Neuron, Vol. 29, 729–738, March, 2001, Copyright ©2001 by Cell Press

Endogenous Cannabinoids Mediate Retrograde Signals from Depolarized Postsynaptic Neurons to Presynaptic Terminals

Takako Ohno-Shosaku, Takashi Maejima, and Masanobu Kano*
Department of Physiology
Kanazawa University School of Medicine
Kanazawa 920-8640
Japan

Summary

Endogenous cannabinoids are considered to function as diffusible and short-lived modulators that may transmit signals retrogradely from postsynaptic to presynaptic neurons. To evaluate this possibility, we have made a paired whole-cell recording from cultured hippocampal neurons with inhibitory synaptic connections. In about 60% of pairs, a cannabinoid agonist greatly reduced the release of the inhibitory neurotransmitter GABA from presynaptic terminals. In most of such pairs but not in those insensitive to the agonist, depolarization of postsynaptic neurons and the resultant elevation of intracellular Ca^{2+} concentration caused transient suppression of inhibitory synaptic currents, which is mainly due to reduction of GABA release. This depolarization-induced suppression was completely blocked by selective cannabinoid antagonists. Our results reveal that endogenous cannabinoids mediate retrograde signals from depolarized postsynaptic neurons to presynaptic terminals to cause the reduction of transmitter release.

Introduction

Cannabinoid receptors are the molecular targets for marijuana and hashish and constitute a major family of G protein–coupled seven-transmembrane domain receptors. They consist of type 1 (CB1) and type 2 (CB2) receptors that differ in their distributions (Matsuda et al., 1990; Munro et al., 1993) (Felder and Glass, 1998, for review). While the CB2 is expressed in the immune system of the periphery (Klein et al., 1998, for review), the CB1 is rich in various regions of the CNS (Herkenham et al., 1991; Matsuda et al., 1993; Tsou et al., 1998; Egertova and Elphick, 2000). Accumulated evidence supports that the CB1 plays an important role in the modulation of synaptic transmission (Di Marzo et al., 1998, for review) and plasticity (Stella et al., 1997; Misner and Sullivan, 1999; Auclair et al., 2000) in the CNS. Two putative endogenous cannabinoid ligands, anandamide (Devane et al., 1992) and sn-2 arachidonylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995), have been identified. These are lipid in nature and are synthesized from membrane phospholipid (Di Marzo et al., 1998; Piomelli et al., 2000, for reviews). It is reported that 2-AG is produced in hippocampal slices in response to stimulation of excitatory fibers in a Ca^{2+}-dependent manner (Stella et al., 1997). It is also reported that anandamide is released in the striatum by activation of D2-like dopamine receptors (Giuffrida et al., 1999). Endogenous cannabinoids are removed by uptake from the site of their action (Di Marzo et al., 1994; Beltramo et al., 1997; Piomelli et al., 1999; Beltramo and Piomelli, 2000). An enzyme that catalyzes the hydrolysis of anandamide and 2-AG, fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996; Goparaju et al., 1998), is strongly expressed in the areas with a high level of the CB1 subtype (Egertova et al., 1998). It is therefore likely that endogenous cannabinoids are diffusible but short-lived modulators that function locally near the site of their production. Retrograde messengers, which are produced in the postsynaptic neurons and influence the presynaptic terminals, have been proposed to modulate synaptic transmission. Endogenous cannabinoids are a good candidate for such retrograde messengers.

The hippocamus is one of the brain regions that contain a high level of CB1 receptors (Herkenham et al., 1991; Matsuda et al., 1993; Tsou et al., 1998; Egertova and Elphick, 2000). In the hippocampus, CB1 receptors (Katona et al., 1999; Tsou et al., 1999) are abundantly expressed in subpopulations of inhibitory interneurons on their presynaptic terminals. The enzyme FAAH is also richly expressed in the proximity to the locations of CB1 receptors (Egertova et al., 1998). A putative endogenous ligand for CB1 receptors, 2-AG (Sugiura et al., 1999), is produced in hippocampal slices in response to stimulation of excitatory fibers (Stella et al., 1997). The activation of CB1 receptors is reported to suppress the release of the inhibitory neurotransmitter γ-amino butyric acid (GABA) from the presynaptic terminals (Katona et al., 1999; Hoffman and Lupica, 2000; Irving et al., 2000). Thus, GABAergic inhibitory synapses of hippocampal neurons provide a good experimental model to study the actions of endogenous cannabinoids on synaptic transmission.

Using hippocampal cultures from the rat, we examined whether endogenous cannabinoids are involved in the suppression of inhibitory synaptic transmission following depolarization of postsynaptic neurons. Our previous results suggest that the main cause of this suppression is the reduction of GABA release from presynaptic terminals and some retrograde messenger must be involved (Ohno-Shosaku et al., 1998). We also demonstrated that the effect of depolarization can spread to the synapses of neighboring nondepolarized neurons, suggesting that some diffusible factor must play a role (Ohno-Shosaku et al., 2000). The results presented here strongly suggest that endogenous cannabinoids mediate retrograde signals from depolarized postsynaptic neurons to presynaptic terminals.

Results

Cannabinoid Agonist Causes Depression of GABA Release

We began by confirming that a selective cannabinoid agonist acted on CB1 receptors on the inhibitory presyn-

*To whom correspondence should be addressed (e-mail: mkano@med.kanazawa-u.ac.jp).

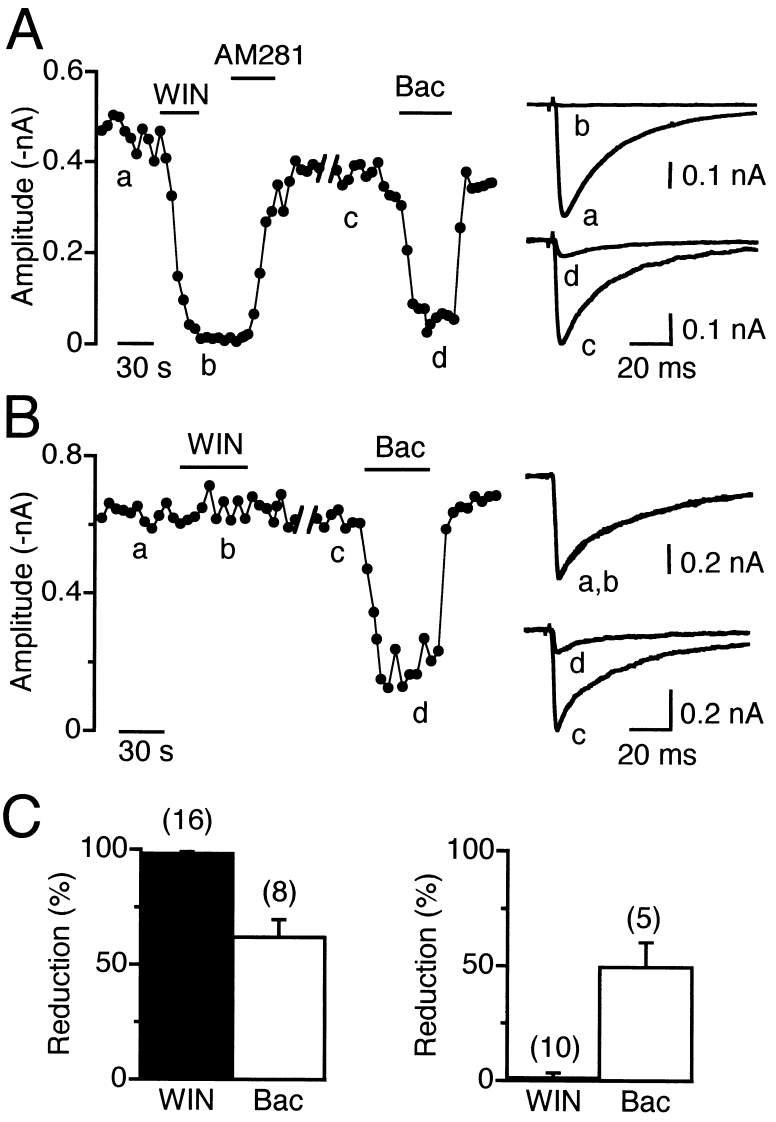

Figure 1. Heterogeneity of the Effects of WIN55,212-2 on IPSCs

(A and B) WIN55,212-2 significantly depressed IPSCs in a neuron pair in (A), while it was ineffective in another pair in (B). Time course of the change in the IPSC amplitude (left) and traces of IPSCs acquired at the indicated time points (right) are shown. The bath was perfused with the solution containing WIN55,212-2 (0.1 μM), AM 281 (0.3 μM), or baclofen (10 μM) for the period indicated by the horizontal bar.

