Analysis of the Effects of Cannabinoids on Synaptic Transmission between Basket and Purkinje Cells in the Cerebellar Cortex of the Rat

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ABSTRACT

The hypothesis of the present work was that activation of CB₁ cannabinoid receptors inhibits GABAergic neurotransmission between basket and Purkinje cells in the cerebellar cortex. The aim was to test this hypothesis under near-physiological conditions. Action potentials of basket cells and spontaneous inhibitory postsynaptic currents (sIPSCs) in synaptically coupled Purkinje cells were recorded simultaneously in rat brain slices. The cannabinoid agonists (R)-(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl] pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-methanone mesylate (WIN 55212-2) and (–)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)-phenyl]-trans-4-[3-hydroxy-propyl]-cyclohexanol (CP55940) decreased the amplitude of sIPSCs occurring simultaneously with basket cell action potentials and lowered the success rate of synaptic transmission. These effects were prevented by the CB₁ receptor antagonist N-piperidino-5-(4-chlorophenyl)-1-[2,4-dichloro-phenyl]-4-methyl-3-pyrazolecarboxamide (SR141716). Depolarization of Purkinje cells also led to suppression of neurotransmission; prevention of this suppression by CP55940 and SR141716 indicates that endocannabinoids released from Purkinje cells were involved. WIN 55212-2 lowered the amplitude of autoreceptor currents recorded in basket cells (autoreceptor currents are due to the action of GABA released from axon terminals on GABA_A autoreceptors of the same axon terminals); this is novel proof of the presynaptic action of cannabinoids. Autoreceptor current experiments also indicated that endogenous cannabinoids are not released by basket cell axon terminals. A presynaptic action is additionally supported by the observation that WIN 55212-2 lowered the frequency of miniature IPSCs recorded in the presence of tetrodotoxin and the calcium ionophore ionomycin. In conclusion, activation of CB₁ receptors by exogenous cannabinoids and by endogenous cannabinoids released by Purkinje cells presynaptically inhibits GABAergic neurotransmission between basket and Purkinje cells. This was demonstrated under near-physiological conditions: transmitter release was elicited by action potentials generated by spontaneously firing intact presynaptic neurons.

Mammals possess two G protein-coupled cannabinoid receptors, CB₁ and CB₂ receptors (Howlett et al., 2002). They are the primary targets of the natural cannabinoid agonist Δ⁹-tetrahydrocannabinol and of the endogenous cannabinoids (endocannabinoids) anandamide and 2-arachidonylglycerol. The CB₂ receptor is localized in peripheral non-neuronal tissues. The CB₁ receptor is widely distributed in the central and the peripheral nervous system (Mailleux and Vanderhaeghen, 1992; Matsuda et al., 1993; Tsou et al., 1998). High concentrations are found in the cerebral cortex, hippocampus, caudate-putamen, globus pallidus, substantia nigra pars reticulata, and cerebellum.

Most of the CB₁ receptors in the cerebellum are found in the cortex, and their exact neuronal localization can be deduced by comparing the localization of CB₁ receptor mRNA (in situ hybridization; Mailleux and Vanderhaeghen, 1992; Matsuda et al., 1993) and CB₁ receptor protein (receptor autoradiography and immunohistochemistry; Mailleux and Vanderhaeghen, 1992; Tsou et al., 1998; Diana et al., 2002). The output neurons of the cerebellar cortex, the Purkinje cells, do not synthesize CB₁ receptors. Most of the CB₁ receptors in the cortex are localized in axon terminals of parallel and climbing fibers; these fibers constitute the excita-

ABBRIVIATIONS: CB, cannabinoid; IPSC, inhibitory postsynaptic current; ACSF, artificial cerebrospinal fluid; DNQX, 6,7-dinitroquinoxaline-2,3-dione; AP5, α₂-aminophosphonoacetic acid; sIPSC, spontaneous inhibitory postsynaptic current; mIPSC, miniature inhibitory postsynaptic current; QX-314, N-ethyl-2-iodocaine Cl; PRe, initial reference value determined before drug application; CP55940, (–)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)-phenyl]-trans-4-[3-hydroxy-propyl]-cyclohexanol; SR141716, N-piperidino-5-(4-chlorophenyl)-1-[2,4-dichloro-phenyl]-4-methyl-3-pyrazolecarboxamide; WIN 55212-2, (R)-(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl] pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-1-(1-naphthalenyl)-methanone mesylate; DMSO, dimethyl sulfoxide; SOL, solvent.
tory input of Purkinje cells. The neurons providing GABAergic input to the Purkinje cells, basket and stellate cells, also synthesize CB₁ receptors (Fig. 1A), as indicated by the observation that “nearly all cells in the molecular layer ... expressed moderately intense (CB₁ mRNA in situ hybridization) signals” (Matsuda et al., 1993). These CB₁ receptors occur in the axon terminals of basket cells, as shown by the presence of CB₁ receptor “immunoreactivity surrounding the Purkinje cell bodies especially their basal part showing a triangular cap-like appearance” (Tsou et al., 1998; see also Diana et al., 2002).

