Cannabinoids Depress Inhibitory Synaptic Inputs Received by Layer 2/3 Pyramidal Neurons of the Neocortex

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INTRODUCTION

Cortical synapses are under the continuous influence of converging chemical modulators, arising from extracortical afferents as well as from cells within the cerebral cortex. A number of recent observations suggest that the endogenous cannabinoids may represent a novel class of intrinsic modulators in this brain region. First, the Gi/o protein–linked type 1 cannabinoid receptor (CB1R) is abundantly expressed throughout the cortical mantle with high levels in superficial layers 2 and 3 (Egertova and Elphick 2000; Egertova et al. 1998; Marsicano and Lutz 1999). Second, cortical neurons are capable of expressing CB1R antagonist AM251, confirming a CB1R-mediated inhibition. Pairing evoked inhibitory postsynaptic currents (IPSCs) at short interstimulus intervals while applying WIN55,212-2 resulted in an increase in paired-pulse facilitation suggesting that the probability of GABA release was reduced. A presynaptic site of cannabinoid action was verified by an observed decrease in the probability of GABAergic inhibitory postsynaptic currents evoked by extracellular stimulation within layer 2/3. The suppression of this inhibition was blocked and reversed by the highly selective CB1R antagonist AM251, confirming a CB1R-mediated inhibition. When Ca2+ was added or Ca2+ was omitted from the recording solution, the remaining fraction of Ca2+-independent IPSCs did not respond to WIN55,212-2. These data suggest that cannabinoids are capable of suppressing the inhibition of neocortical pyramidal neurons by depressing Ca2+-dependent GABA release from local interneurons.

METHODOLOGY

Swiss-Webster mice (postnatal day 12 to 20 [P12–20]; Charles River) were rapidly decapitated following CO2 asphyxiation according to procedures approved by the University of Connecticut Health Center Animal Care Committee. Brains were rapidly dissected into ice-cold saline containing (in mM) 125.0 NaCl, 2.5 KCl, 1.25 NaH2PO4, 25.0 NaHCO3, 2.0 CaCl2, 1.0 MgCl2, and 20.0 glucose, and gassed with 95% O2–5% CO2 (pH 7.3, 317 ± 3 mmol · kg−1, mean ± SE) and sectioned (300 μm) in the coronal plane. Cortical slices containing auditory fields (Frisina and Walton 2001; Paxinos and Franklin 2001) were incubated for 30–45 min in

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32°C saline before being transferred to a recording chamber perfused with oxygenated saline (22–23°C). Ionotropic glutamate receptors were blocked with 6,7-dinitroquinoxaline-2,3-dione (DNQX, 10 μM, Tocris, Bristol, UK) and 3-[(R)-2-carboxypiperazin-4-yl]-propyl-1-phosphonic acid (CPP, 2 μM, Tocris). Layer 2/3 pyramidal neurons were visualized at ×400 (Olympus LUMPlanFL, 0.80NA) with infrared-DIC optics. These neurons responded to depolarizing current injection with regular, frequency-adapting spikes (Trettel and Levine 2001), characteristic of cortical pyramidal cells (Connors and Gutnick 1990; McCormick et al. 1985). All recordings used in these analyses were made in the whole cell voltage-clamp configuration with borosilicate glass micropipettes (R<sub>i</sub> = 3–5 MΩ) filled with (in mM) 120.0 CsCl, 10.0 HEPES, 1.0 CaCl<sub>2</sub>, 1.5 MgCl<sub>2</sub>, 4.0 Na<sub>2</sub>-ATP, 0.3 Na-GTP, and 5.0 QX-314 (pH 7.3, 290 ± 4 mmol·kg⁻¹). Signals were filtered at 2.9 kHz and digitized at ≈6 kHz using a HEKA EPC9 amplifier and a PCl-16 interface board (Heka Elektronik, Darmstadt, Germany). On breaking into whole cell configuration, a brief series of voltage ramps (50 ms, 2 mV/ms) were applied to promote the activity-dependent block of Na<sup>+</sup> conductances by QX-314 (Sigma, St. Louis, MO). Series resistance (R<sub>i</sub>) was then compensated to 60% or greater at 10–100 μs lag (8.7 ± 0.4 MΩ uncompensated R<sub>i</sub>, n = 44). During the course of the experiments, leak currents were subtracted on-line (P/4), and the input resistance (R<sub>i</sub>) was monitored continuously with 5-mV hyperpolarizing voltage steps (50 ms). Neurons were rejected from analyses 1) if R<sub>i</sub> was >23 MΩ at the time of break-in or >10.5 MΩ after compensation, 2) if R<sub>i</sub> changed by >15% during the course of an experiment, or 3) if R<sub>i</sub> fell below 100 MΩ. All drugs were delivered through the bath perfusion system at 2–3 ml/min. WIN55,212-2 (Sigma), AM251 (Gift from Dr. A. Makriyannis, University of Connecticut), and DNQX were stored in 10-mM aliquots in DMSO at −20°C. WIN55,212-2 and AM251 were delivered in saline containing 0.01% BSA; final DMSO concentration did not exceed 0.03%.