(C) Summary data for neuron pairs with (left) and without (right) WIN55,212-2 sensitivity. Percentage reductions of IPSC amplitudes by 0.1 μM WIN55,212-2 (WIN) and 10 μM baclofen (Bac) were calculated relative to the control values before agonist application. For the following figures, summary data for the reduction of IPSC amplitudes are presented similarly.

aptic terminals. We recorded inhibitory postsynaptic currents (IPSCs) from 26 neuron pairs in hippocampal cultures. These synaptic currents were mediated by $GABA_A$ receptors because they were suppressed by bicuculline. In 16 of 26 pairs, bath application of a selective cannabinoid agonist, WIN55,212-2 (0.1 μM), induced a remarkable suppression of IPSCs (Figure 1A). On the average, the amplitude of IPSCs was depressed to 1.7% of the control by WIN55,212-2 (Figure 1C). The inhibitory transmission was recovered after an addition of a selective cannabinoid antagonist, AM 281 (0.3 μM), to the bath. In the remaining ten pairs, WIN55,212-2 failed to suppress IPSCs (Figure 1B). It seems unlikely that this insensitivity is caused by some damages of synapses, because a $GABA_B$ receptor agonist, baclofen (10 μM), was effective in all the pairs tested, irrespective of the sensitivity to WIN55,212-2 (Figures 1A and 1B). These data clearly indicate that the activation of cannabinoid receptors causes a suppression of the inhibitory transmission in more than half of neuron pairs.

The presynaptic locus for the action of cannabinoids was suggested by the following experiments in which the effect of WIN55,212-2 (0.1 μM) was examined on both IPSCs and inhibitory autaptic currents (IACs) in the same neuron pairs. We used 18 pairs with the synaptic connectivity illustrated in Figure 2A (inset). In a pair where IPSCs were greatly suppressed by WIN55,212-2, IACs were also depressed (Figure 2A, middle). In another pair where IPSCs were insensitive to WIN55,212-2, IACs were also not affected (Figure 2A, bottom). The extent of suppression of IACs was strongly correlated with that of IPSCs (Figure 2B). Because IACs and IPSCs in a given neuron pair reflect the activity of presynaptic terminals of the same neuron (Figure 2A, inset), the summary data in Figure 2B suggest that the presynaptic neuron is responsible for the sensitivity to WIN55,212-2.

The presynaptic locus for the action of WIN55,212-2 was further confirmed by monitoring the change in paired-pulse ratio that presumably reflects the state of the readily releasable transmitter pool at presynaptic terminals. In our culture conditions, the paired-pulse ratio was not significantly changed by the $GABA_B$ receptor antagonist CGP55845A (0.82 ± 0.06 in control; 0.83 ± 0.04 in 2 μM CGP55845A; n = 9). Our results are consis-

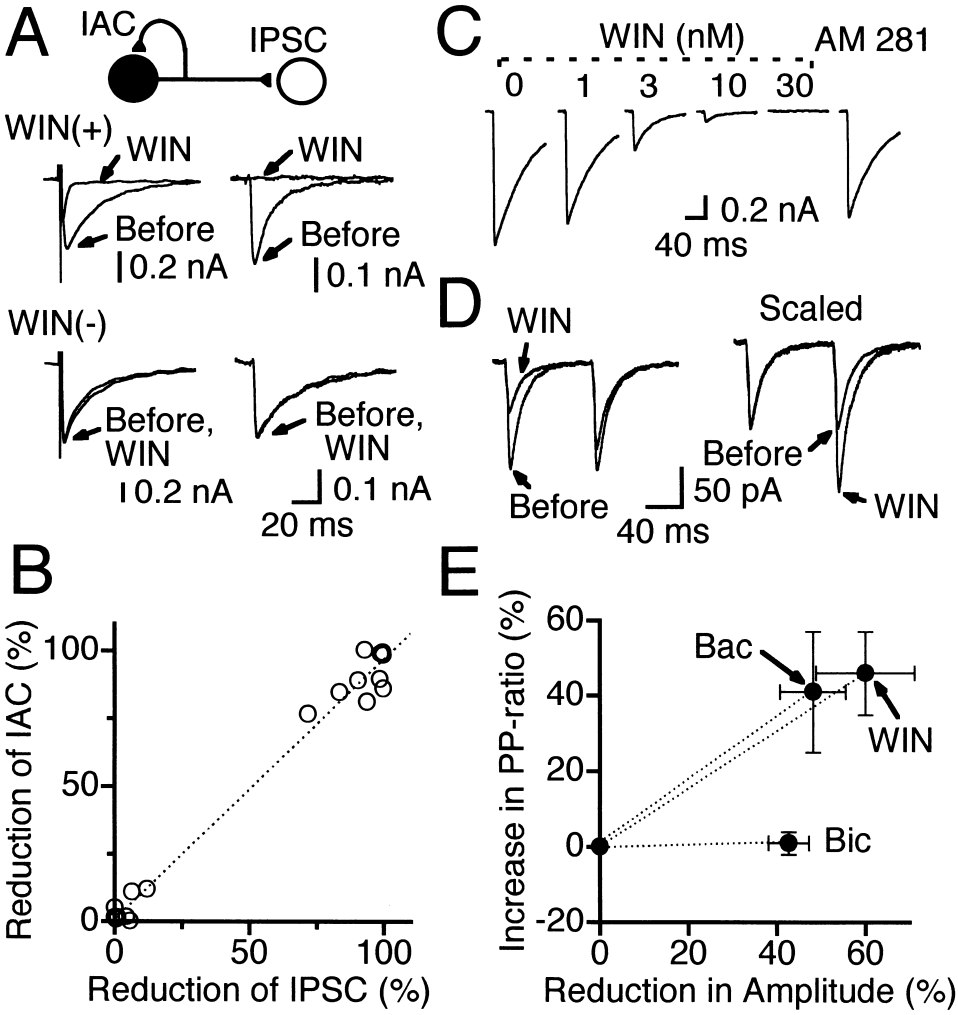

Figure 2. WIN55,212-2 Causes Suppression of GABA Release from Presynaptic Neurons

(A) Examples of simultaneous recordings of IACs (left) and IPSCs (right) from WIN55,212-2-sensitive (upper) or WIN55,212-2-insensitive (lower) pairs. Traces acquired before (Before) and during (WIN) application of WIN55,212-2 (0.1 μM) are superimposed. For the following figures, sample traces of IPSCs are shown similarly.

(B) Correlation between the effects of WIN55,212-2 on IACs and IPSCs.

(C) Effects of different concentrations (1, 3, 10, and 30 nM) of WIN55,212-2 on IPSCs. IPSC traces were sequentially recorded from the same neuron.

(D) Example of IPSCs evoked by paired stimuli with an interpulse interval of 100 ms. Each trace is the average of 10–12 consecutive IPSCs. Traces scaled to the first IPSCs are shown on the right.

