

The Cannabinoid CB1 Receptor Mediates Retrograde Signals for Depolarization-Induced Suppression of Inhibition in Cerebellar Purkinje Cells

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Action potential firing or depolarization of the postsynaptic neuron can induce a transient suppression of inhibitory synaptic inputs to the depolarized neuron in the cerebellum and hippocampus. This phenomenon, termed depolarization-induced suppression of inhibition (DSI), is initiated postsynaptically by an elevation of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and is expressed presynaptically as a suppression of the transmitter release. It is, therefore, thought that some retrograde signal must exist from the depolarized postsynaptic neurons to the presynaptic terminals. Recent studies on hippocampal neurons have revealed that endogenous cannabinoids (endocannabinoids) play a key role as a retrograde messenger. There are, however, conflicting reports that glutamate may be a candidate retrograde messenger for cerebellar DSI that acts on presynaptic group II metabotropic glutamate receptors (mGluRs). In this study, we examined whether endo-

cannabinoids mediate retrograde signal for cerebellar DSI. We recorded IPSCs from Purkinje cells by stimulating putative basket cell axons in mouse cerebellar slices. DSI was readily induced in evoked IPSCs by a depolarizing pulse train. We found that DSI was completely occluded by a cannabinoid agonist, WIN55,212-2, was totally eliminated by a specific antagonist of the type 1 cannabinoid (CB1) receptor, SR141716A, and was deficient in the CB1 knock-out mouse. In contrast, a group II mGluR-specific agonist, (2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine, did not completely occlude DSI, and an mGluR antagonist, (RS)- α -methyl-4-carboxyphenylglycine, had no depressant effect on DSI. These results clearly indicate that the CB1 receptor mediates retrograde signal for DSI in cerebellar Purkinje cells.

Key words: inhibitory transmission; cerebellum; Purkinje cell; retrograde signal; synaptic modulation; cannabinoid receptor

The synapse is a main target of various neuromodulators in the CNS. In cerebellar Purkinje cells (Llano et al., 1991) and hippocampal neurons (Pitler and Alger, 1992; Ohno-Shosaku et al., 1998), action potential firing or postsynaptic depolarization causes a transient suppression of inhibitory synaptic inputs to the depolarized neuron. This phenomenon, termed depolarization-induced suppression of inhibition (DSI), is initiated postsynaptically by an elevation of cytoplasmic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and is expressed presynaptically as a suppression of the transmitter release (Llano et al., 1991; Pitler and Alger, 1992; Ohno-Shosaku et al., 1998), suggesting the existence of some retrograde messenger that mediates DSI. It is reported that glutamate is a candidate retrograde messenger of DSI in the cerebellum (Glitsch et al., 1996) and the hippocampus (Morishita et al., 1998). It is proposed that glutamate is released from the postsynaptic neuron in a Ca^{2+} -dependent manner, acts on presynaptic metabotropic glutamate receptors (mGluRs) and suppresses GABA release. However, a widely used mGluR antagonist, (RS)- α -methyl-4-carboxyphenylglycine (MCPG), is either

not effective in cerebellar Purkinje cells (Glitsch et al., 1996) or only partially effective in hippocampal neurons (Morishita et al., 1998) to antagonize DSI. It is therefore conceivable that glutamate may not be the major mediator, if any, for DSI.

Recent studies have provided strong evidence that endogenous cannabinoids (endocannabinoids) mediate retrograde signals for DSI in hippocampal neurons (Maejima et al., 2001a; Montgomery and Madison, 2001; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001; Wilson et al., 2001). These studies indicate that endocannabinoids are released from depolarized hippocampal neurons in a Ca^{2+} -dependent manner and act on cannabinoid receptors at inhibitory presynaptic terminals. In the cerebellum, a DSI-like phenomenon at excitatory synapses (depolarization-induced suppression of excitation; DSE) is also mediated by endocannabinoids (Kreitzer and Regehr, 2001a; Maejima et al., 2001b). Cannabinoid receptors are the molecular targets for the active component Δ^9 -tetrahydrocannabinol of marijuana and hashish. They consist of type 1 (CB1) and type 2 (CB2) receptors (Matsuda et al., 1990; Munro et al., 1993) of which the CB1 is rich in various regions of the CNS (Herkenham et al., 1990, 1991). The CB1 receptors are strongly expressed at the basket cell terminals around Purkinje cell somata (Egertova and Elphick, 2000). Application of a cannabinoid agonist to cerebellar slices is shown to suppress spontaneous IPSCs in Purkinje cells (Takahashi and Linden, 2000). These results suggest that endocannabinoids mediate DSI of cerebellar Purkinje cells via CB1 receptors on inhibitory presynaptic terminals.

To test this hypothesis, we recorded IPSCs from mouse Pur-

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kinje cells by stimulating putative basket cell axons. We found that DSI was completely occluded by a cannabinoid agonist, WIN55,212-2, and was abolished by a specific CB1 receptor antagonist, SR141716A. Furthermore, DSI was deficient in Purkinje cells of the CB1 knock-out mouse. In contrast, a group II mGluR-specific agonist, (2*S*,2'*R*,3'*R*)-2-(2',3'-dicarboxycyclopropyl)glycine (DCG IV), did not completely occlude DSI and MCPG had no effect on DSI. These results clearly indicate that the retrograde signal for DSI is mediated by endocannabinoids in cerebellar Purkinje cells.

MATERIALS AND METHODS

Electrophysiological recording. All experiments were performed according to the guidelines laid down by the animal welfare committee of Kanazawa University. Parasagittal cerebellar slices (250- μ m-thick) were prepared from C57BL/6 mice aged 8–13 d postnatally, as described (Kano et al., 1995, 1997). Whole-cell recordings were made from visually identified Purkinje cells, using an upright microscope (BX50WI; Olympus, Tokyo, Japan). Resistance of the patch pipette was 3–6 M Ω when filled with the standard intracellular solution composed of (in mM): 140 CsCl, 10 HEPES, 1 EGTA, 0.1 CaCl₂, 4.6 MgCl₂, 4 Na-ATP, and 0.4 Na-GTP, pH 7.3, adjusted with CsOH. The pipette access resistance was compensated by 70–80%. The composition of the standard bathing solution was (in mM): 125 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgSO₄, 1.25 NaH₂PO₄, 26 NaHCO₃, and 20 glucose, which was bubbled continuously with a mixture of 95% O₂ and 5% CO₂. The bath solution was supplemented with 10 μ M 6-cyano-7-nitroquinoxaline-2,3-dione and 10 μ M (RS)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid for recording IPSCs. Membrane currents were recorded with an Axopatch-1D (Axon Instruments, Foster City, CA) amplifier. Glass pipettes filled with the standard extracellular solution were used to stimulate putative basket cell axons in the deep molecular layer close to the Purkinje cell layer. The PULSE software (HEKA Elektronik, Lambrecht/Pfalz, Germany) was used for stimulation and data acquisition. Two successive stimulus pulses with an interstimulus interval of 50 msec were repeated every 3 sec. Purkinje cells were usually held at a membrane potential of –70 or –80 mV. The signals were filtered at 3 kHz and digitized at 20 kHz. The decay phase of IPSCs was fitted with a single exponential by using PULSE-FIT software (HEKA). In the end of each experiment, we applied 10 μ M bicuculline to the bath and confirmed that the recorded synaptic currents were GABA_A receptor-mediated IPSCs. All agonists and antagonists (Tocris Cookson, Ballwin, MO, except SR141716A) were applied to the bath. For the perfusion of solutions containing WIN55,212-2, or SR141716A, different tubes were used to avoid contamination. SR 141716A was a generous gift from Sanofi Recherche.