The presence of CB₁ receptors in basket cell terminals forming synaptic contacts with Purkinje cells suggested that activation of these receptors modulates GABAergic neurotransmission between basket and Purkinje cells. Indeed, it had been demonstrated before the beginning of our study that exogenous and endogenous cannabinoids depress spontaneous and miniature GABAergic inhibitory postsynaptic currents (IPSCs) recorded in Purkinje cells (Takahashi and Linden, 2000; Kreitzer and Regehr, 2001a). The objective of the present study was to carry out a comprehensive analysis of the effects of exogenous and endogenous cannabinoids on neurotransmission between basket and Purkinje cells under near-physiological conditions. To this end, neurotransmission was studied by simultaneous recording of spontaneous action potentials in basket cells and of postsynaptic GABAergic inhibitory currents in synaptically coupled Purkinje cells (Fig. 1A). In addition, the presynaptic action of cannabinoids was studied with the novel method of autoreceptor current analysis (Pouzat and Marty, 1999; Than and Szabo, 2002).

Materials and Methods

The experiments conformed to the rules of the German law regulating the use of animals in biomedical research. The methods were similar to those described previously (Szabo et al., 1998; Wallmich-rath and Szabo, 2002).

Brain Slices. Nine- to 18-day-old Wistar rats were anesthetized with halothane and decapitated. The brains were rapidly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF) of the following composition: 126 mM NaCl, 1.2 mM NaH₂PO₄, 3 mM KCl, 5 mM MgCl₂, 1 mM CaCl₂, 26 mM NaHCO₃, 20 mM glucose, and 4 mM Na-lactate, pH 7.3 to 7.4 (after the solution was gassed with 95% O₂, 5% CO₂). Sagittal slices (200 or 250 μm) were cut with a vibrating tissue slicer from the cerebellar vermis. The slices were stored in a Gubb chamber containing ACSF of the following composition: 126 mM NaCl, 1.2 mM NaH₂PO₄, 3 mM KCl, 1 mM MgCl₂, 2.5 mM CaCl₂, 26 mM NaHCO₃, 10 mM glucose, and 4 mM Na-lactate, pH 7.3 to 7.4 (after the solution was gassed with 95% O₂, 5% CO₂). To support regeneration processes in neurons, the temperature was raised to 35°C for 45 min. Thereafter, the slices were stored at room temperature until patch clamping started up to 6 h later.

For patch-clamp recording, slices were fixed at the glass bottom of a superfusion chamber with a nylon grid on a platinum frame and superfused with ACSF at room temperature at a flow rate of 1.5 ml/min⁻¹. The ACSF was of the following composition: 126 mM NaCl, 1.2 mM NaH₂PO₄, 3 mM KCl, 1 mM MgCl₂, 2.5 mM CaCl₂, 26 mM NaHCO₃, and 10 mM glucose, pH 7.3 to 7.4 (after the solution was gassed with 95% O₂, 5% CO₂). The superfusion ACSF contained
6,7-dinitroquinoxaline-2,3-dione (DNQX, 10 μM) and 6-cyano-7-nitroquinoxaline-2,3-dione (AP5; 25 μM) to suppress fast glutamatergic neurotransmission.

Neurons in slices were visualized with infrared videomicroscopy. The slices were trans-illuminated with infrared light and viewed with a Zeiss Axioskop FS-2 microscope (Carl Zeiss, Göttingen, Germany) equipped with differential interference contrast optics and a videocamera. We recorded from Purkinje cells that were easily identifiable due to their large size. We recorded from basket cells that had diameters of 7 to 10 μm, were localized in the inner half of the molecular layer, and were spontaneously active. We call these cells “basket cells,” although we know that basket and stellate cells cannot be clearly distinguished according to anatomical criteria (Sultan and Bower, 1998).

**Patch-Clamp Recording Techniques.** Pipettes were pulled from borosilicate glass and had resistances of 2 to 3 MΩ (Purkinje cells) and 4 to 5 MΩ (basket cells) when filled with intracellular solution. Patch-clamp recordings were obtained with an EPC-9 double amplifier (HEKA Elektronik, Lambrecht, Germany). Data were evaluated with the TIDA for Windows software (HEKA Elektronik). Series resistance compensation of 50% was usually applied. Data were filtered at 1 to 2.9 kHz and stored with sampling rates at least twice the filtering frequency. Series resistance was measured before and after recordings, and experiments with major changes in series resistance (>20%) were discarded.

**Recording of Spontaneous Inhibitory Postsynaptic Currents (sIPSCs) and Miniature Inhibitory Postsynaptic Currents (mIPSCs).** sIPSCs and mIPSCs in Purkinje cells were recorded in whole cell configuration at a holding potential of −60 mV with pipettes containing 142 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, 1 mM EGTA, 4 mM ATP-Na2, and 2 mM N-ethyl-dicyclohexyl CI (QX-314; pH 7.4). Tetrodotoxin (3 × 10−7 M) was used to isolate mIPSCs. sIPSCs and mIPSCs were identified and analyzed using the MiniAnalysis software (version 5.2.6; Synaptosoft, Decatur, GA).

**Recording of Neuronal Firing.** Spontaneous firing of basket cells was recorded in the cell-attached mode at a holding potential of 0 mV. In most of the experiments, the pipettes contained 145 mM K gluconate, 0.1 mM CaCl2, 2 mM MgCl2, 5 mM HEPES, 1.1 mM EGTA, 5 mM ATP-Mg, and 0.3 mM GTP-Tris, pH 7.4. To match conditions of the recent study by Kreitzer et al. (2002), in one series of experiments bicuculline (2 × 10−5 M) was included in the superfusion buffer and the pipettes contained 165 mM NaCl, 3 mM CaCl2, 2 mM MgCl2, 1.25 mM NaH2PO4, 10 mM HEPES, and 25 mM glucose, pH 7.4. Action currents corresponding to action potentials were identified from their frequency determined using the MiniAnalysis software.