(E) The relationships between the reduction in the first IPSC amplitude and the increase in the paired-pulse ratio induced by applications of 1–3 nM WIN55,212-2 (WIN; n = 7), 2–3 μM baclofen (Bac; n = 6), and 1–3 μM bicuculline (Bic; n = 7).

tent with previous reports (Wilcox and Dichter, 1994; Jensen et al., 1999) that $GABA_B$ autoreceptors at presynaptic terminals do not contribute to the paired-pulse synaptic plasticity. In a dose range of WIN55,212-2 (1–3 nM) where the amplitudes of synaptic currents were suppressed to about 40% of the control (Figure 2C), WIN55,212-2 induced a clear increase in the paired-pulse ratio of IPSCs (Figure 2D). On the average, the paired-pulse ratio increased by about 50% in the presence of WIN55,212-2, which was similar to the change caused by a $GABA_B$ receptor agonist, baclofen, in the same preparation (Figure 2E). In contrast, the suppression due to blockade of postsynaptic $GABA_A$ receptors by bicuculline caused no change in paired-pulse ratio (Figure 2E). WIN55,212-2 (0.1 μM) did not change the postsynaptic sensitivity to iontophoretically applied GABA. The average response in the presence of WIN55,212-2 (0.1 μM) was 95.5% ± 2.3% (mean ± SEM; n = 4) of the control. These results lead us to conclude that the cannabinoid agonist suppresses the release of GABA by activating cannabinoid receptors on the presynaptic terminals.

Depolarization-Induced Calcium Elevation of the Postsynaptic Neuron Induces Suppression of GABA Release from the Presynaptic Neuron

We applied a depolarizing voltage pulse (to 0mV for 5 s) to the postsynaptic neuron of a pair and transiently elevated the intracellular calcium concentration ($[Ca^{2+}]_i$) (Figure 3A). Effects of the same depolarizing voltage

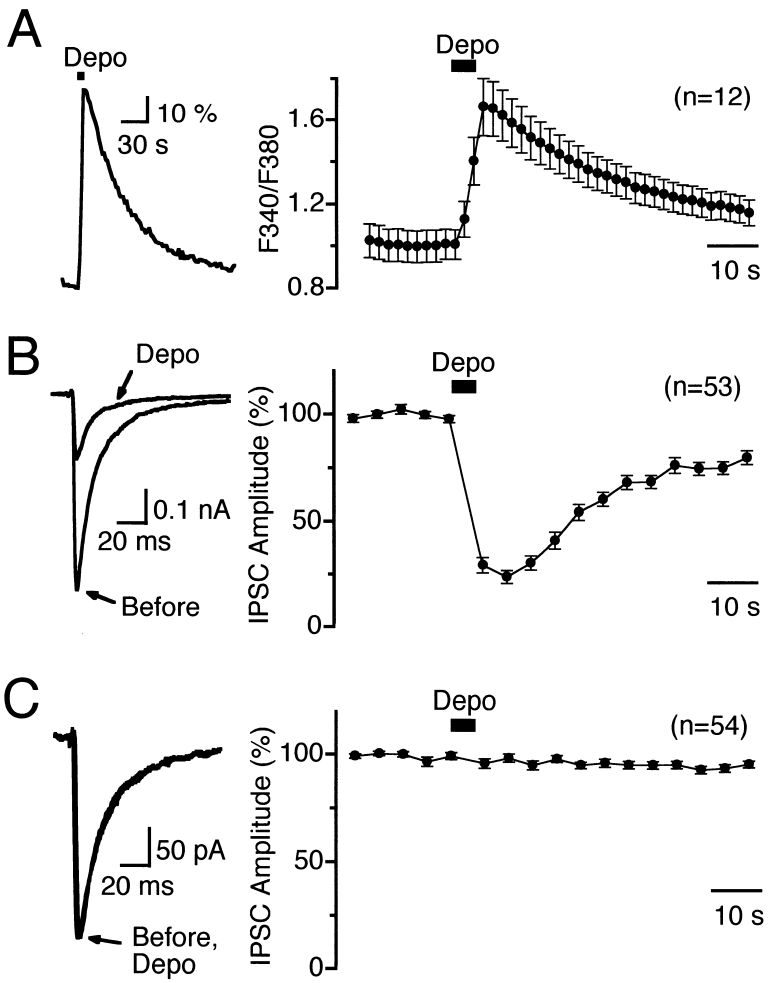

Figure 3. Depolarization-Induced Postsynaptic $[Ca^{2+}]_i$ Elevation and Suppression of IPSCs

(A) An example (left) and the averaged data (right) of depolarization-induced $[Ca^{2+}]_i$ elevation. The postsynaptic neuron was depolarized from −80mV to 0mV for 5 s (Depo). The averaged Ca^{2+} levels, expressed as the ratio (F340/F380), are plotted as a function of time.

(B and C) An example (left) and the averaged data (right) of depolarization-induced changes in IPSC amplitudes for neuron pairs sensitive (B) or insensitive (C) to depolarization. Traces of IPSCs acquired before (Before) and 6 s after (Depo) the depolarization are superimposed. The amplitude of IPSCs was normalized to the value before depolarization in each pair, and the averaged amplitudes are plotted as a function of time.

pulses on IPSCs were examined in 107 neuron pairs. In 53 neuron pairs, the amplitude of IPSCs decreased transiently with a time course similar to the elevation of $[Ca^{2+}]_i$ (Figure 3B). However, in the rest of 54 pairs, the depolarization induced no significant suppression of IPSCs (Figure 3C). The suppression of IPSCs induced by depolarization was not due to depolarization of the membrane itself but due to the elevation of $[Ca^{2+}]_i$ for the following two reasons. First, the same depolarizing pulse failed to depress IPSCs when the recording pipette contained a fast Ca^{2+} buffer, BAPTA (30 mM). The average response after depolarization was 100.4% ± 2.6% (mean ± SEM; n = 10) of the control. Second, the depolarization was ineffective when the Ca^{2+} inflow was eliminated by perfusing the neurons with a Ca^{2+}-free solution during depolarization (data not shown; Ohno-Shosaku et al., 1998).

The depolarization caused a slight decrease in the response of the postsynaptic neuron to iontophoretically applied GABA in neron pairs, irrespective of the presence (Figures 4A and 4B, left) or the absence (Figure 4B, right) of the depolarization-induced suppression of IPSCs. This slight decrease in the GABA responsiveness was presumably due to the Ca^{2+}-induced transient inactivation of $GABA_A$ receptors (Inoue et al., 1986; Chen and Wong, 1995). Nevertheless, the reduction of the IPSC amplitude was much greater than that of the GABA responsiveness in the neuron pairs exhibiting the depolarization-induced suppression of IPSCs (Figures 4A and 4B, left), indicating that the suppression of IPSCs was largely not due to the decrease in postsynaptic responsiveness to GABA. Furthermore, the suppression of IPSCs accompanied a clear increase in the paired-pulse ratio (data not shown; Ohno-Shosaku et al., 1998). These results indicate that the depolarization of postsynaptic neuron and resultant $[Ca^{2+}]_i$ elevation induces suppression of the GABA release from the presynaptic neuron. Therefore, some substance must be released from the postsynaptic neuron and act retrogradely on presynaptic terminals to reduce GABA release.

