mM $Ca^{2+}{}_o$ (Than and Szabo, 2002; Harvey and Stephens, 2004). To test whether this suppression is selective for Ca^{2+}-enhanced mIPSCs, we combined loading of BAPTA-AM and removal of external Ca^{2+} to minimize Ca^{2+}-enhanced mIPSCs (Fig. 8*D*). Under this condition, the magnitude of baclofen-induced suppression in a Ca^{2+}-free, EGTA-containing solution was significantly smaller than that in a 2 mM $Ca^{2+}{}_o$ solution (normalized mIPSC frequency with baclofen was 69.0 ± 8.0% in 0 mM $Ca^{2+}{}_o$ plus BAPTA-AM, $n = 6$; 32.9 ± 2.9% in 2 mM $Ca^{2+}{}_o$ solution, $n = 6$; $p < 0.01$) (Fig. 8*D*). These results indicate that $GABA_B$ receptor activation also preferentially suppresses Ca^{2+}-enhanced mIPSCs. However, in contrast to the cannabinoid agonists, baclofen produces small but significant suppression of mIPSC frequency even when an application of Ca^{2+}-free solution is combined with BAPTA-AM loading.

Discussion

In the present study, we found that mEPSC and mIPSC in PCs had fundamentally similar responsiveness to cannabinoid receptor activation, although the apparent effect was different at the normal $[Ca^{2+}]_o$. In both cases, cannabinoid agonists preferentially suppressed miniature responses triggered by $[Ca^{2+}]_i$ rise, although they did not affect those independent of $[Ca^{2+}]_i$ rise. The difference in cannabinoid sensitivity between mIPSC and mEPSC could be derived from the differential incidence of Ca^{2+}-enhanced miniature events. Although Ca^{2+}-enhanced mIPSCs regularly occur with 2 mM $Ca^{2+}{}_o$, as a result of Ca^{2+} release from internal stores (Llano et al., 2000; Harvey and Stephens, 2004), Ca^{2+}-enhanced mEPSCs infrequently occur at the normal $[Ca^{2+}]_o$. Importantly, VDCCs did not contribute to generation of Ca^{2+}-enhanced events in either case (Chen and Regehr, 1997; Llano et al., 2000; Harvey and Stephens, 2004). These results suggest that the occurrence of Ca^{2+}-enhanced miniature events determines the cannabinoid sensitivity of miniature events in PCs.

Heterogeneity of miniature events and cannabinoid sensitivity

In PCs, mIPSC is suppressed by cannabinoid agonists, whereas mEPSC is not at the normal $[Ca^{2+}]_o$ (Takahashi and Linden, 2000). This result has been regarded as evidence that release machinery is a target for presynaptic depression of the inhibitory inputs but not for that of the excitatory ones. However, our results indicate that mEPSCs become sensitive to cannabinoid agonists when the occurrence of Ca^{2+}-enhanced mEPSCs is facilitated by raising the $[Ca^{2+}]_i$ in the presynaptic terminals (Fig. 5). Conversely, mIPSCs became insensitive to cannabinoid agonists when the occurrence of Ca^{2+}-enhanced mIPSCs was suppressed (Fig. 7). These results suggest that, at least in PCs, the cannabinoid sensitivity of miniature events depends on the proportion of Ca^{2+}-enhanced component in the total miniature events. Therefore, the aforementioned interpretation that the apparent cannabinoid insensitivity excludes possible inhibitory effect on release machinery is not the case at least for PC synapses.