**Evaluation of Neurotransmission between Basket and Purkinje Cells.** Action currents due to firing of presynaptic basket cells were recorded in cell-attached configuration and sIPSCs in postsynaptic Purkinje cells in whole cell configuration (Fig. 1, A and B). For evaluation, 100 action potentials were selected in the presynaptic recording using the MiniAnalysis software. Subsequently, simultaneous postsynaptic current segments were selected by the evaluator (these segments are called “action potential-coupled postsynaptic currents”). If a postsynaptic peak (amplitude >10 pA) was detected in a 3-ms time window beginning 0.5 ms after the downward peak of a presynaptic action potential, the transmission event was termed a “success.” In contrast to a “failure,” synaptic transmission is characterized in the present work by the amplitude of action potential-coupled postsynaptic currents (only successes were evaluated) and by the success rate of neurotransmission.

**Recording of Autoreceptor Currents in Basket Cells.** GABAergic autoreceptor currents originating in axon terminals of basket cells were recorded principally as described by Pouzat and Marty (1999) (Fig. 7). When recorded in the whole cell patch-clamp mode, autoreceptor currents show a strong run-down within 10 to 15 min (Pouzat and Marty, 1999; Than and Szabo, 2002). Diana et al. (2002) observed that run-down of transmitter release from axon terminals of basket cells can be prevented by approaching the cells in the perforated patch mode. Accordingly, we decided to study autoreceptor currents in the perforated, bicuculline mode. The pipettes contained 140 mM KCl, 0.1 mM CaCl2, 4.6 mM MgCl2, 10 mM HEPES, 1 mM EGTA, 4 mM ATP- Na2, and 0.4 mM GTP-Tris, pH 7.4; amphotericin B (300 μg/ml) was added for establishing perforated patches. The holding potential was −70 mV. Autoreceptor currents were elicited by depolarizing the soma of the neurons by 40 to 70 mV for 1 to 2 ms.

**Protocols and Statistics.** Except in autoreceptor current experiments, recordings started 15 to 30 min after establishment of the whole cell or cell-attached configuration. Zero time in the figures is the time when recording began. Solvent and drug superfusion is indicated in the figures. Parameters (sIPSCs, mIPSCs, and neuronal firing) were usually recorded in 1- to 2-min epochs. Values of parameters during superfusion with solvent or drugs were expressed as percentages of the initial reference values (PRE; the PRE period is indicated in the figures).

In autoreceptor current experiments, 20 to 60 min was necessary for reaching the perforated patch configuration. Zero time in the figures refers to the time when the perforated patch configuration had been established. Autoreceptor currents were elicited every 6 s. Depending on the drug applied, currents were averaged every 1 to 2 min (10–20 recordings). Current amplitudes were expressed as percentages of the initial reference values (PRE).

Means ± S.E.M. are given throughout. The nonparametric two-tailed Mann-Whitney and Wilcoxon signed rank tests were used to identify significant differences between groups (drug versus solvent) and within groups (drug versus PRE), respectively. Significance of differences in cumulative probability distributions of amplitudes and interevent intervals of mIPSCs were evaluated with the Kolmogorov-Smirnov test. p < 0.05 was taken as the limit of statistical significance.

**Drugs.** Drugs were obtained from the following sources: QX-314 (Alamone Labs, Jerusalem, Israel); ionomycin (Calbiochem, San Diego, CA); (−)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol (CP55940) (Pfizer, Groton, CT; N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide (SR141716; recently named rimonabant) (Sanofi, Montpellier, France); amphotericin B, ATP-Mg, ATP- Na2, bicuculline, and tetrodotoxin (Sigma Chemie, Deisenhofen, Germany); and DNQX, AP5, and (R)-(−)-2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2-d:1,4-benzoxazin-7-yl](1-naphthyl)ethanone mesylate (WIN 55212-2) (Torres Cooke, Bristol, England).

The cannabinoid ligands WIN 55212-2, CP55940, and SR141716 were dissolved in dimethyl sulfoxide (DMSO). Stock solutions were stored at −20°C. Dilutions were made with superfusion buffer; the final concentration of DMSO in the superfusion fluid was 1 ml/l. Control solutions [SOL (solvent) in the figures] always contained the appropriate concentration of DMSO. DNQX, bicuculline, and ionomycin were also dissolved in DMSO. AP5 and tetrodotoxin were dissolved in distilled water. Stock solutions were stored at −20°C. Further dilutions were made with superfusion buffer.

**Results**

**Basic Properties of Synaptic Transmission between Basket and Purkinje Cells.** To study the effect of activation of cannabinoid receptors on neurotransmission between basket and Purkinje cells, activities of synaptically connected pairs of neurons were simultaneously recorded (Fig. 1A). Action currents (henceforth called action potentials) due to spontaneous firing of basket cells were registered in cell-attached configuration. sIPSCs in neighboring Purkinje cells were recorded in whole cell configuration.