Endogenous Cannabinoid Mediates the Retrograde Effect

We examined a correlation between the sensitivities of IPSCs to the postsynaptic depolarization and to the cannabinoid agonist. In 26 pairs, effects of the postsynaptic depolarization and WIN55,212-2 on IPSCs were sequentially examined. In 12 of 26 pairs, the depolarization induced a transient suppression of IPSCs. In all of these pairs, WIN55,212-2 suppressed IPSCs almost completely (Figures 5A, 5Da, and 5Ea). In the remaining 14 pairs, the postsynaptic depolarization failed to sup-

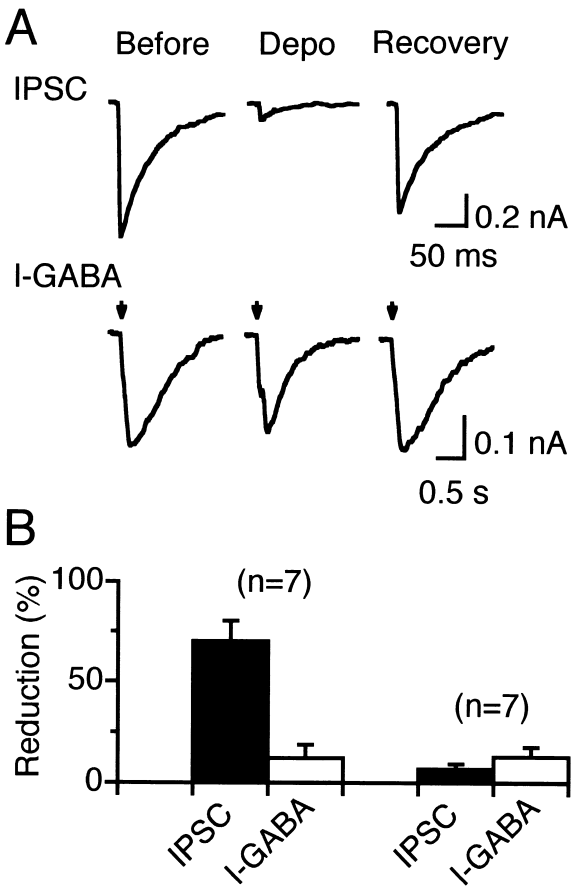

Figure 4. Depolarization of Postsynaptic Neurons Has No Effect on Their Responses to Applied GABA

(A) Examples of IPSCs (upper) and GABA-evoked currents (lower) recorded from the same neuron before, 6 s (Depo), and 56 s (Recovery) after the depolarization.

(B) Averaged data for depolarization-induced changes in the amplitudes of IPSCs and GABA-evoked currents obtained from neuron pairs sensitive (left) or insensitive (right) to depolarization.

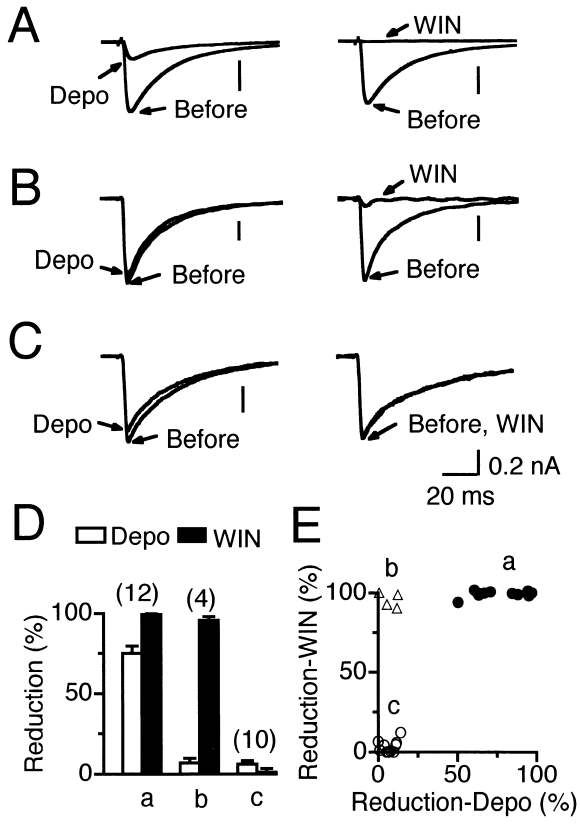

Figure 5. Correlation between the Effects of Depolarization and WIN55,212-2 on IPSCs

(A–D) Examples of IPSCs (A, B, and C) and the averaged data (D) for neuron pairs sensitive to both depolarization and WIN55,212-2 (A and Da), those insensitive to depolarization but sensitive to WIN55,212-2 (B and Db), and those insensitive to both of them (C and Dc).

(E) Scatter plot showing the relationship between the effect of WIN55,212-2 (ordinate) and that of depolarization (abscissa). Data points marked with (a) (filled circles), (b) (open triangles), and (c) (open circles) are from the corresponding three groups of neuron pairs in (D).

press IPSCs. In four of these pairs, WIN55,212-2 markedly suppressed IPSCs (Figures 5B, 5Db, and 5Eb). In the rest of ten pairs, WIN55,212-2 was totally ineffective (Figures 5C, 5Dc, and 5Ec). Importantly, we could not find any pairs where the depolarization induced suppression of IPSCs but WIN55,212-2 had no effect (Figures 5D and 5E). These results strongly suggest that only the presynaptic terminals that are sensitive to cannabinoid agonists can undergo suppression of GABA release in response to the depolarization of postsynaptic neurons.

We have reported previously that not only excitatory but also inhibitory postsynaptic neurons can exhibit depolarization-induced suppression of IPSCs (Ohno-Shosaku et al., 1998). In the present study, the type of postsynaptic neuron could be identified in 56 neuron pairs. Among 22 pairs with excitatory postsynaptic neurons, 10 pairs exhibited depolarization-induced suppression of IPSCs. On the other hand, among 34 pairs with inhibitory postsynaptic neurons, 14 pairs exhibited depolarization-induced suppression of IPSCs. These results indicate that both excitatory and inhibitory postsynaptic neurons can produce the retrograde signal in response to depolarization that may act on presynaptic cannabinoid receptors.

We next examined the effects of selective cannabinoid antagonists, AM 281 and SR141716A, on the depolarization-induced suppression of IPSCs. In these experiments, only the neuron pairs sensitive to the depolarization were used. We confirmed that the suppression can be elicited repeatedly without any run-down of its magnitude with time (up to 1 hr). In the normal external solution, the repeated depolarizations suppressed IPSCs to the same extent (Figure 6A, control 1 and 2), showing no run-down of the magnitude of suppression. Then, depolarization-induced changes in IPSC amplitudes were measured before and after application of AM 281 (0.3 μM) or SR141716A (0.3 μM) in each pair. These antagonists totally reversed the presynaptic inhibition induced by WIN55,212-2 (see Figure 1A). In all the pairs tested with AM 281, the depolarization-induced suppression was totally eliminated (Figure 6B). Essentially the same results were obtained with

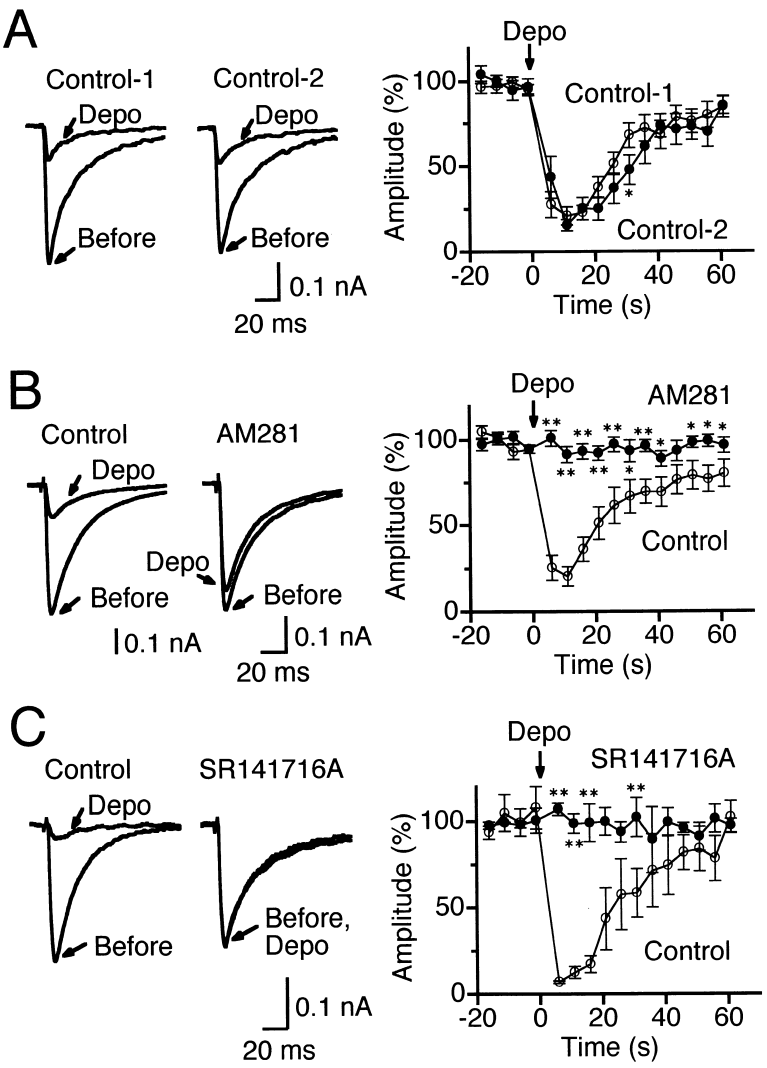

Figure 6. Blockade of Depolarization-Induced Suppression of IPSCs by Cannabinoid Antagonists