Induction and estimation of DSI. To induce DSI, a series of depolarizing pulses (10 pulses of 100 msec duration from –70 or –80 mV to 0 mV, repeated at 1 Hz) was applied to Purkinje cells. The depolarizing pulse train was applied repeatedly. However, the results from the first pulse train were excluded to minimize contamination with the rebound potentiation (Kano et al., 1992; Vincent et al., 1992). DSI was estimated as the percentage of the mean amplitude of five consecutive IPSCs after the pulse train (acquired between 3 and 18 sec after the end of pulse) relative to that of five IPSCs just before the pulse train. The depression caused by drugs was estimated as the percentage of the mean amplitudes of 10 consecutive IPSCs during drug application relative to that before application. Averaged data from different experiments are presented as mean $\pm$ SEM.

CB1 receptor knock-out mouse. CB1 receptor knock-out mice were generated as described (Zimmer et al., 1999). Briefly, the coding region of the CB1 gene was replaced between amino acids 32 and 448 with PGK-neo in embryonic stem cells. Chimeric mice derived from these cells were bred with C57BL/6J animals. Homozygous mutants (CB1^{–/–}) and wild-type (CB1^{+/+}) mice were produced with heterozygous intermatings. In the present investigation, juvenile mutant mice with both sexes aged 9 and 12 d were used. Animals were housed in groups under standard laboratory conditions (12 hr light/dark cycle) with food and water available *ad libitum*.

Ca²⁺ imaging. Purkinje cells were loaded for at least 20 min with a Ca²⁺ indicator (Magnesium Green; Molecular Probes, Eugene, OR; 500 μ M) through patch pipette filled with the cesium-based intracellular solution that was composed of (in mM): 140 CsCl, 10 HEPES, 1 EGTA, 0.1 CaCl₂, 0.3 MgCl₂, 4 Na-ATP, and 0.4 Na-GTP, pH 7.3, adjusted with

CsOH. Fluorescence images were acquired by using a high-speed confocal laser-scanning microscope (Oz; NORAN Instruments Inc., Middleton, WI). The Ca²⁺-dependent fluorescence signals from selected regions of interest were background-corrected and expressed as increases in fluorescence divided by the prestimulus fluorescence values ($\Delta F/F_0$) using Igor Pro software (Wavemetrics, Lake Oswego, OR).

RESULTS

Depolarization-induced suppression of evoked IPSCs in mouse Purkinje cells

Previous studies on DSI of cerebellar Purkinje cells were mostly performed on spontaneous IPSCs in the rat (Llano et al., 1991; Glitsch et al., 1996). We began by examining whether DSI can be induced for IPSCs evoked by stimulating putative basket cell axons in Purkinje cells of the mouse cerebellum. When a series of depolarizing pulses (10 pulses of 100 msec duration from –70 to 0 mV, repeated at 1 Hz) were applied to Purkinje cells, the subsequent IPSCs were suppressed transiently (Fig. 1*A*). We found that DSI can be induced repeatedly in the same Purkinje cell without any run-down of its magnitude (Fig. 1*A,D*). DSI was accompanied by a clear change in the paired-pulse ratio (Fig. 1*B,C*), confirming that DSI is caused by the decrease in GABA release from the inhibitory synaptic terminals. The average data from seven cells indicate that the suppression of IPSCs reached $26.2 \pm 8.1\%$ of control immediately after the depolarizing pulse train and then recovered gradually within 60 sec to the control level (Fig. 1*E*). These results indicate that DSI is readily induced for evoked IPSCs in mouse cerebellar slices by the depolarization pulse protocol similar to that used for spontaneous IPSCs in the rat.

The group II metabotropic glutamate receptors are not involved in DSI

Previous studies suggest that DSI is mediated by glutamate or glutamate-like substance, which is thought to be released from depolarized postsynaptic neurons, acts on presynaptic mGluRs and suppresses GABA release (Glitsch et al., 1996; Morishita et al., 1998; Glitsch and Jack, 2001). In cerebellar Purkinje cells, the group II mGluRs (mGluR2 and mGluR3) on inhibitory presynaptic fibers are reported to be responsible for DSI (Glitsch et al., 1996; Glitsch and Jack, 2001). We examined whether the DSI of evoked IPSCs in mouse Purkinje cells also involves the group II mGluRs. In the Purkinje cell shown in Figure 2*A*, bath application of a selective group II mGluR agonist, DCG IV (0.3 μ M), induced a reversible suppression of evoked IPSC amplitude to 67% of the control (Fig. 2*Aa,Ad*). DSI was not completely occluded by the DCG IV-induced suppression (Fig. 2*Aa,Ab,Ae,Af*). The level of the maximum suppression during DSI in the control (Fig. 2*Ab*) was almost identical to that in the presence of DCG IV (Fig. 2*Af*). In the same Purkinje cell, bath application of MCPG (3 mM), a group I and II-specific mGluR antagonist, blocked the depressant effect of DCG IV on evoked IPSCs (Fig. 2*Ag,Ah*). In contrast, MCPG (3 mM) had no effect on DSI (Fig. 2*Ai,Aj*). DCG IV clearly increased the paired-pulse ratio of evoked IPSCs (Fig. 2*B,C*), indicating that DCG IV caused reduction of GABA release presumably acting on mGluR2/3 at the inhibitory presynaptic fibers. The IPSC amplitude was depressed to $56.8 \pm 4.5\%$ and $41.1 \pm 4.9\%$ of control by DCG IV at 0.1 and 0.3 μ M, respectively ($n = 5$; $p < 0.05$; paired t test). However, DCG IV at 3 μ M did not induce further depression ($36.7 \pm 6.1\%$ of control) when compared with that at 0.3 μ M ($n = 5$; $p > 0.05$; paired t test), indicating that the depressant effect of DCG IV saturated at 0.3 μ M. The summary graph in Figure 2*D* shows that DSI was