In the example shown in Fig. 1B1, every presynaptic action potential was followed by a successful postsynaptic event. The mean success rate of neurotransmission in the group of
neurons we studied was 83 ± 5% (n = 20; PRE): for 100 presynaptic action potentials in basket cells, a synchronous successful postsynaptic event in Purkinje cells occurred, on average, 83 times. The amplitude of successful action potential-coupled postsynaptic currents was 219 ± 39 pA (n = 20; PRE). These properties of synaptic transmission are similar to those observed previously for the basket cell-Purkinje cell synapse (Than and Szabo, 2002).

**Activation of CB1 Cannabinoid Receptors Leads to Inhibition of Synaptic Transmission between Basket and Purkinje Cells.** The synthetic CB1/CB2 cannabinoid receptor agonist WIN 55212-2 (10⁻⁵ M) changed neurotransmission in two ways: it lowered the success rate (compare Fig. 1B1, 1B2, and 1E), and it lowered the amplitude of successful action potential-coupled postsynaptic currents (Fig. 1, C and D). The inhibitory effects of WIN 55212-2 developed slowly, they reached maximum 10–15 min after beginning of the superfusion of the drug. Cannabinoids generally act slowly in brain slices, probably because they are strongly lipophilic (Levenes et al., 1998; Wallmichrath and Szabo, 2002). During the second part of the experiments, the CB1 cannabinoid receptor antagonist SR141716 (10⁻⁶ M) was superfused in addition to WIN 55212-2 (Fig. 1, D and E). SR141716 antagonized the effects of WIN 55212-2 on the amplitude of action potential-coupled postsynaptic currents (Fig. 1D) and the success rate (Fig. 1E).

In Fig. 2, the effects of WIN 55212-2 (10⁻⁵ M) are compared with those of solvent. The amplitude of action potential-coupled postsynaptic currents and the success rate of neurotransmission remained fairly constant in solvent-treated slices (Fig. 2). WIN 55212-2 caused significant inhibition also if it was compared with solvent. After 15 min of superfusion, the effects of WIN 55212-2 tended to be weaker; this could be due to desensitization of the CB1 receptor. In addition, we tested the effects of CP55940, another synthetic CB1/CB2 cannabinoid receptor agonist. The chemical structure of CP55940 differs markedly from that of WIN 55212-2. CP55940 (10⁻⁶ M) depressed the amplitude of action potential-coupled postsynaptic currents (Fig. 2A). The success rate of neurotransmission was not changed by CP55940 at this concentration (Fig. 2B). CP55940 influenced neurotransmission less than WIN 55212-2, probably because its concentration was lower and because it is only a partial agonist in some neuronal test systems (Shen et al., 1996).

The effects of the CB1 cannabinoid receptor antagonist SR141716 were studied in an additional series of experiments. Superfused alone, SR141716 (10⁻⁶ M) did not change neurotransmission. Thus, 15 min after the beginning of SR141716 superfusion, the amplitude of action potential-coupled postsynaptic currents was 79 ± 9% of PRE (n = 3); the success rate of neurotransmission was 100 ± 1% of PRE (n = 3). This observation indicates that under the present experimental conditions, endogenous cannabinoids (endocannabinoids) do not inhibit transmission between basket and Purkinje cells. During the second part of these experiments, WIN 55212-2 (10⁻⁵ M) was superfused in the continued presence of SR141716. SR141716 completely prevented the effects of WIN 55212-2. Thus, 10 min after the beginning of WIN 55212-2 superfusion, the amplitude of action potential-coupled postsynaptic currents was 104 ± 2% of the value preceding WIN 55212-2 superfusion (n = 3); the success rate of neurotransmission was 99 ± 1% of the value preceding WIN 55212-2 superfusion (n = 3). This interaction (and the interaction between WIN 55212-2 and SR141716 shown in Fig. 1) verifies that CB1 cannabinoid receptors were involved in the inhibition of neurotransmission by WIN 55212-2.

**Effects of Cannabinoids on the Firing of Basket Cells.** The effects of cannabinoids on the firing of basket cells are shown in Fig. 3. Before superfusion of solvent or drug, basket cells fired action potentials at a rate of 3.8 ± 0.7 Hz (n = 21; PRE). The firing rate remained constant also in the presence of the cannabinoid agonists WIN 55212-2 (10⁻⁵ M) and CP55940 (10⁻⁶ M) and the antagonist SR141716 (10⁻⁶ M) (Fig. 3, A and B). In Fig. 3C, the effects of cannabinoids on the firing rate of basket cells and their effects on synaptic transmission between basket and Purkinje cells are compared. WIN 55212-2 and CP55940 lowered the amplitude of action potential-coupled postsynaptic currents (they inhibited synaptic transmission) but they did not influence the firing of basket cells. Thus, activation of CB1 cannabinoid receptors led to selective inhibition of neurotransmission; effects on the somadendritic region of the presynaptic neuron were not elicited. The blockade of CB1 receptors changed neither neurotransmission nor action potential generation.
Depolarization of the Postsynaptic Neuron Inhibits Synaptic Transmission between Basket and Purkinje Cells. Depolarization of Purkinje cells leads to depression of their GABAergic input (Llano et al., 1991). Depolarization of Purkinje cells for 5 s (Fig. 4A) inhibited synaptic transmission also in our experiments: the amplitude of action potential-coupled postsynaptic currents (Fig. 4B) and the success rate of neurotransmission (Fig. 4C) decreased. In contrast, the firing rate of basket cells was not changed (Fig. 4D).