(A) Examples of IPSCs (left) and the summary (right) of the results showing that the depolarization-induced suppression can be elicited repeatedly without any run-down of its magnitude. Traces acquired before and 6 s after the first (control-1) or the second (control-2) depolarization in the normal external solution are shown. Averaged time courses of the changes in IPSC amplitudes induced by the first (open circles) and the second (closed circles) depolarization (n = 10).

(B and C) Examples of IPSCs (left) and the summary (right) of the results showing the blockade of the depolarization-induced suppression by 0.3 μM AM 281 ([B]; n = 11) and 0.3 μM SR141716A ([C]; n = 3). IPSC traces and averaged time courses of the depolarization-induced changes in IPSC amplitudes are shown in the similar manners to (A). The asterisks attatched to data points represent statistically significant differences from the control (asterisk, $p < 0.05$; double asterisks, $p < 0.01$; paired t test).

SR141716A (Figure 6C). These results indicate that endogenous cannabinoids mediate retrograde signals from depolarized postsynaptic neurons to presynaptic terminals to suppress the GABA release.

Neither Metabotropic Glutamate Receptor nor $GABA_B$ Receptor Mediate the Retrograde Effect

We then examined a possibility that glutamate or related excitatory amino acids are released from postsynaptic neuron and activate metabotropic glutamate receptors (mGluRs) at presynaptic terminals. In fact, in slice preparations of the cerebellum and the hippocampus, glutamate is reported to mediate the depolarization-induced suppression of inhibition (Glitsch et al., 1996; Morishita et al., 1998)—a phenomenon analogous to that examined in the present study. We found, however, that the suppression of IPSCs was not affected by a selective group I/II mGluR antagonist, (RS)-α-Methyl-4-carboxyphenylglycine (MCPG) (3–5 mM; Figures 7A and 7B), a group I mGluR antagonist, (RS)-1-Aminoindan-1,5-dicarboxylic acid (AIDA) (0.5 mM; data not shown), a mGluR5-specific antagonist, 2-Methyl-6-(phenylethynyl)pyridine (MPEP) (4 μM; Figures 7A and 7B), or a group II/III mGluR antagonist, (RS)-α-Cyclopropyl-4-phosphonophenylglycine (CPPG) (0.1 mM; Figures 7A and 7B). It should be noted that the mGluR antagonists used here significantly blocked the presynaptic inhibition induced by respective mGluR agonists (Figure 7C). We then asked whether GABA released from the postsynaptic neuron activates $GABA_B$ receptors at presynaptic terminals. As shown in Figure 1, baclofen (10 μM) effectively induced presynaptic inhibition in all neuron pairs tested. However, a potent and selective $GABA_B$ receptor antagonist, CGP55845A (2 μM), had no effect on the depolarization-induced suppression of IPSCs (Figures 7A and 7B), although it totally eliminated the presynaptic inhibition induced by baclofen (Figure 7C). These results indicate that neither glutamate (or related excitatory amino acids) nor GABA is likely to function as a retrograde messenger, at least in our culture conditions.

Postsynaptic Action Potentials Induce Suppression of Inhibitory Synaptic Transmission

Finally, we examined whether action potentials in the postsynaptic neuron can induce the suppression of inhibitory synaptic transmission. In four neuron pairs that

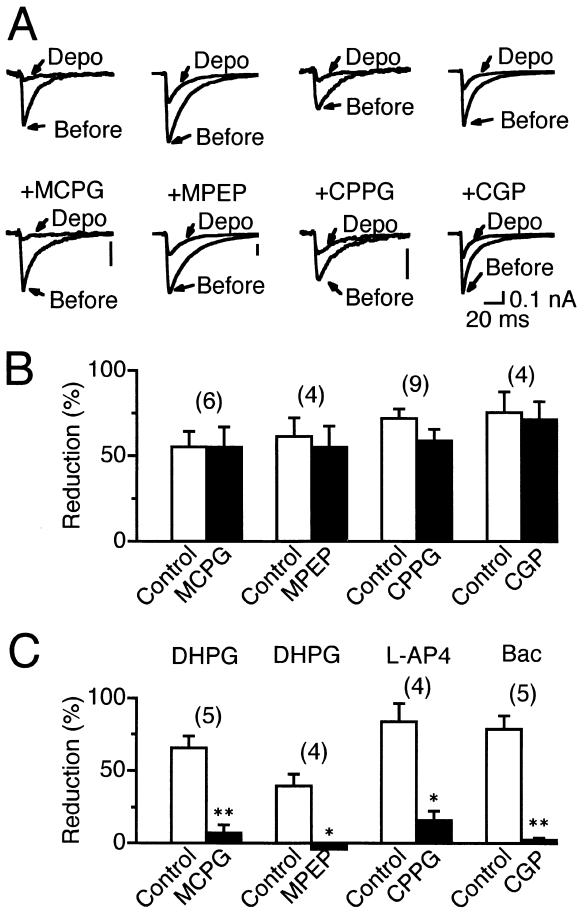

Figure 7. Neither mGluR nor $GABA_B$ Mediate the Retrograde Effect

(A and B) Examples of IPSCs (A) and the summary data (B) for the effects of mGluR antagonists (3–5 mM MCPG, 4 μM MPEP, 0.1 mM CPPG) and a $GABA_B$ receptor antagonist (2 μM CGP55845A) on the depolarization-induced suppression. IPSC traces were acquired in the absence (upper) or presence (lower) of the indicated antagonists. (C) These antagonists (2 mM MCPG, 4 μM MPEP, 0.1 mM CPPG, and 2 μM CGP55845A) blocked the suppressing effects of the corresponding agonists (50 μM DHPG, 10 μM L-AP4, and 10 μM baclofen) on IPSCs. The asterisks represent statistically significant differences from the control (asterisk, $p < 0.05$; double asterisks, $p < 0.01$; paired t test).

exhibited depolarization-induced suppression of IPSCs, trains of action potentials were elicited in the postsynaptic neuron by repetitively injecting short depolarizing current pulses (5 ms, 1 nA) at 50 Hz for 5 or 3 s under the current-clamp mode (Figure 8). In the neuron pair shown in Figure 8A, the action potential train for 5 s caused a transient suppression of IPSCs (Figures 8Ac and 8Ad) that was quite similar to the suppression caused by the depolarization (Figures 8Aa and 8Ab). The subsequent action potential train for 3 s also caused a weaker but clear transient suppression of IPSCs (Figures 8Ae and 8Af). After incubation of the culture with AM 281 (0.3 μM), the action potential train for 5 s (Figures 8Ag and 8Ah) and that for 3 s (Figures 8Ai and 8Aj), as well as the depolarization (Figures 8Ak and 8Al), became totally inneffective to suppress IPSCs. The similar results were obtained in all of the four neuron pairs tested (summarized in Figure 8B). These results indicate that the endocannabinoid-mediated suppression of inhibition can occur under a physiologically relevant condition and suggest that this form of synaptic plasticity may play a role in the modulation of neural excitability in vivo.