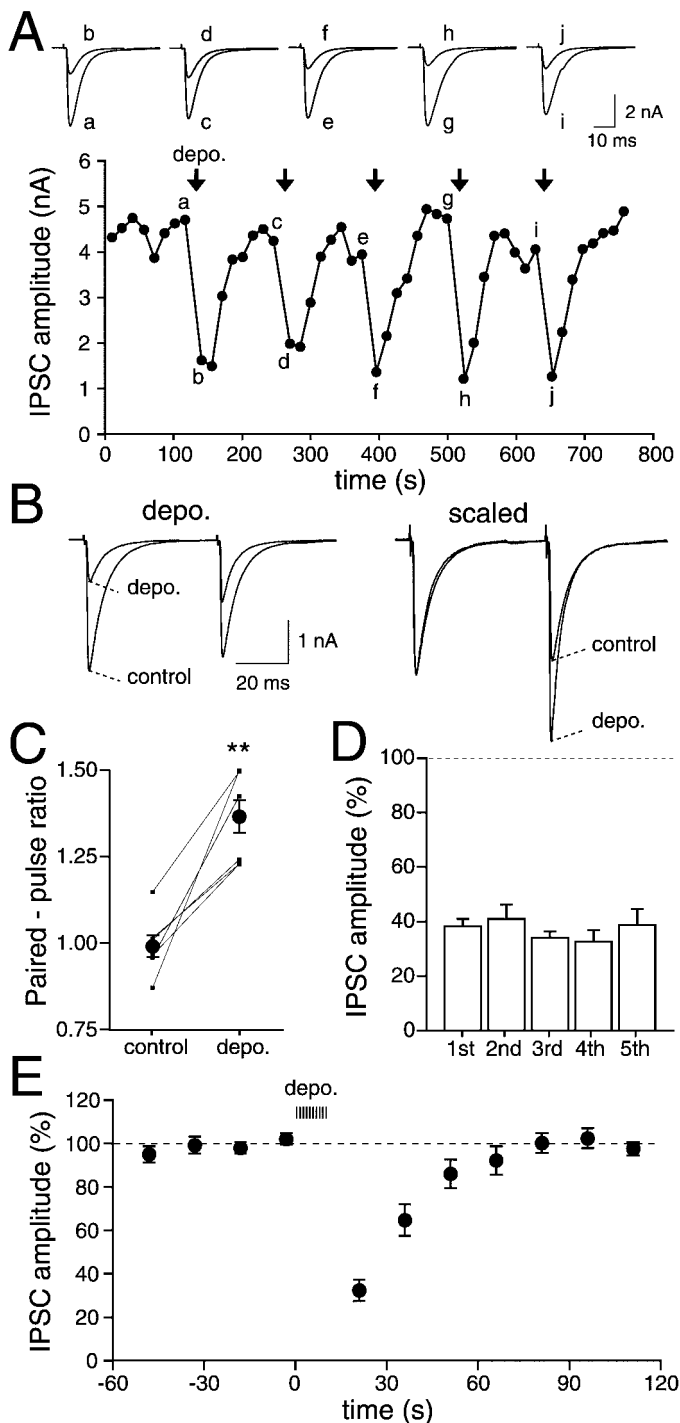


Figure 1. DSI of evoked IPSCs in mouse Purkinje cells. *A*, Top, Example of IPSC traces from a Purkinje cell by stimulating putative basket cell axons. Traces were acquired at the stimulating time points in the graph of the bottom panel. Each trace is the average of five consecutive IPSCs. Bottom, Time course of the change in IPSC amplitudes. IPSCs were evoked at 0.33 Hz. Each data point represents the mean of five consecutive IPSCs. Depolarizing pulse trains (10 pulses of 100 msec duration from -70 to 0 mV, repeated at 1 Hz) were applied to Purkinje cells at the time indicated by arrows. In the following figures, sample IPSC traces and the time course of the change in IPSC amplitudes from a representative Purkinje cell are illustrated in a similar manner as this figure. *B*, Example of IPSC traces (average of five consecutive responses) in response to paired stimuli (50 msec interval). Records obtained before (control) and after (depo.) the depolarizing pulse train are superimposed. In the right panel, the first IPSC after depolarization is scaled to the amplitude of the

not occluded by DCG IV ($0.3 \mu\text{M}$), and the effect of DCG IV ($0.3 \mu\text{M}$) was effectively antagonized by MCPG (3 mM) (Fig. 2*D*). The summary data from six Purkinje cells show that neither the magnitude nor the time course of DSI was affected by MCPG (3 mM) (Fig. 2*E*). These results clearly indicate that the group II mGluRs are not involved in DSI of evoked IPSCs in mouse Purkinje cells.

CB1 receptors mediate DSI

Recent studies clearly demonstrated that DSI in the hippocampus is mediated by endocannabinoids that are released from depolarized postsynaptic neurons and act retrogradely on presynaptic CB1 receptors (Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001; Wilson et al., 2001). We examined whether this scheme is also applied to the DSI in the mouse cerebellum. In the Purkinje cell shown in Figure 3*A*, bath application of a cannabinoid agonist, WIN55,212-2 ($5 \mu\text{M}$), suppressed evoked IPSC amplitude to 32% of the control (Fig. 3*Aa,Ad*). DSI was completely occluded by WIN55,212-2 (Fig. 3*Aa,Ab,Ae,Af*). The effect of WIN55,212-2 was completely reversed by a selective CB1 receptor antagonist, SR141716A ($1 \mu\text{M}$) (Fig. 3*Ag,Ah*). WIN55,212-2 clearly increased the paired-pulse ratio of evoked IPSCs (Fig. 3*B,C*), indicating that WIN55,212-2 caused reduction of GABA release acting on CB1 receptors at the inhibitory presynaptic fibers. The summary graph in Figure 3*D* shows that DSI was completely occluded by WIN55,212-2, and the effect of WIN55,212-2 was totally antagonized by SR141716A ($1 \mu\text{M}$). These data suggest that DSI and the WIN55,212-2-induced suppression share the common presynaptic mechanisms.