In the hippocampus, it has also been reported that the cannabinoid sensitivity of miniature synaptic events changes depending on recording conditions (Hoffman and Lupica, 2000; Wilson and Nicoll, 2001; Varma et al., 2002). Miniature mIPSCs of hippocampal pyramidal neurons are normally insensitive to WIN55,212-2. However, mIPSCs facilitated by a high external K^+ concentration are suppressed by WIN55,212-2 (Hoffman and Lupica, 2000; Wilson and Nicoll, 2001; Varma et al., 2002). Because the mIPSC facilitation triggered by a high external K^+ concentration is also suppressed by Cd^{2+}, the above results have been taken as evidence that VDCCs are the main target of the cannabinoid-mediated presynaptic depression. However, because Cd^{2+} blocks not only VDCCs themselves but also the subsequent Ca^{2+} influx and enhancement of vesicle release, the experiment with Cd^{2+} is inconclusive. In the present study, mEPSCs triggered by $[Ca^{2+}]_i$ rise were suppressed by WIN55,212-2 (Fig. 5). Therefore, if mIPSCs of hippocampal pyramidal cells have similar properties to the mEPSCs of cerebellar PCs, the possibility remains that cannabinoid agonists inhibit the Ca^{2+}-enhancd mIPSCs elicited by Ca^{2+} influx through VDCCs. Moreover, it has been shown that mIPSCs recorded in layer 2/3 pyramidal neurons in the cerebral cortex are generally sensitive to WIN55,212-2, but they become insensitive when VDCC or subsequent Ca^{2+} influx is blocked (Trettel and Levine, 2002). These data can also be interpreted in a similar way that cannabinoid agonists inhibit Ca^{2+}-enhanced mIPSCs.

Suppression of miniature events by activation of $GABA_B$ receptor or group III mGluR

In the present study, baclofen and L-AP-4 suppressed mEPSC frequency with 5 mM $Ca^{2+}{}_o$ but not with 2 mM $Ca^{2+}{}_o$. This indicates that activation of $GABA_B$ receptor or group III mGluR of PFs also has a tendency to suppress mEPSCs triggered by $[Ca^{2+}]_i$ rise. $GABA_B$ receptor, group III mGluR, and cannabinoid receptor are all coupled to $G_{i/o}$-protein and may share common signaling cascades for presynaptic depression in PF terminals. However, a previous report has shown that mEPSCs recorded in PCs can be suppressed by baclofen (Dittman and