Discussion

We have demonstrated electrophysiologically that a cannabinoid agonist, WIN55,212-2, greatly reduced GABA release from presynaptic terminals in about 60% of cultured hippocampal neurons. Depolarization of the postsynaptic neuron and the resultant elevation of $[Ca^{2+}]_i$ caused transient suppression of inhibitory synaptic currents in about 50% of hippocampal neurons, which is mainly due to reduction of GABA release from the synaptic terminals. The neuron pairs that underwent depolarization-induced suppression of IPSCs were all sensitive to WIN55,212-2. Conversely, the neuron pairs in which the depolarization induced no change in IPSCs were mostly insensitive to WIN55,212-2. We have found that selective cannabinoid antagonists, AM 281 and SR141716A, totally eliminated the depolarization-induced suppression. In contrast, widely used antagonists against mGluRs and a potent antagonist against $GABA_B$ receptor were totally ineffective. Taken together, these results strongly suggest that endogenous cannabinoids produced in postsynaptic neurons act on cannabinoid receptors on the presynaptic terminals to suppress GABA release.

Two endogenous cannabinoids, anandamide (Devane et al., 1992) and 2-AG (Mechoulam et al., 1995; Sugiura et al., 1995), have been identified. Biochemical data indicate that these molecules 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). Several pathways for their Ca^{2+}-dependent biosynthesis have also been identified (Cadas et al., 1996, 1997; Bisogno et al., 1999), which accounts for the "on demand" synthesis of endogenous cannabinoids in stimulated cells (Di Marzo et al., 1998; Mechoulam et al., 1998; Piomelli et al., 2000). We have found that the postsynaptic elevation of $[Ca^{2+}]_i$ and the presynaptic suppression of synaptic currents had quite similar time courses. It is therefore likely that endogenous cannabinoids are produced and released from the postsynaptic neurons during the elevation of $[Ca^{2+}]_i$, and they disappear quickly by simple diffusion, uptake (Di Marzo et al., 1994; Beltramo et al., 1997; Piomelli et al., 1999; Beltramo and Piomelli, 2000), or enzymatic degradation (Cravatt et al., 1996; Goparaju et al., 1998).

In slice preparations from the cerebellum (Llano et al., 1991) and the hippocampus (Pitler and Alger, 1992), the depolarization-induced suppression of inhibitory transmission (DSI) has been reported to be mediated by glutamate or a glutamate-like substance (Glitsch et al., 1996; Morishita et al., 1998). It is proposed that glutamate or a glutamate-like substance is released from postsynaptic neuron in a Ca^{2+}-dependent manner and it acts on mGluRs on the inhibitory presynaptic terminals to reduce GABA release (Glitsch et al., 1996; Morishita et al., 1998). In the present study, however, we never observed any significant effects of widely used mGluR antagonists on the depolarization-induced suppression. Moreover, a selective and potent $GABA_B$ receptor antagonist,

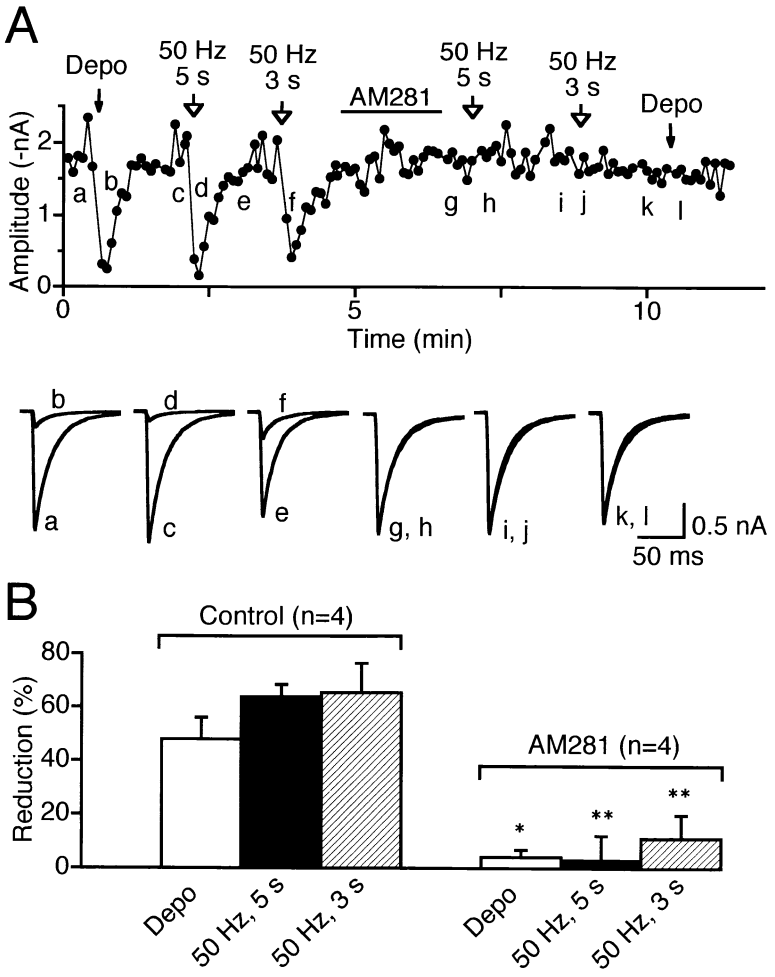

Figure 8. Action Potential Trains in the Postsynaptic Neurons Cause Cannabinoid Receptor-Mediated Suppression of IPSCs

(A) An example showing that suppression of IPSCs can be induced by action potential trains (50 Hz, 5 and 3 s) in the postsynaptic neuron, as well as by a continuous depolarization (0mV, 5 s). Bath application of a cannabinoid antagonist (0.3 μM AM 281) totally abolished the effects of both action potential trains and depolarization. The time course of the change in IPSC amplitudes (upper) and the IPSC traces (lower) acquired at the indicated time points are shown.

(B) Averaged data for the reduction of IPSC amplitudes induced by the depolarization and the action potential trains (50 Hs, 5 and 3 s) before (left) and after (right) the blockade of cannabinoid receptors (AM 281). The asterisks represent statistically significant differences from the control (asterisk, $p < 0.05$; double asterisks, $p < 0.01$; paired t test). For these experiments, the internal solution containing 0.2 mM EGTA was used.

CGP55845A, also had no effect on the suppression. These results clearly indicate that neither glutamate nor GABA is a major mediator of depolarization-induced suppression in our preparation. The reasons for this discrepancy are not clear. The difference in preparation may certainly be a reason. However, because the DSI in the cerebellum was little affected by a widely used mGluR antagonist, MCPG, (Glitsch et al., 1996) and DSI in the hippocampus was blocked only partly by MCPG, even with a high concentration (5 mM) (Morishita et al., 1998), we suspect that glutamate may not be the sole mediator of DSI in slice preparations. In fact, Wilson and Nicoll showed very recently that depolarization-induced suppression of inhibition could be blocked by cannabinoid antagonists in the hippocampal slice preparation (Wilson and Nicoll, 2001).

In our culture preparation, about 50% of neuron pairs did not display depolarization-induced suppression. This deficiency may result from the inability of the postsynaptic neuron to produce endogenous cannabinoids (postsynaptic deficiency) or from the insensitivity of the presynaptic terminals to cannabinoids (presynaptic deficiency). Among 14 neuron pairs that did not display depolarization-induced suppression, WIN55,212-2 markedly suppressed IPSCs in four pairs. In these pairs, the site of deficiency would be postsynaptic. On the other hand, WIN55,212-2 was ineffective in the rest of ten pairs. The site of deficiency in these pairs would be either pre- or postsynaptic. Reasons for the postsynaptic deficiency, however, are unknown at this stage. On the other hand, a reason for the presynaptic deficiency may be the heterogeneity of the CB1 receptor localization. An immunocytochemical study on rat hippocampal culture indicates that CB1 receptors are located on presynaptic terminals of the majority but not all of GABAergic neurons (Irving et al., 2000). Another study on hippocampal slice (Katona et al., 1999) shows that the CB1 immunoreactivity is high in cholecystokinin-containing GABAergic terminals but is undetectable in parvalbumin-containing terminals. It is therefore likely that the WIN55,212-2-sensitive terminals correspond at least partly to the cholecystokinin-containing GABAergic terminals, whereas the WIN55,212-2-insensitive terminals correspond to the parvalbumin-containing terminals.