We then examined whether SR141716A can block DSI of evoked IPSCs in the mouse cerebellum. In the Purkinje cell shown in Figure 4*A*, the depolarizing pulse trains induced typical DSI of evoked IPSCs (Fig. 4*Aa,Ab,Ac,Ad*). Subsequent application of the same pulse trains after perfusion of SR141716A ($1 \mu\text{M}$)-containing external solution caused no change in IPSCs (Fig. 4*Ae–Ah*). The summary data from six Purkinje cells clearly show that DSI was completely abolished by SR141716A ($1 \mu\text{M}$) (Fig. 4*B*).

To confirm the involvement of CB1 receptors in DSI, we examined the cerebellum of the CB1 knock-out mouse. It is recently reported that hippocampal DSI is deficient in the CB1 knock-out mouse (Wilson et al., 2001). We found no perceptible difference in the kinetics and paired-pulse plasticity of IPSCs in Purkinje cells from the CB1 knock-out mouse. However, the depolarizing pulse train caused no change in the amplitude (Fig. 5*A,E*) or paired-pulse ratio (Fig. 5*B,C*) of IPSCs, indicating that DSI is totally deficient in the CB1 knock-out mouse. The group II mGluRs were functionally intact in the CB1 knock-out mouse because DCG IV caused a reversible inhibition of IPSCs that was very similar to that of the wild-type mouse (Fig. 5*D,E*). Further-

first IPSC of the control and superimposed. In the following figures, sample IPSC traces to paired stimuli from a representative Purkinje cell are illustrated in a similar manner as this figure. *C*, Changes in paired-pulse ratio induced by the depolarizing pulse train for individual Purkinje cells ($n = 6$). Data from the same Purkinje cells were connected. Closed symbols with error bars represent average values (mean $\pm$ SEM). *D*, Reduction of IPSC amplitude induced by the depolarizing pulse trains applied at 120 sec intervals to the same Purkinje cells ($n = 4$). *E*, Averaged time course of the changes in IPSC amplitudes induced by the depolarizing pulse train ($n = 7$). * $p < 0.05$; ** $p < 0.01$ (paired t test).

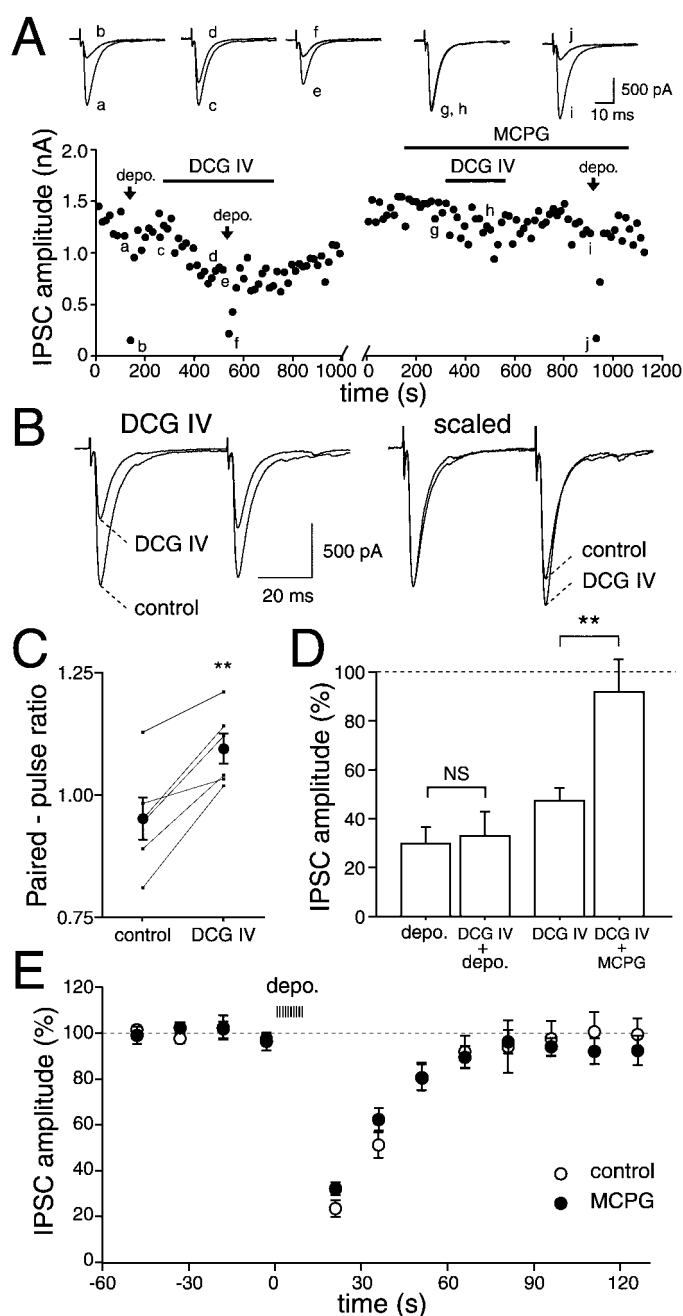


Figure 2. Group II mGluRs do not mediate DSI. *A*, Bath-applied DCG IV ($0.3 \mu\text{M}$) induces a suppression of evoked IPSCs but the suppression does not completely occlude DSI. In the same Purkinje cell, the depressant effect of DCG IV on IPSCs is blocked by a group I and II-specific mGluR antagonist, MCPG (3 mM). In contrast, DSI is totally insensitive to MCPG (3 mM). *B*, Bath-applied DCG IV ($0.3 \mu\text{M}$) induces a clear increase in the paired-pulse ratio of evoked IPSCs. *C*, Changes in paired-pulse ratio induced by DCG IV ($0.3 \mu\text{M}$) for individual Purkinje cells ($n = 6$; $**p < 0.01$; paired t test). *D*, Summary bar graph for the changes in IPSC amplitudes induced by the depolarizing pulse train (depo.), the depolarizing pulse train plus $0.3 \mu\text{M}$ DCG IV (DCG IV + depo.), $0.3 \mu\text{M}$ DCG IV, and $0.3 \mu\text{M}$ DCG IV in the presence of 3 mM MCPG (DCG IV + MCPG). Data from six Purkinje cells. $**p < 0.01$; NS; $p > 0.05$ (paired t test). *E*, Averaged time courses of DSI in the control (open symbols) and after bath-application of 3 mM MCPG (closed symbols). Data from six Purkinje cells.