To determine the mechanism of the inhibition evoked by depolarization of Purkinje cells, we carried out experiments in the presence of the cannabinoid agonist CP55940 (10^{-6} M) and the CB₁ antagonist SR141716 (10^{-6} M). CP55940 (10^{-6} M) inhibited neurotransmission already before depolarization of Purkinje cells. Thus, the amplitude of action potential-coupled postsynaptic currents (147 ± 40 pA; n = 5) and the success rate of neurotransmission (71 ± 10%; n = 5) were lower in the CP55940-treated group than in the solvent-treated group (298 ± 73 pA; 93 ± 4%; n = 12). In the presence of CP55940, depolarization of Purkinje cells no longer inhibited synaptic transmission (Fig. 4, B and C). In the presence of SR141716 (10^{-6} M), the amplitude of action potential-coupled postsynaptic currents (255 ± 126 pA; n = 4) and the success rate of neurotransmission (95 ± 2%; n = 4) were similar to the values determined in the solvent-treated group. SR141716A also prevented the depolarization-induced inhibition of synaptic transmission (Fig. 4, B and C). The abolition of the depolarization-induced inhibition of synaptic transmission by CP55940 and SR141716 supports the idea that endocannabinoids released from the depolarized postsynaptic neuron were involved in the inhibition of synaptic transmission.

Mechanism of the Inhibition of Synaptic Transmission by Cannabinoids: Analysis of mIPSCs. Exogenous (Figs. 1 and 2) and endogenous cannabinoids (Fig. 4) inhibited synaptic transmission between basket and Purkinje cells. We carried out three kinds of experiments to determine whether this was due to presynaptic inhibition of GABA release from axon terminals of basket cells or to interference with the effects of released GABA on postsynaptic Purkinje cells. At first, we carried out a traditional analysis of mIPSCs.

mIPSCs were isolated by tetrodotoxin (3 × 10^{-7} M). During the initial reference period (PRE), the frequency and amplitude of mIPSCs were 1.9 ± 0.4 Hz and 130 ± 25 pA (n = 23), respectively. In control experiments with solvent, the frequency and amplitude of mIPSCs remained constant (Fig. 5E).

WIN 55212-2 (10^{-5} M) lowered the frequency of mIPSCs (Fig. 5, A, C, and E). In contrast, it did not change the amplitude of mIPSCs (Fig. 5, B, D, and E). Lack of effect of WIN 55212-2 on the amplitude of mIPSCs indicates that the cannabinoid did not interfere with the effect of released GABA on postsynaptic Purkinje cells. This is indirect evidence for a presynaptic mode of action of cannabinoids at inhibiting synaptic transmission. The direct evidence for the inhibition of GABA release from terminals of basket cells is the decrease in the frequency of mIPSCs produced by WIN 55212-2.

The decrease in the frequency of mIPSCs shows that WIN 55212-2 inhibited transmitter release independently of actions on voltage-dependent sodium and calcium channels.
Spontaneous transmitter release in the absence of calcium influx through voltage-dependent calcium channels can be due to calcium release from intracellular stores (Emptage et al., 2001). Thus, lowering of mIPSC frequency by WIN 55212-2 can be attributed to inhibition of calcium release from intracellular stores or to inhibition of the vesicular release machinery. In the second series of experiments, we sought to answer the question whether vesicular release was inhibited when it was activated by calcium. Vesicular release was activated in the presence of tetrodotoxin by superfusion with the calcium ionophore ionomycin.

Superfusion with ionomycin (2 × 10^{-6} M) led to a continuous increase in the frequency of mIPSCs in the group of neurons that received solvent in addition; the amplitude of mIPSCs was not changed (Fig. 6E). Ionomycin increased the frequency, but not the amplitude, of mIPSCs also in the
group that later received WIN 55212-2 (Fig. 6, A–E). WIN 55212-2 (10^-5 M) lowered the frequency, but not the amplitude, of ionomycin-stimulated mIPSCs (Fig. 6, A–E). Twelve minutes after the beginning of WIN 55212-2 superfusion, the frequency of mIPSCs returned to the initial PRE level (Fig. 6E). Thus, WIN 55212-2 suppressed vesicular release when it was activated by calcium.

**Mechanism of the Inhibition of Synaptic Transmission by Cannabinoids: Analysis of Autoreceptor Currents.** To further analyze the mechanism of the synaptic inhibition by cannabinoids, we took advantage of an interesting property of basket cells. GABA released from axon terminals of basket cells activates GABAA autoreceptors on the terminals, and the resulting autoreceptor current can be recorded via a pipette in the soma of the cells (Pouzat and Marty, 1999) (see Fig. 7 for explanation of the generation of the autoreceptor current). To determine whether cannabinoids inhibit GABA release from axon terminals of basket cells, we analyzed their effects on the autoreceptor current.