Because CB1 receptors are widespread in the CNS, endogenous cannabinoids may be of general importance in neural functions. For example, Kreitzer and Regehr report in this issue that postsynaptic depolarization of cerebellar Purkinje cells causes transient presynaptic suppression of excitatory inputs that is mediated by CB1 receptors (Kreitzer and Regehr, 2001). Moreover, the elevation of $[Ca^{2+}]_i$, which can trigger the production

of endogenous cannabinoids, may result from multiple sources. These include Ca^{2+} inflow through voltage-gated Ca^{2+} channels and through Ca^{2+}-permeable channels, such as NMDA receptors, and Ca^{2+} release from the internal stores. It is therefore highly likely that the retrograde modulation by endogenous cannabinoids is an important and widespread mechanism in the brain, by which the activity of postsynaptic neurons can influence the presynaptic functions.

Experimental Procedures

Culture

Cells were mechanically dissociated from the whole hippocampus of newborn rats, including the CA1, CA2, and CA3 subfields, as well as the dentate gyrus. The hippocampal cells were plated onto culture dishes that had been pretreated with poly L-ornithine (0.01%). The cells were incubated in the DMEM/F-12 medium (GIBCO-BRL, NY) supplemented with putrescine (0.1 mM), sodium selenite (30 nM), L-glutamine (1.4 mM), gentamicin (10 μg/ml), insulin (5 μg/ml), and fetal calf serum (10%). Cultures were maintained at 36°C in 5% CO_2 for 7–14 days. All experiments were performed at room temperature. The external solution contained (in mM) NaCl, 140; KCl, 2.5; $MgCl_2$, 1; $CaCl_2$, 2; HEPES, 10; glucose, 10; and kynurenic acid, 1 (pH 7.3, adjusted with NaOH). The bath was perfused with the normal solution or the solution containing drugs at a flow rate of 1–3 ml/min. For the perfusion with a solution containing WIN55,212-2, AM281, or SR141716A, a separate perfusion tube was used to avoid contamination. The standard internal solution contained (in mM) K-gluconate, 120; KCl, 15; $MgCl_2$, 6; EGTA, 5; HEPES, 10; KOH, 20; and Na_2ATP, 5 (pH 7.3, adjusted with KOH). In some experiments, the internal solution containing 30 mM BAPTA or 0.2 mM EGTA instead of 5 mM EGTA was used. The electrode resistance ranged from 3 to 5 MΩ when filled with the internal solution.

Electrophysiology

Each neuron of a pair was whole-cell voltage clamped, and the membrane potential of each neuron was held at −80mV. The presynaptic neuron was stimulated by applying positive voltage pulses (80mV, 2 ms) at 0.2 Hz, and IPSCs were measured from the postsynaptic neuron, with a patch-clamp amplifier (EPC-7/List or EPC-9/HEKA). In some experiments, IACs were also recorded from the stimulated neuron. To monitor the GABA sensitivity of the postsynaptic neuron, GABA was applied to the dendrites through a pipette filled with 100 mM GABA, using 200 nA current pulses (50–100 ms) and 50 nA retaining currents.

Suppression of synaptic currents was induced by a depolarizing pulse (to 0mV for 5 s) to the postsynaptic neuron of the pair from which inhibitory synaptic currents were recorded. The magnitudes of depolarization-induced suppression were measured as the percentage of the mean amplitude of three consecutive synaptic currents after depolarization (acquired between 6 and 16 s after the end of the pulse) relative to that of five synaptic currents before the depolarization. The depression caused by drugs was estimated as the percentage of the mean amplitudes of 5 to 12 consecutive synaptic currents during drug application relative to that before application. The synapse was judged to be positive for depolarization-induced suppression or sensitive to the drug when the amplitude of synaptic currents was reduced to less than 85% of the control. Averaged data from different experiments are presented as mean ± SEM. WIN55,212-2, AM 281, MCPG, MPEP, AIDA, and CPPG were purchased from Tocris Cookson, and baclofen was purchased from Sigma. SR141716A and CGP55845A were generous gifts from Sanofi Recherche (France) and Novartis Pharma (Switzerland), respectively.

Ca^{2+} Measurements

Neurons were loaded for at least 10 min with a Ca^{2+} indicator (200 μM; fura-2) through patch pipette. Fluorescence images for excitations of 340 nm (F340) and 380 nm (F380) were acquired from the soma at 0.5 Hz by using a cooled-CCD camera system (IMAGO, TILL Photonics). The Ca^{2+} signal was expressed as the F340 to F380 ratio.

Acknowledgments

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan (to T. O.-S. and M. K.), by the Human Frontier Science Program (to M. K.), and by Special Coordination Funds for Promoting Science and Technology from the Science and Technology Agency of Japan (to M. K.).

Received November 20, 2000; revised January 28, 2001.

References

Auclair, N., Otani, S., Soubrie, P., and Crépel, F. (2000). Cannabinoids modulate synaptic strength and plasticity at glutamatergic synapses of rat prefrontal cortex pyramidal neurons. J. Neurophysiol. *83*, 3287–3293.

Beltramo, M., and Piomelli, D. (2000). Carrier-mediated transport and enzymatic hydrolysis of the endogenous cannabinoid 2-arachidonylglycerol. Neuroreport *11*, 1231–1235.

Beltramo, M., Stella, N., Calignano, A., Lin, S.Y., Makriyannis, A., and Piomelli, D. (1997). Functional role of high-affinity anandamide transport, as revealed by selective inhibition. Science *277*, 1094–1097.

Bisogno, T., Sepe, N., Melck, D., Maurelli, S., De-Petrocellis, L., and Di Marzo, V. (1997). Biosynthesis, release and degradation of the novel endogenous cannabimimetic metabolite 2-arachidonoylglycerol in mouse neuroblastoma cells. Biochem. J. *322*, 671–677.

Bisogno, T., Melck, D., De Petrocellis, L., and Marzo, U.D. (1999). Phosphatidic acid as the biosynthetic precursor of the endocannabinoid 2-arachidonoylglycerol in intact mouse neuroblastoma cells stimulated with ionomycin. J. Neurochem. *72*, 2113–2119.

Cadas, H., Gaillet, S., Beltramo, M., Venance, L., and Piomelli, D. (1996). Biosynthesis of an endogenous cannabinoid precursor in neurons and its control by calcium and cAMP. J. Neurosci. *16*, 3934–3942.

Cadas, H., di Tomaso, E., and Piomelli, D. (1997). Occurrence and biosynthesis of endogenous cannabinoid precursor, N-arachidonoyl phosphatidylethanolamine, in rat brain. J. Neurosci. *17*, 1226–1242.

Chen, Q.X., and Wong, R.K.S. (1995). Suppression of GABAA receptor responses by NMDA application in hippocampal neurones acutely isolated from the adult guinea-pig. J. Physiol. *482*, 353–362.

Cravatt, B.F., Giang, D.K., Mayfield, S.P., Boger, D.L., Lerner, R.A., and Gilula, N.B. (1996). Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. Nature *384*, 83–87.

Devane, W.A., Hanus, L., Breuer, A., Pertwee, R.G., Stevenson, L.A., Griffin, G., Gibson, D., Mandelbaum, D., Etinger, A., and Mechoulam, R. (1992). Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science *258*, 1946–1949.

Di Marzo, V., Fontana, A., Cadas, H., Schinelli, S., Cimino, G., Schwartz, J.-C., and Piomelli, D. (1994). Formation and inactivation of endogenous cannabinoid anandamide in central neurons. Nature *372*, 686–691.