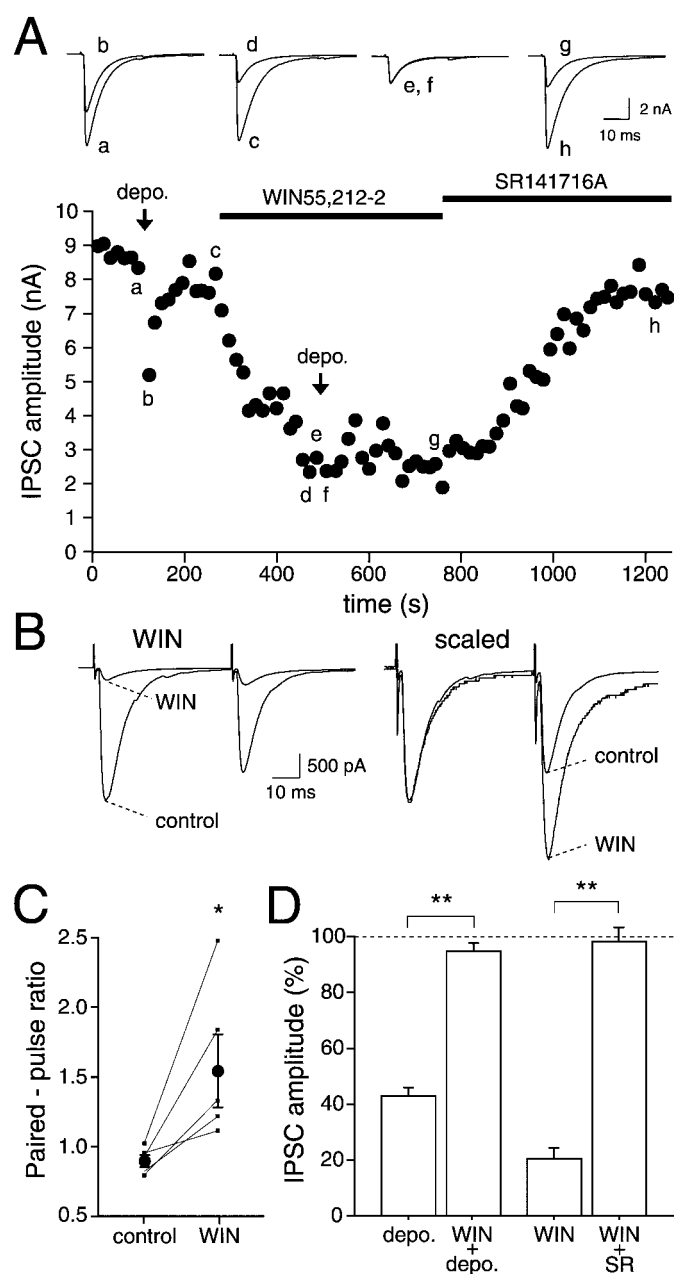


Figure 3. A cannabinoid agonist, WIN55,212-2, completely occludes DSI. *A*, Bath-applied WIN55,212-2 ($5 \mu\text{M}$) induces a strong suppression of evoked IPSCs, which completely occludes DSI. A selective CB1 receptor antagonist, SR141716A ($1 \mu\text{M}$), reverses the effect of WIN55,212-2. *B*, Bath-applied WIN55,212-2 ($5 \mu\text{M}$) induces a clear increase in the paired-pulse ratio of evoked IPSCs. *C*, Changes in paired-pulse ratio induced by WIN55,212-2 ($5 \mu\text{M}$) for individual Purkinje cells ($n = 5$; $*p < 0.05$; paired t test). *D*, Summary bar graph for the changes in IPSC amplitudes induced by the depolarizing pulse train (depo.), the depolarizing pulse train plus $5 \mu\text{M}$ WIN55,212-2 (WIN + depo.), $5 \mu\text{M}$ WIN55,212-2 (WIN), and $5 \mu\text{M}$ WIN55,212-2 in the presence of $1 \mu\text{M}$ SR141716A (WIN + SR). Data from six Purkinje cells. $*p < 0.05$; $**p < 0.01$ (paired t test).

more, we verified that the inhibitory synapses of the CB1 knock-out mouse had no cannabinoid sensitivity because WIN55,212-2 ($5 \mu\text{M}$) had no effect on IPSCs (Fig. 5*D,E*). These results indicate that DSI is mediated by endocannabinoids that act on presynaptic CB1 receptors.

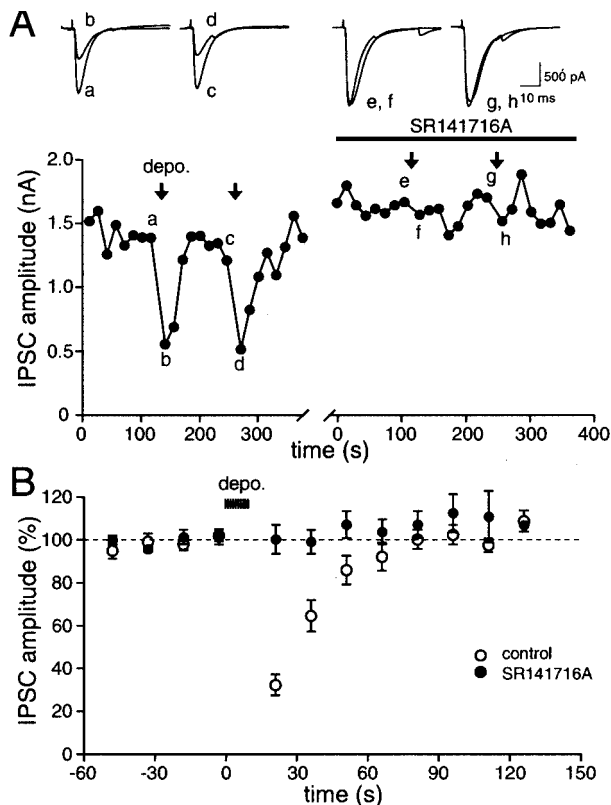


Figure 4. SR141716A totally eliminates DSI. *A*, Repeated application of the depolarizing pulse train at the times indicated by arrows (*depo.*) induces typical DSI with similar amplitudes in a Purkinje cell. After perfusion of SR141716A (1 μM)-containing external solution, application of the same pulse train causes no change in IPSCs. *B*, Averaged time courses of DSI in the control (*open symbols*) and after bath-application of 1 μM SR141716A (*closed symbols*). Data from six Purkinje cells.