Basket cells were patched in the perforated patch mode; the holding potential was -70 mV. Autoreceptor currents were elicited by depolarizing the neurons by 40 to 70 mV for 1 to 2 ms. The amplitude of the autoreceptor currents was 56 ± 9 pA (n = 16). The latency between the downward deflection of the Na^+ current and the time point at which the maximum of the autoreceptor current was 8.4 ± 0.3 ms (n = 16). Width of the current at half-amplitude was 34 ± 4 ms (n = 16). The decay time constant was 35 ± 4 ms (n = 16). Finally, the success rate of autoreceptor currents was high (>90%; see Figs. 8A and 9A). The slow kinetic and the high success rate are typical for autaptic currents recorded in basket cells (Pouzat and Marty, 1998). Fitting to the mechanism of the autoreceptor currents (Fig. 7), they were eliminated when voltage-dependent sodium and calcium channels and GABA_A receptors were blocked by tetrodotoxin (3 × 10^-7 M) (Fig. 8, A and B), cadmium (5 × 10^-5 M) (Fig. 8C), and bicuculline (2 × 10^-5 M) (Fig. 8D), respectively.

**Fig. 6.** Effects of SOL and WIN 55212-2 (WIN) on mIPSCs recorded in the presence of tetrodotoxin and ionomycin (iono). Tetrodotoxin (3 × 10^-7 M) was present throughout. After an initial reference period (PRE), superfusion of ionomycin (2 × 10^-6 M) commenced (E). One of the groups received later SOL, the other group WIN (10^-5 M). A, original tracings from an experiment with WIN, B, averaged mIPSCs from an experiment with WIN (same experiment as in A). C and D, cumulative probability distribution plots of interevent intervals and amplitudes of mIPSCs from an experiment with WIN (same experiment as in A). The effects of ionomycin (ionomycin versus SOL) and WIN (WIN versus ionomycin) on the interevent interval were significant (Kolmogorov-Smirnov test, p < 0.05). E, means ± S.E.M. of six (SOL) and four (WIN) experiments. The original recordings in A to D were obtained at time points 1 to 3 shown in E. Significant difference from SOL: *p < 0.05.

**Fig. 7.** Autoreceptor current in basket cells. A, events involved in the generation of the autoreceptor current. The basket cell was approached in the perforated patch mode. Depolarization of the soma initiated action potentials, which in turn elicited GABA release from the terminals. The released GABA activated GABA_A autoreceptors, and the resulting chloride current was recorded with the pipette on the soma. B, current recording in a typical experiment.
Autoreceptor currents remained remarkably stable for 30 min in control experiments in which solvent was superfused (Fig. 9E). Recorded in the whole cell patch-clamp mode, autoreceptor currents show a strong run-down within 10 to 15 min (Pouzat and Marty, 1999; Than and Szabo, 2002). The stability in the present experiments was most probably due to recording in the perforated patch-clamp mode. With this stability, it was possible to study the effects of the slowly acting cannabinoids.

The effects of WIN 55212-2 on the autoreceptor current are shown in Fig. 9. In one especially long recording, effects of WIN 55212-2 (10⁻⁵ M) were studied alone and in the presence of SR141716 (10⁻⁶ M); in this experiment, effects on the sodium current were also evaluated (Fig. 9, A-D). WIN 55212-2 (10⁻⁵ M) markedly inhibited the autoreceptor current (Fig. 9, A and B), without changing the sodium current (Fig. 9, C and D). When, during the second part of the experiment, the antagonist SR141716 (10⁻⁶ M) was superfused together with WIN 55212-2, the amplitude of the autoreceptor current returned to the initial reference level (Fig. 9, A and B). This latter observation points to the involvement of CB₁ cannabinoid receptors. The statistical evaluation in Fig. 9E shows that the effect of WIN 55212-2 was concentration-dependent. Similarly to the inhibitory effect of WIN 55212-2 on the amplitude of action potential-coupled postsynaptic currents (Fig. 1D) and the success rate of neurotransmission (Fig. 1E), the inhibition of the autoreceptor current developed slowly: 10 to 15 min was necessary for reaching maximal inhibition (Fig. 9, A and B).

Dopolarizing voltage jumps (Fig. 4) and action potential series (Ohno-Shosaku et al., 2001) can elicit the release of endocannabinoids from postsynaptic neurons that in turn inhibit transmitter release from the presynaptic terminal. We considered the hypothesis that depolarization of the presynaptic terminal can elicit endocannabinoid synthesis also in the terminal and the generated endocannabinoids inhibit transmitter release from the terminal itself. To test this hypothesis, we studied the effect of a rapid series of action potentials on autoreceptor currents and observed the effect of the CB₁ antagonist SR141716 (Fig. 10). Application of 250 action potentials at a frequency of 50 Hz potentiated autoreceptor currents for 1 to 2 min (Fig. 10). The potentiation was very similar if the experiment was carried out in the presence of SR141716 (10⁻⁶ M) (Fig. 10). The reason for the potentiation is not known. However, the lack of effect of the CB₁ antagonist makes it unlikely that endocannabinoids released by the series of action potentials from the axon terminal influenced transmitter release from the presynaptic terminal.

**Discussion**

We studied GABAergic neurotransmission between basket and Purkinje cells under near-physiological conditions, i.e., transmitter release was elicited by action potentials generated by the spontaneously firing intact presynaptic neuron. Activation of CB₁ cannabinoid receptors by exogenous agonists inhibited the transmission between the two neurons. Several experiments, including analysis of autoreceptor currents, indicated that the neurotransmission was inhibited with a presynaptic mechanism. Endocannabinoids released from depolarized Purkinje cells also produced presynaptic inhibition. In contrast, depolarized basket cell axon terminals did not release endocannabinoids.