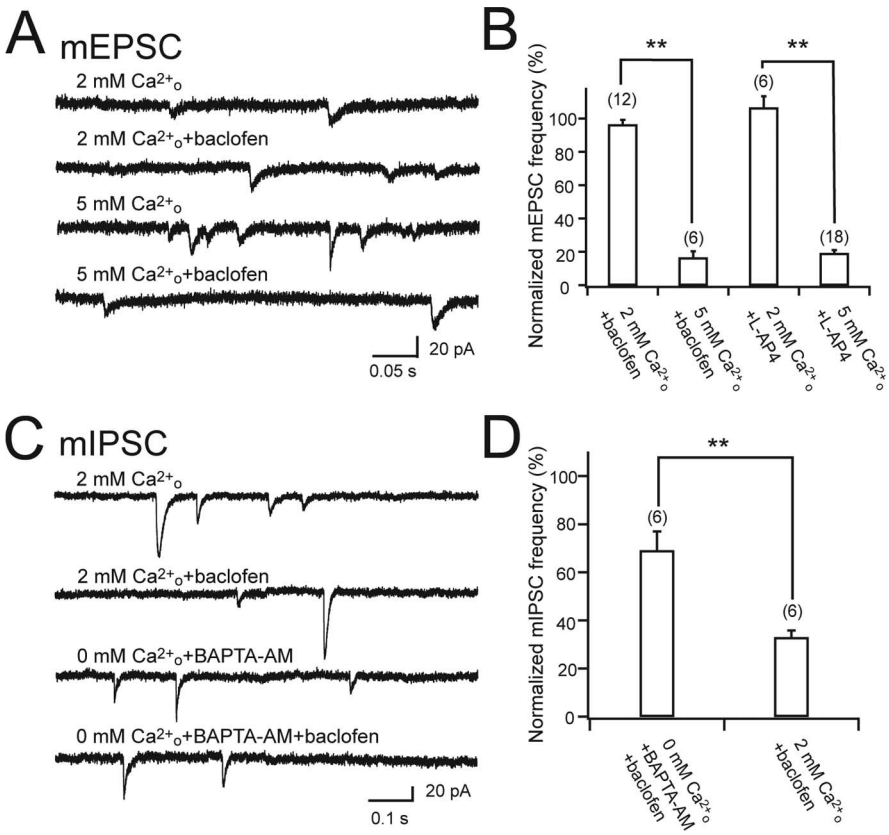

Figure 8. Effects of baclofen and L-AP-4 on miniature synaptic events. ***A***, Representative data showing the effect of baclofen on frequency of mEPSCs. Baclofen had no effect with 2 mM Ca^{2+}_o but suppressed mEPSC frequency with 5 mM Ca^{2+}_o. ***B***, Summary bar graph showing the effect of baclofen and L-AP-4 with 2 and 5 mM Ca^{2+}_o. Data are normalized to the baseline frequency before application of baclofen or L-AP-4 and shown as mean ± SEM for the indicated number of experiments. $^{**}p < 0.01$, *t* test. ***C***, Representative data showing the effect of baclofen on frequency of mIPSCs. Baclofen markedly suppressed mIPSC frequency with 2 mM Ca^{2+}_o. The effect of baclofen was much weaker than that with 0 mM Ca^{2+}_o plus BAPTA-AM. ***D***, Summary bar graph showing the effect of baclofen with 2 mM Ca^{2+}_o ($n = 6$) and with 0 mM Ca^{2+}_o plus BAPTA-AM ($n = 6$). Data are normalized to the baseline frequency before application of baclofen and shown as mean ± SEM for the indicated number of experiments. $^{**}p < 0.01$, *t* test.

Regehr, 1996). This apparent discrepancy from our results may be attributed to species difference (between rat and mouse) or to the differential incidence of Ca^{2+}-enhanced events at the normal $[Ca^{2+}]_o$. Because a previous study (Dittman and Regehr, 1996) reports an mEPSC frequency (~3 Hz) that is significantly higher than our data (~0.5 Hz), we assume that a considerable portion of mEPSCs recorded in their experiment might be Ca^{2+}-enhanced events and therefore sensitive to baclofen.

We showed that mIPSC frequency was suppressed by baclofen in the presence of 2 mM Ca^{2+}_o, and the depressant effect was significantly reduced by Ca^{2+} removal together with BAPTA-AM loading (Fig. 8*C*,*D*). This indicates that baclofen preferentially suppresses Ca^{2+}-enhanced mIPSCs. Previous reports have shown that the effect of baclofen on mIPSCs is not affected by Cd^{2+} (Than and Szabo, 2002; Harvey and Stephens, 2004), indicating that VDCCs are not involved in the baclofen-induced suppression of mIPSCs. Importantly, in PCs, the mIPSCs facilitated by ionomycin are suppressed by baclofen (Than and Szabo, 2002), whereas those triggered by α-LTX are not (Harvey and Stephens, 2004). Together, the mIPSC depression by $GABA_B$ receptor activation may have common nature with the cannabinoid-mediated one. However, a recent report demonstrates that perfusion with a Ca^{2+}-free solution or presynaptic loading of BAPTA-AM does not influence the effect of baclofen on mIPSCs in PCs (Harvey and Stephens, 2004). A possible reason for this apparent discrepancy from our result is imperfect blockade of Ca^{2+}-enhanced miniature events. We found that BAPTA-AM loading alone was not sufficient to suppress Ca^{2+}-enhanced mIPSCs because the subsequent $[Ca^{2+}]_o$ removal further suppressed mIPSC frequency.