Neither SR141716A nor genetic deletion of CB1 receptors affect depolarization-induced Ca^{2+} transients

The data presented so far indicate that presynaptic CB1 receptors are essential for DSI. It is, however, still theoretically possible that SR141716A or genetic deletion of CB1 receptors reduced the elevation of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) of Purkinje cells during the depolarizing pulse train and thereby blocked the induction of DSI. To exclude this possibility, we examined whether the Ca^{2+} transients by the depolarizing pulse train are affected by SR141716A. We recorded from Purkinje cells with an internal solution containing a low affinity Ca^{2+} indicator (Magnesium Green; 500 μM) and measured the Ca^{2+} transients at the Purkinje cell somata (Eilers et al., 1995) as indicated in Figure 6*A*. The depolarizing pulse train induced DSI of evoked IPSCs (Fig. 6*B*) and transient elevation of $[\text{Ca}^{2+}]_i$ at the soma (Fig. 6*D*). After perfusion of SR141716A, DSI was eliminated (Fig. 6*C*), whereas the Ca^{2+} transients remained unchanged (Fig. 6*E*). The summary data show that SR141716A blocked DSI (the DSI amplitudes are $57.2 \pm 3.1\%$ in normal saline and $86.6 \pm 4.2\%$ in SR141716A; $n = 5$; $p < 0.01$; t test) but had no effect on the peak Ca^{2+} transients induced by the depolarizing pulse train (the $\Delta F/F_0$ values are 104.9 ± 4.2 in normal saline and 96.5 ± 3.8 in SR141716A; $n = 6$; $p > 0.05$; t test). Furthermore, we confirmed that the depolarization-induced Ca^{2+} transients are intact in the CB1 knock-out mouse. The

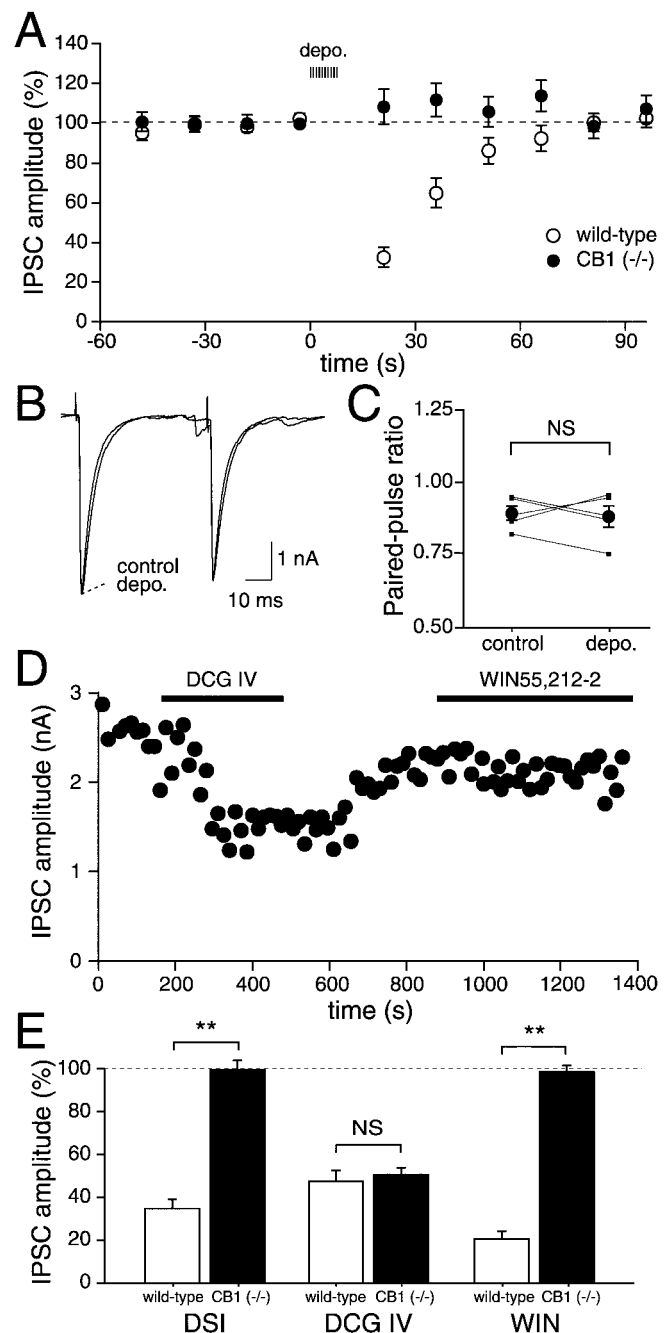


Figure 5. DSI is deficient in the CB1 receptor knock-out mouse. *A*, Averaged time courses of DSI in the wild-type (*open symbols*, $n = 7$; the same data illustrated in Fig. 1*E*) and CB1 knock-out (*closed symbols*, $n = 5$) mice. *B*, Example of IPSC traces (average of five consecutive responses) in response to paired stimuli (50 msec interval) from a CB1 knock-out mouse recorded before (*control*) and after (*depo.*) the depolarizing pulse train. *C*, Changes in paired-pulse ratio induced by the depolarizing pulse train for individual Purkinje cells from CB1 knock-out mice ($n = 5$; NS > 0.05; paired t test). *D*, Time course of the change in IPSC amplitudes from a representative CB1 knock-out Purkinje cell. Bath-applied DCG IV (0.3 μM) induces a suppression of evoked IPSCs, whereas WIN55,212-2 (5 μM) had no effect. *E*, Summary bar graph for the changes in IPSC amplitudes induced by the depolarizing pulse train (*DSI*), 0.3 μM DCG IV (*DCG IV*), and 5 μM WIN55,212-2 (*WIN*) for the wild-type (*open columns*) and CB1 knock-out (*filled columns*) mice. The data for wild-type mice and are the same illustrated in Figure 2*D* (*DSI* and *DCG IV*) and 3*D* (*WIN*). Data from 6 wild-type and five CB1 knock-out Purkinje cells. ** $p < 0.01$; NS; $p > 0.05$ (t test).