WIN 55212-2 is an aminoalkylindole derivative and CP55940 is a bicyclic analog of Δ⁹-tetrahydrocannabinol.
Both of these synthetic cannabinoids possess affinity for CB₁ and CB₂ receptors but have low affinity for other neurotransmitter receptors and ion channels (Pertwee, 1999; Howlett et al., 2002). Similar inhibition of neurotransmission by these two chemically different agonists strongly suggests involvement of cannabinoid receptors in their effects. Abolishment of the inhibitory effects of WIN 55212-2 by the CB₁-selective antagonist SR141716 (Pertwee, 1999; Howlett et al., 2002) points to involvement of CB₁ receptors. This observation is congruent with anatomical evidence: CB₂ receptors are generally absent in the brain and CB₁ receptors are specifically present in the presynaptic axon terminals of basket cells (see Introduction).

Four observations indicate that the cannabinoids inhibited neurotransmission between basket and Purkinje cells with a presynaptic mechanism. First, the success rate of neurotransmission was decreased by WIN 55212-2, probably because WIN 55212-2 inhibited the quantal release process. Second, WIN 55212-2 did not change the amplitude of mIPSCs recorded in Purkinje cells. This observation excludes the possibility that the cannabinoid interferes with the activation of postsynaptic GABAₐ receptors by synaptically released GABA. Third, WIN 55212-2 decreased the frequency of mIPSCs, an indication of an inhibition of the synaptic vesicle release mechanism. Fourth, a further kind of proof for the presynaptic action of the cannabinoids is derived from the autoreceptor current experiments (see below). A further argument for a presynaptic mechanism of action is the anatomical localization of the CB₁ receptor in basket cell axon terminals, but its absence in postsynaptic Purkinje cells (see Introduction).

Autoreceptor currents are elicited by somatic depolarization and are due to the action of GABA released from axon terminals on GABAₐ autoreceptors of the same axon terminals. In accord with this mechanism, they were abolished by tetrodotoxin, cadmium, and bicuculline. WIN 55212-2 concentration dependently inhibited the autoreceptor currents and the effect was blocked by the CB₁-selective antagonist SR141716. Inhibition of the autoreceptor current by WIN 55212-2 is direct proof, and a novel kind of proof, of the inhibition of GABA release from terminals of basket cells. It is remarkable that another drug that frequently causes presynaptic inhibition, the GABAₐ receptor agonist baclofen, also inhibited autoreceptor currents recorded in basket cells (Than and Szabo, 2002). WIN 55212-2 did not change the voltage-dependent sodium current in basket cells. Inhibition of action potential propagation due to sodium channel blockade is, therefore, not involved in the inhibition of the autoreceptor current, and more generally, in the inhibition of neurotransmission between basket and Purkinje cells.

Theoretically, at least three mechanisms can be involved in presynaptic inhibition of transmitter release by cannabinoids (for review, see Schlicker and Kathmann, 2001): activation of potassium channels (Daniel and Crepel, 2001; Diana and Marty, 2003), inhibition of voltage-dependent calcium channels (Kreitzer and Regehr, 2001b; Diana et al., 2002), and direct inhibition of the vesicular release machinery (Takahashi and Linden, 2000). In our study, WIN 55212-2 lowered the frequency of mIPSCs, also when vesicular release was stimulated by the calcium ionophore ionomycin. This observation indicates that the cannabinoid directly inhibits the vesicular release machinery. Involvement of potassium and...
calcium channels was not investigated in the present study; their modulation probably also contributes to the inhibition of GABA release from terminals of basket cells by cannabinoids.

The CB₁ receptor antagonist SR141716 itself did not change ongoing neurotransmission between basket and Purkinje cells, indicating that under basal conditions endocannabinoids were not released. SR141716 prevented, however, the depolarization-evoked inhibition of neurotransmission. This indicates that activation of CB₁ receptors by endocannabinoids is involved in the depolarization-evoked inhibition of neurotransmission. This assumption is supported by occlusion of the inhibition by the cannabinoid agonist CP55940: in the presence of CP55940-evoked presynaptic inhibition, endocannabinoids could not produce additional inhibition. Similar involvement of endocannabinoids in retrograde neuronal signaling was observed recently at several synapses in the brain (Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001; for reviews, see Wilson and Nicoll, 2002; Freund et al., 2003). In the autoreceptor current experiments, we addressed the question whether endocannabinoids are also released from axon terminals. A series of action potentials did not depress the following autoreceptor currents; this indicates that axon terminal CB₁ receptors are activated only by endocannabinoids released from postsynaptic neurons and not by endocannabinoids released from axon terminals. Our axon stimulation conditions (250 action potentials at 50 Hz) were appropriate: stimulation with these parameters elicited strong endocannabinoid release from the somadendritic region of neurons (Ohno-Shosaku et al., 2001).