Di Marzo, V., Melck, D., Bisogno, T., and De Petrocellis, L. (1998). Endocannabinoids: endogenous cannabinoid receptor ligands with neuromodulatory action. Trends Neurosci. *21*, 521–528.

Egertova, M., and Elphick, M.R. (2000). Localisation of cannabinoid receptors in the rat brain using antibodies to the intracellular C-terminal tail of CB1. J. Comp. Neurol. *422*, 159–171.

Egertova, M., Giang, D.K., Cravatt, B.F., and Elphick, M.R. (1998). A new perspective on cannabinoid signalling: complementary localization of fatty acid amide hydrolase and the CB1 receptor in rat brain. Proc. R. Soc. Lond. B Biol. Sci. *265*, 2081–2085.

Felder, C.C., and Glass, M. (1998). Cannabinoid receptors and their endogenous agonists. Annu. Rev. Pharmacol. Toxicol. *38*, 179–200.

Giuffrida, A., Parsons, L.H., Kerr, T.M., Rodríguez de Fonseca, F., Navarro, M., and Piomelli, D. (1999). Dopamine activation of endoge-

nous cannabinoid signaling in dorsal striatum. Nature Neurosci. *2*, 358–363.

Glitsch, M., Llano, I., and Marty, A. (1996). Glutamate as a candidate retrograde messenger at interneurone-Purkinje cell synapses of rat cerebellum. J. Physiol. *497*, 531–537.

Goparaju, S.K., Ueda, N., Yamaguchi, H., and Yamamoto, S. (1998). Anandamide amidohydrolase reacting with 2-arachidonoylglycerol, another cannabinoid receptor ligand. FEBS Lett. *422*, 69–73.

Herkenham, M., Lynn, A.B., Johnson, M.R., Melvin, L.S., de Costa, B.R., and Rice, K.C. (1991). Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study. J. Neurosci. *11*, 563–583.

Hoffman, A.F., and Lupica, C.R. (2000). Mechanisms of cannabinoid inhibition of GABAA synaptic transmission in the hippocampus. J. Neurosci. *20*, 2470–2479.

Inoue, M., Oomura, Y., Yakushiji, T., and Akaike, N. (1986). Intracellular calcium ions decrease the affinity of the GABA receptor. Nature *324*, 156–158.

Irving, A.J., Coutts, A.A., Harvey, J., Rae, M.G., Mackie, K., Bewick, G.S., and Pertwee, R.G. (2000). Functional expression of cell surface cannabinoid CB1 receptors on presynaptic inhibitory terminals in cultured rat hippocampal neurons. Neuroscience *98*, 253–262.

Jensen, K., Lambert, J.D.C., and Jensen, M.S. (1999). Activity-dependent depression of GABAergic IPSCs in cultured hippocampal neurons. J. Neurophysiol. *82*, 42–49.

Katona, I., Sperlágh, B., Sík, A., Käfalvi, A., Vizi, E.S., Mackie, K., and Freund, T.F. (1999). Presynaptically located CB1 cannabinoid receptors regulate GABA release from axon terminals of specific hippocampal interneurons. J. Neurosci. *19*, 4544–4558.

Klein, T.W., Newton, C., and Friedman, H. (1998). Cannabinoid receptors and immunity. Immnol. Today *19*, 373–381.

Kreitzer, A.C., and Regehr, W.G. (2001). Retrograde inhibition of presynaptic calcium influx by endogenous cannabinoids at excitatory synapses onto Purkinje cells. Neuron, this issue, 717–727.

Llano, I., Leresche, N., and Marty, A. (1991). Calcium entry increases the sensitivity of cerebellar Purkinje cells to applied GABA and decreases inhibitory synaptic currents. Neuron *6*, 565–574.

Matsuda, L.A., Lolait, S.J., Brownstein, M.J., Young, A.C., and Bonner, T.I. (1990). Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature *346*, 561–564.

Matsuda, L.A., Bonner, T.I., and Lolait, S.J. (1993). Localization of cannabinoid receptor mRNA in rat brain. J. Comp. Neurol. *327*, 535–550.

Mechoulam, R., Ben-Shabat, S., Hanus, L., Ligumsky, M., Kaminsky, N.E., Schatz, A.R., Gopher, A., Almog, S., Martin, B.R., Compton, D.R., et al. (1995). Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. Biochem. Pharmacol. *50*, 83–90.

Mechoulam, R., Fride, E., and Di Marzo, V. (1998). Endocannabinoids. Eur. J. Pharmacol. *359*, 1–18.

Misner, D.L., and Sullivan, J.M. (1999). Mechanism of cannabinoid effects on long-term potentiation and depression in hippocampal CA1 neurons. J. Neurosci. *19*, 6795–6805.

Morishita, W., Kirov, S.A., and Alger, B.E. (1998). Evidence for metabotropic glutamate receptor activation in the induction of depolarization-induced suppression of inhibition in hippocampal CA1. J. Neurosci. *18*, 4870–4882.

Munro, S., Thomas, K.L., and Abu-Shaar, M. (1993). Molecular characterization of a peripheral receptor for cannabinoids. Nature *365*, 61–65.

Ohno-Shosaku, T., Sawada, S., and Yamamoto, C. (1998). Properties of depolarization-induced suppression of inhibitory transmission in cultured rat hippocampal neurons. Pflügers Arch. *435*, 273–279.

Ohno-Shosaku, T., Sawada, S., and Kano, M. (2000). Heterosynaptic expression of depolarization-induced suppression of inhibition (DSI) in rat hippocampal cultures. Neurosci. Res. *36*, 67–71.

Piomelli, D., Beltramo, M., Glasnapp, S., Lin, S.Y., Goutopoulos, A., Xie, X.Q., and Makriyannis, A. (1999). Structural determinants for recognition and translocation by the anandamide transporter. Proc. Natl. Acad. Sci. USA *96*, 5802–5807.

Piomelli, D., Giuffrida, A., Calignano, A., and Rodriguez de Fonseca, F. (2000). The endocannabinoid system as a target for therapeutic drugs. Trends Pharmacol. Sci. *21*, 218–224.

Pitler, T.A., and Alger, B.E. (1992). Postsynaptic spike firing reduces synaptic GABAA responses in hippocampal pyramidal cells. J. Neurosci. *12*, 4122–4132.

Stella, N., Schweitzer, P., and Piomelli, D. (1997). A second endogenous cannabinoid that modulates long-term potentiation. Nature *388*, 773–778.

Sugiura, T., Kondo, S., Sukagawa, A., Nakane, S., Shinoda, A., Itoh, K., Yamashita, A., and Waku, K. (1995). 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. Biochem. Biophys. Res. Commun. *215*, 89–97.

Sugiura, T., Kodaka, T., Nakane, S., Miyashita, T., Kondo, S., Suhara, Y., Takayama, H., Waku, K., Seki, C., Baba, N., and Ishima, Y. (1999). Evidence that the cannabinoid CB1 receptor is a 2-arachidonoylglycerol receptor. J. Biol. Chem. *274*, 2794–2801.

Tsou, K., Brown, S., Sañudo-Reña, J.C., Mackie, K., and Walker, J.M. (1998). Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system. Neuroscience *83*, 393–411.

Tsou, K., Mackie, K., Sañudo-Reña, J.C., and Walker, J.M. (1999). Cannabinoid CB1 receptors are localized primarily on cholecystokinin-containing GABAergic interneurons in the rat hippocampal formation. Neuroscience *93*, 969–975.

Wilcox, K.S., and Dichter, M.A. (1994). Paired pulse depression in cultured hippocampal neurons is due to a presynaptic mechanism independent of GABAB autoreceptor activation. J. Neurosci. *14*, 1775–1788.

Wilson, R.I., and Nicoll, R.A. (2001). Endogenous cannabinoids mediate retrograde signaling at hippocampal snyapses. Nature *410*, 588–592.