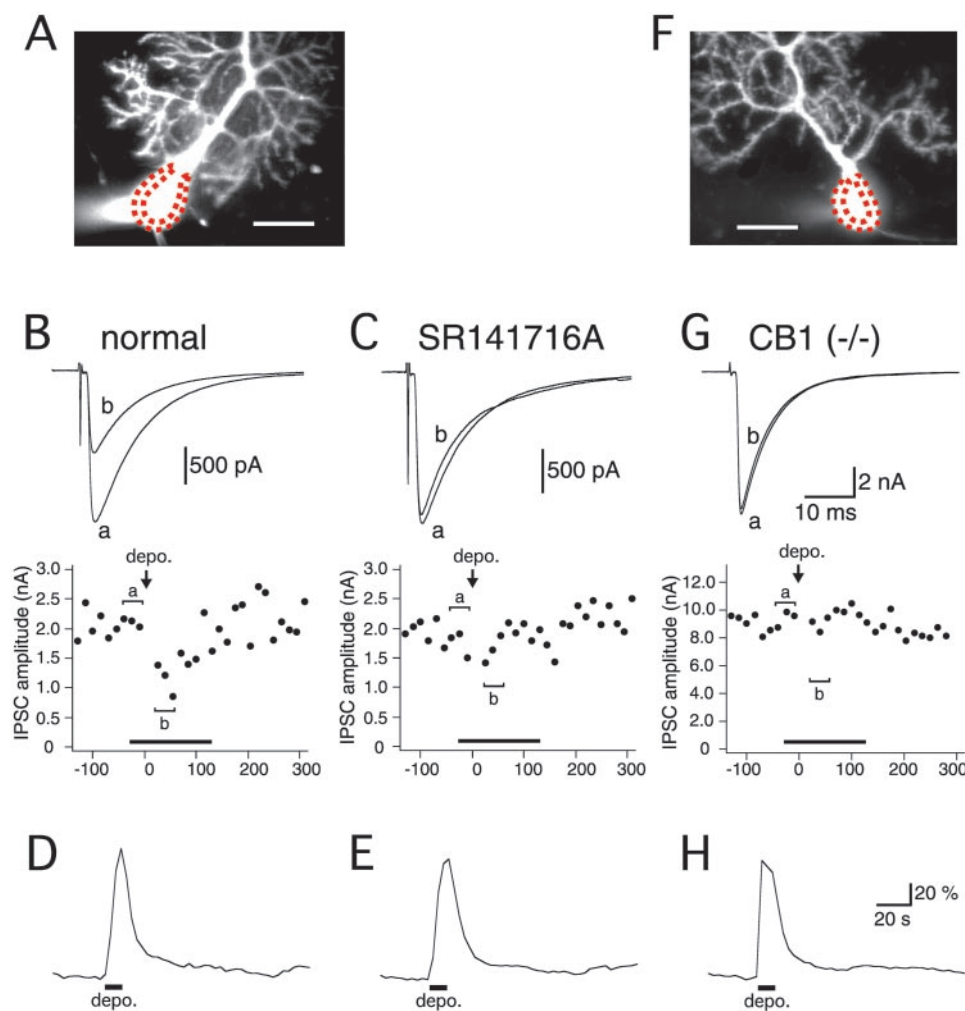


Figure 6. Neither SR141716A nor genetic deletion of CB1 receptors affect depolarization-induced Ca^{2+} transients. *A, F*, Reconstructed images of Purkinje cells from a wild-type mouse (*A*) and a CB1 knock-out mouse (*F*). The area depicted with dotted red line in each image indicates the somatic submembrane shell [Eilers et al., 1995 from which the Ca^{2+} signals shown in *D* and *E* (for *A*) or in *H* (for *F*)] are measured. Scale bars, 20 μm . *B, C, G*, Sample traces of IPSCs and time course of the change in IPSC amplitude caused by the depolarizing pulse train (*depo.*) for the wild-type Purkinje cell shown in *A* (for *B, C*) and for the CB1 knock-out Purkinje cell shown in *F* (for *G*). Records were obtained in normal external solution (*B, G*) and after bath application of SR141716A (1 μM) (*C*). *D, E, H*, Ca^{2+} signals from the regions depicted in *A* (for *D, E*) and in *F* (for *H*) obtained in normal external solution (*D, H*) and after bath application of SR141716A (1 μM) (*E*). The periods during which the Ca^{2+} signals by the depolarizing pulse train (*depo.*) are measured are depicted with horizontal bars in *B, C*, and *G*.

depolarizing pulse train induced no DSI (Fig. 6*G*) but elicited transient elevation of $[\text{Ca}^{2+}]_i$ (Fig. 6*H*) measured at the soma (Fig. 6*F*). The summary data from nine Purkinje cells of CB1 knock-out mice shows no DSI ($89.4 \pm 4.0\%$; $n = 9$; $p < 0.01$; compared with the wild-type; t test) and intact depolarization-induced Ca^{2+} transients ($\Delta F/F_0$; 95.9 ± 5.6 ; $n = 9$; $p > 0.05$; compared with the wild-type; t test). These results lead us to conclude that DSI in the mouse cerebellum is mediated by endocannabinoids that are released from depolarized Purkinje cells and act retrogradely on CB1 receptors at inhibitory presynaptic terminals.

DISCUSSION

We report that DSI can readily be induced in IPSCs evoked by stimulating putative basket cell axons in mouse cerebellar Purkinje cells. Although a group II mGluR-specific agonist, DCG IV, caused a reversible presynaptic suppression of IPSCs, DCG IV with a saturating dose (0.3 μM) did not completely occlude DSI. A mGluR antagonist, MCPG (3 mM), completely blocked the presynaptic effect of DCG IV, whereas it had no effect on DSI. These results clearly indicate that group II mGluRs on inhibitory presynaptic fibers do not play a major role in DSI. On the other hand, a cannabinoid receptor agonist, WIN55,212-2 (5 μM), caused a presynaptic suppression of IPSCs and completely occluded DSI. A CB1-specific cannabinoid antagonist, SR141716A (1 μM), totally abolished DSI. Furthermore, DSI was deficient in

the cerebellum of the CB1 receptor knock-out mouse. These results clearly indicate that DSI in the mouse cerebellum is mediated by endocannabinoids that are released from depolarized Purkinje cells and act retrogradely on CB1 receptors at inhibitory presynaptic terminals.

Glutamate is not a major mediator of DSI

In slices from the cerebellum and hippocampus, it was considered previously that DSI is mediated by glutamate or a glutamate-like substance (Glitsch et al., 1996; Morishita et al., 1998). In Purkinje cells of the rat cerebellum, DSI of spontaneous IPSCs was reduced when the GABA release was inhibited by saturating doses of DCG IV (Glitsch et al., 1996). Both DSI and DCG IV-induced inhibition of miniature IPSC were inhibited by L-AP-3, which has complex effects on several ionotropic and metabotropic glutamate receptors. However, neither DSI nor DCG IV-induced inhibition was blocked by MCPG, a widely used mGluR antagonist that specifically blocks both group I and II mGluRs. From these results, Glitsch et al. (1996) propose that glutamate or a glutamate-like substance is released from depolarized rat Purkinje cells and acts on an unusual type of group II mGluR on inhibitory presynaptic fibers that is sensitive to DCG IV but totally insensitive to MCPG (Glitsch et al., 1996, 2000). In contrast, we have demonstrated in mouse Purkinje cells that DCG IV-induced presynaptic inhibition was almost completely blocked by MCPG (3 mM), whereas DSI was totally insensitive to the