Activation of the CB₁ cannabinoid receptor led to selective inhibition of GABA release from basket cell terminals; the firing rate of basket cells was not changed. This was true for the synthetic agonists WIN 55212-2 and CP55940 (Fig. 3) and for the endogenous cannabinoid released by depolarized Purkinje cells (Fig. 4). The GABA<sub>γ</sub> receptor agonist baclofen, studied under the same conditions as the cannabinoid agonists, decreased simultaneously GABA release from axon terminals of basket cells and the firing rate of basket cells (see Fig. 3 in Than and Szabo, 2002). Thus, compared with baclofen, the cannabinoid agonists act preferentially at presynaptic terminals. We believe that this preferential presynaptic action is typical for cannabinoids, even though under optimal experimental conditions weak somadendritic effects occur (see next paragraph). A preferential presynaptic action of cannabinoid agonists in our preparation is also supported by the localization of the CB₁ receptor: whereas CB₁ receptors are easily visible in basket cell terminals, “GABAergic interneuron (basket cell) somata do not express CB₁Rs” (Diana et al., 2002). Preferential localization of CB₁ receptors in axon terminals was also observed in hippocampal neurons (Twitchell et al., 1997; Irving et al., 2000).

In the presence of bicuculline and using pipettes containing a NaCl-based solution, WIN 55212-2 weakly inhibited the firing of basket cells, similarly as in the experiments of Kreitzer et al. (2002). It is not completely understood why this modification of conditions changed the somadendritic effect of WIN 55212-2. One possible explanation is that in the absence of bicuculline, a direct inhibitory effect of the cannabinoid on basket cell firing was counteracted by an inhibition of the GABAergic input to the basket cells by the cannabinoid (disinhibition). Basket cells receive inhibitory input from neighboring basket and stellate cells, and we expect that cannabinoids presynaptically inhibit GABAergic neurotransmission not only at the basket-Purkinje cell synapse but also at the basket-basket cell and stellate-basket cell synapses.

Effects of exogenous and endogenous cannabinoids on the GABAergic input of Purkinje cells were analyzed in several recent publications. Cannabinoids depressed spontaneous and miniature IPSCs recorded in Purkinje cells (Takahashi and Linden, 2000; Kreitzer and Regehr, 2001a) and IPSCs elicited by electrical stimulation in the molecular layer (Yoshida et al., 2002). The connection between basket and Purkinje cells was studied also directly, by simultaneous recording from basket cells in the perforated patch mode and Purkinje cells in the whole cell mode (Diana et al., 2002; Diana and Marty, 2003). Synaptic transmission elicited by depolarization of basket cells was inhibited by exogenous and endogenous cannabinoids in these latter experiments.

Similarly to these above-mentioned studies, we also showed that exogenous and endogenous cannabinoids presynaptically depress synaptic transmission between basket and Purkinje cells. The distinguishing feature of our study is that this was shown under near-physiological conditions. Thus, the presynaptic cell was intact and synaptic transmission was elicited by spontaneous action potentials at physiological frequencies. Inhibition of neurotransmission that is due to inhibition of action potential propagation at axon branching points and at terminal axons could also occur in our model. We obtained novel and conclusive evidence for a presynaptic mode of action by analyzing autoreceptor currents. We observed, again by using the possibilities offered by autoreceptor current analysis, that endocannabinoids are not released by depolarized axon terminals. Finally, being able to simultaneously observe presynaptic and somadendritic effects, we showed that cannabinoids act preferentially at the axon terminal; their action on the somadendritic region of the same neuron is much weaker.

Interestingly, activation of CB₁ receptors in the cerebellar cortex modulates not only inhibitory but also excitatory neurotransmission. Thus, exogenous cannabinoid agonists and endocannabinoids released from Purkinje cells inhibit glutamatergic neurotransmission between parallel fibers and Purkinje cells and between climbing fibers and Purkinje cells (Levenes et al., 1998; Takahashi and Linden, 2000; Kreitzer and Regehr, 2001b).

Activation of CB₁ receptors inhibits neurotransmission not only in the cerebellum. Inhibition of glutamatergic, GABAergic, glycinerergic, cholinergic, noradrenergic, and serotonergic neurotransmission was shown in many regions of the central and peripheral nervous systems (Gifford and Ashby, 1996; Shen et al., 1996; Nakazi et al., 2000; Katona et al., 2001; Szabo et al., 2000, 2001; Hoffman et al., 2003; for reviews, see Schlicker and Kathmann, 2001; Freund et al., 2003). At present, presynaptic inhibition of neurotransmitter release is the best characterized neuropharmacological effect of cannabinoids, probably because it is the most important effect of cannabinoids on the nervous system. To our knowledge, the present study demonstrated cannabinoid-evoked presynaptic inhibition under conditions closest to physiological.

Cannabinoids cause static and gait ataxia in dogs and mice, and this is attributed to cerebellar dysfunction (Fränkel, 1903; Walton et al., 1938; Dar, 2000; Patel and
Hillard, 2001). Although activation of CB₁ receptors also inhibits the excitatory input of Purkinje cells, it is thought that inhibition of the inhibitory input of the Purkinje cells leads to cerebellar ataxia (Patel and Hillard, 2001). Accordingly, the presynaptic inhibition of neurotransmission between basket and Purkinje cells, as observed in the present study, may be the basic mechanism behind the cerebellar ataxia produced by cannabinoids.

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References

Daniel H and Crepel F (2001) Control of Ca²⁺ influx by cannabinoid and metabotropic glutamate receptors in rat cerebellar cortex requires K⁺ channels. J Physiol (Lond) 537:793–800.


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