Baclofen appeared to have a slight effect on mIPSCs even after Ca^{2+}_o removal combined with BAPTA-AM loading. This might be because either baclofen suppresses the basal mIPSCs or the Ca^{2+}-enhanced mIPSCs still remain under such condition. Then, why does Ca^{2+}_o removal exert different effects after $GABA_B$ receptor and cannabinoid receptor activation? We found that the depressant effect of baclofen on mIPSCs was stronger than that of WIN55,212-2 in the presence of 2 mM Ca^{2+}_o (32.9 ± 2.9% with baclofen; 56.9 ± 5.2% with WIN55,212-2) (Figs. 7, 8*C*,*D*). Therefore, it could be possible that coupling efficacy of $GABA_B$ receptor to the GABA release mechanism is stronger than that of cannabinoid receptor to the release mechanism.

Possible contribution to synaptic transmission

In the cerebellum, basket cell to PC transmission is partially mediated by Ca^{2+} release from intracellular stores (Bardo et al., 2002; Galante and Marty, 2003). In addition, mIPSCs with 2 mM Ca^{2+}_o are also mediated by Ca^{2+} release from internal stores (Llano et al., 2000). In contrast, Ca^{2+} release does not contribute to evoked or miniature excitatory synaptic responses in PCs (Carter et al., 2002). In contrast, in the hippocampus, Ca^{2+} release is reported to contribute to excitatory but not to inhibitory transmission (Emptage et al., 2001; Bouchard et al., 2003). Several previous reports have consistently shown that, at normal external Ca^{2+} and K^+ concentrations, mIPSCs in the hippocampus are not affected by cannabinoid agonists or endocannabinoids released by DSI protocol (Pitler and Alger, 1994; Alger et al., 1996; Hajos et al., 2000), whereas mEPSCs are clearly suppressed (Misner and Sullivan, 1999; Sullivan, 1999). Therefore, it is conceivable that, at the normal $[Ca^{2+}]_o$, mEPSCs in the hippocampus may involve Ca^{2+}-enhanced events and thus sensitive to cannabinoid agonists, whereas mIPSCs may be almost devoid of Ca^{2+}-enhanced events and thus insensitive to cannabinoid agonists. Together, these lines of evidence suggest that Ca^{2+}-enhanced miniature synaptic events are specifically suppressed by activation of cannabinoid receptors and presumably also of other $G_{i/o}$-coupled receptors in various brain regions.

Because synaptic transmission by an action potential is triggered by rapid $[Ca^{2+}]_i$ rise in the presynaptic terminals through VDCCs, released vesicles are regarded as Ca^{2+}-dependent ones. Therefore, certain extent of presynaptic depression of action potential-evoked transmitter release by $G_{i/o}$-coupled receptors may be attributed to the suppression of Ca^{2+}-dependent release machinery. Activation of $G_{i/o}$-coupled receptors also reduces

Ca^{2+} influx into presynaptic terminals presumably through inhibition of VDCCs (Dittman and Regehr, 1996; Diana et al., 2002; Brown et al., 2004), which can also contribute to the suppression of transmitter release.

A previous report suggests that presynaptic depression by cannabinoid agonist is mainly induced by suppression of Ca^{2+} influx at the PF–PC synapse (Brown et al., 2004). In contrast, presynaptic depression by baclofen at the same synapse is induced by both reduced Ca^{2+} influx and inhibition of release machinery (Dittman and Regehr, 1996). These results suggest that the contribution of the modulation of Ca^{2+}-dependent release machinery to presynaptic depression may vary depending on the classes of $G_{i/o}$-coupled receptors. At the inhibitory synapses of PCs, Diana and Marty (2003) estimated that reduction of mIPSC frequency contributed to 13.4% of DSI. In addition, mIPSC amounted to ~47% of spontaneous IPSC in PCs (Diana and Marty, 2003). Therefore, modulation of Ca^{2+}-enhanced mIPSCs seems to have important roles in controlling inhibitory synaptic transmission to PCs. Because Ca^{2+}-enhanced miniature events are present at synapses in various brain regions (Llano et al., 2000; Bardo et al., 2002; Simkus and Stricker, 2002), specific suppression of these events by cannabinoid receptor and other $G_{i/o}$-coupled receptors may be an important mechanism for modulating synaptic transmission.

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