same dose of MCPG. Our results indicate that mouse inhibitory presynaptic fibers have a usual type of group II mGluR that is sensitive to DCG IV and is blocked by MCPG. The group II mGluR does not play a major role in DSI in mouse Purkinje cells. The reasons for the discrepancy between the results of Glitsch et al. (1996) and ours are not clear. They showed that L-AP-3 acted both as an antagonist and as a partial agonist of the group II mGluR in the rat (Glitsch et al., 1996). L-AP-3 reduced the basal miniature IPSC frequency by ~28%. They report that DSI was reduced by L-AP-3 from $51.3 \pm 2.6\%$ in the control to $30.0 \pm 4.0\%$. Therefore, the reduction of DSI may reflect the partial occlusion of DSI by the inhibition of GABA release caused by the agonistic action of L-AP-3 on group II mGluRs. They reported that MCPG (1 mM) failed to inhibit the effects of DCG IV. However, they used relatively high doses (1–5 μM) of DCG IV, which is ~10 times higher than the saturating dose of DCG IV (0.3 μM) for mouse Purkinje cells. Because MCPG is a competitive mGluR antagonist, its effect must be critically dependent on the agonist concentration. In fact, we observed that MCPG had only a weak antagonistic action against higher doses of DCG IV. Taken together, we conclude that glutamate or glutamate-like substance is not a major mediator of DSI in cerebellar Purkinje cells. Glitsch and Jack (2001) recently reported that DSI is reduced only slightly by LY-341495, a highly specific and potent antagonist of group II mGluR. They now conclude that activation of group II mGluRs play a relatively minor role in the induction of cerebellar DSI.

We have reported recently that activation of postsynaptic mGluR1 in Purkinje cells causes reversible suppression of excitatory transmitter release from presynaptic terminals that is mediated by CB1 receptors (Maejima et al., 2001b). This indicates that endocannabinoids are released from Purkinje cells in response to activation of postsynaptic mGluR1. The mGluR1-dependent mechanism may work in a synergistic manner with the Ca^{2+} -dependent pathway for the release of endocannabinoids from Purkinje cells. The depolarization-induced release of endocannabinoids may be enhanced when postsynaptic mGluR1-dependent mechanism is active. It is, therefore, possible that the amplitude of DSI is influenced by the ambient glutamate concentration around Purkinje cells.

Endocannabinoids mediate retrograde modulation of synaptic transmission

It is recently revealed that endocannabinoids mediate the retrograde signal of DSI in the hippocampus (Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001). In cultured hippocampal neurons, the cannabinoid receptor agonist WIN55,212-2 mimics DSI and cannabinoid receptor antagonists, AM 281 and SR141716A completely eliminated DSI (Ohno-Shosaku et al., 2001). Wilson and Nicoll reported essentially the same results in the hippocampal slice (Wilson and Nicoll, 2001). In addition, they found that DSI is completely occluded by the pretreatment of the slices with WIN55,212-2 (Wilson and Nicoll, 2001). Very recently, Kreitzer and Regehr (2001b) reported that DSI of spontaneous IPSCs in the rat cerebellum is not mediated by activation of mGluRs or GABA_B receptors but involves cannabinoid receptors. Furthermore, DSI was absent in the hippocampus of the CB1 receptor knock-out mouse (Wilson et al., 2001). Taken together with the results from the present study, the experimental evidence clearly indicates that endocannabinoids mediate retrograde signal for DSI in the hippocampus and cerebellum.

Kreitzer and Regehr (2001a) reported that depolarization-

induced presynaptic suppression also occurs in excitatory climbing fiber and parallel fiber synapses onto cerebellar Purkinje cells. This phenomenon, termed depolarization-induced suppression of excitation (DSE), was abolished by a CB1 receptor antagonist, AM 251, and was occluded by the pretreatment of the slices with WIN55,212-2 (Kreitzer and Regehr, 2001a). Thus, both DSI and DSE appear to be mediated by endocannabinoids and have mechanisms in common. Postsynaptic depolarization triggers Ca^{2+} influx through voltage-gated Ca^{2+} channels and causes a transient increase in $[\text{Ca}^{2+}]_i$. The $[\text{Ca}^{2+}]_i$ increase will stimulate the biosynthesis of endocannabinoids and cause their release from the depolarized Purkinje cell. In support of this notion, previous biochemical data indicate that the two candidate endocannabinoids, anandamide and 2-arachidonylglycerol (2-AG), are produced and released from neurons in a Ca^{2+} -dependent manner (Di Marzo et al., 1994; Bisogno et al., 1997; Stella et al., 1997). Then, the released endocannabinoids will diffuse the synaptic cleft retrogradely and bind to the CB1 on the inhibitory or excitatory presynaptic terminals contacting onto the depolarized Purkinje cell. The presence of CB1 receptors on inhibitory and excitatory presynaptic fibers in the cerebellum has been verified by immunohistochemical methods (Egertova and Elphick, 2000). Finally, the activation of presynaptic CB1 receptors will suppress the release of GABA or glutamate, presumably by inhibiting voltage-gated Ca^{2+} channels, as shown in neuroblastoma-glioma cells (Mackie and Hille, 1992) and hippocampal neurons (Twitchell et al., 1997; Hoffman and Lupica, 2000). Takahashi and Linden (2000) reported that WIN55,212-2 suppresses the frequency of miniature IPSCs in Purkinje cells. One possibility would be that activation of presynaptic CB1 receptors directly interferes with the release machinery of GABA-containing synaptic vesicles. It is reported that DSI is observed in miniature IPSCs in cerebellar Purkinje cells (Llano et al., 1991; Glitsch et al., 1996; Glitsch and Jack, 2001). These results suggest that endocannabinoids may exert some additional effects on inhibitory presynaptic terminals of the cerebellum to cause DSI.

Recent studies have clarified that endocannabinoid release is triggered by an elevation of $[\text{Ca}^{2+}]_i$ (Kreitzer and Regehr, 2001a; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001) or activation of mGluR1 (Maejima et al., 2001b) in postsynaptic neurons. The elevation of $[\text{Ca}^{2+}]_i$ is caused by the activation of postsynaptic receptors and channels and reflects the activity of postsynaptic neurons. Activation of mGluR1 in Purkinje cells requires repetitive activation of parallel fibers (Batchelor and Garthwaite, 1997; Finch and Augustine, 1998; Takechi et al., 1998), which also reflects the activity of postsynaptic neurons. Presynaptic terminals can sense the postsynaptic activity by detecting released endocannabinoids from postsynaptic neurons with the CB1. Because the CB1 is abundantly expressed in various regions of CNS (Herkenham et al., 1990; Herkenham et al., 1991; Matsuda et al., 1993; Egertova and Elphick, 2000), it is likely that the retrograde modulation mediated by endocannabinoids is a general and important mechanism by which the postsynaptic activity can influence the presynaptic function.

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