Evoked inhibitory postsynaptic currents (eIPSCs) were elicited by applying 50-μs current pulses at 0.1–0.2 Hz through a saline-filled glass micropipette or a bipolar tungsten electrode (World Precision Instruments, Sarasota, FL) positioned 150–200 μm lateral to the recording pipette, within layer 2. The intensity of the stimulation was adjusted so that the average eIPSC amplitude was ~70% of the maximal amplitude for each recording and ranged from 30 to 300 μA. Figure 1A illustrates the current-voltage relationship of the pharmacologically isolated GAB<sub>A</sub>-mediated Cl⁻ conductance (n = 5). Some outward rectification of the eIPSCs could be seen before the dialysis of QX-314 was complete. Postsynaptic GAB<sub>A</sub> responses were blocked by intracellular Cs<sup>+</sup> and QX-314. Action potential–independent IPSCs (mIPSCs) were recorded in the presence of 1 μM TTX and 2.0 mM [Ca<sup>2+</sup>]<sub>i</sub>. For nominally Ca<sup>2+</sup>-free experiments, Ca<sup>2+</sup> ions were replaced with Mg<sup>2+</sup> and 1 mM EGTA. All IPSCs were abolished by the GAB<sub>A</sub> antagonist bicuculline methiodide (BMI, 30 μM, Sigma; see Fig. 1B).

Off-line analysis was carried out using PulseFit (Heka Elektronik) and MiniAnalysis (Synaptosoft, Decatur, GA) software. The effects of the test solutions on eIPSCs were determined by comparing the currents evoked during a 5-min baseline period (BL, 30 sweeps) to those from a 5-min window centered around the termination of the 10-min drug exposure (30 sweeps). The mean amplitudes and the rise and decay times for the eIPSCs were compared using the Student’s t-test. Miniature IPSCs were differentiated from noise by detecting inward peaks in continuous recordings that exceeded an area threshold and had exponential rise and decay time constants. The binwidth used for analyzing mIPSC frequency and kinetics before and during drug exposure was 120 s. Nonparametric Kolmogorov-Smirnov (K-S) statistics were used to compare mIPSC amplitude distributions, and the Student’s t-test and one-way ANOVAs were used for determining significant changes in mIPSC frequency and rise and decay times. The paired-pulse ratio of eIPSCs (PPR = IPSC<sub>2</sub>/IPSC<sub>1</sub>; see Fig. 3A) was determined at 75 ms ISI with analysis bins that were identical to those used for eIPSC analysis (i.e., 30 sweeps) and significance was established using Student’s t-test. Paired-pulse data are reported as the mean PPRs before and after WIN, calculating the mean PPR by dividing the P2 mean by the P1 mean yielded similar results. All data are presented as means ± SE.

**RESULTS**

Whole cell recordings were made from 44 layer 2/3 pyramidal neurons in primary and secondary auditory cortices. At a holding potential of −70 mV and with E<sub>Cl</sub> = −2.4 mV, stimulation within layer 2/3 resulted in a multicomponent, inward postsynaptic current (not shown). Adding 10 μM DNQX and 2 μM CPP to the bath left a BMI-sensitive,
GABA<sub>A</sub>-mediated eIPSC (mean amplitude = -326.8 ± 54.1 pA; n = 26) that reversed polarity near E<sub>GCl</sub> (Fig. 1A, n = 5). Application of the CB1R agonist WIN55,212-2 (3 μM) reduced the amplitude of eIPSCs in 15/15 cells tested (Fig. 1, B and C; 63.7 ± 4.8% of BL; P < 0.0001), and the magnitude of this effect was not correlated with the age of the animal used (P > 0.90). The rise and decay time constants of the eIPSCs were not altered by WIN55,212-2 exposure (P > 0.30, n = 15). The vehicle solution had no effect on eIPSC amplitude (Fig. 1C; n = 3; 99.2 ± 2.6% of BL). The effect of WIN55,212-2 on eIPSC amplitude was blocked by pretreatment with the competitive CB1R antagonist AM251 (5 μM; n = 3; BL = -447.6 ± 60 pA, WIN + AM251 = -466 ± 118 pA; P > 0.70), which alone had no effect on eIPSC amplitude (Fig. 1C; n = 5; P > 0.40). In addition, application of AM251 during WIN55,212-2 exposure in a separate group of cells reversed the depression of eIPSC amplitude to near baseline values (n = 3; see example in Fig. 1B). Because the type-2 cannabinoid receptor is not expressed in the CNS (Munro et al. 1993) and the suppression of eIPSC amplitude by WIN55,212-2 was blocked and reversed by the highly selective CB1R antagonist AM251 (i.e., K<sub>i</sub> = 7.5 nM) (Lan et al. 1999), we conclude that the effect of WIN55,212-2 in this preparation is mediated by CB1R.

The reduction in eIPSC amplitude caused by WIN55,212-2 could involve presynaptic and/or postsynaptic mechanisms. To address this issue we examined the effects of WIN55,212-2 on mIPSCs in saline containing 1 μM TTX (Fig. 2A). In the absence of WIN55,212-2, baseline mIPSC frequency was 2.43 ± 0.5 Hz (n = 10), which was not different from the frequency of mIPSCs recorded in the presence of the vehicle control solution (Fig. 2B; 100.3 ± 3.2% of BL, n = 3, P > 0.70). Adding 3 μM WIN55,212-2 to the bath perfusate reduced the frequency of mIPSCs to 1.57 ± 0.2 Hz (Fig. 2B; n = 7, P < 0.05). WIN55,212-2 had no effect on mIPSC peak amplitude in five of seven cells (see example in Fig. 2C; P > 0.5, K-S) or on rise and decay kinetics (Fig. 2D; BL rise 10–90% = 2.48 ± 0.11 ms, τ<sub>decay</sub> = 9.2 ± 1.3 ms). The reduction in mIPSC frequency with no change in peak amplitude or kinetics suggests that the CB1R-mediated reduction in eIPSCs (Fig. 1, B and C) results from a suppression of presynaptic GABA release from interneuron terminals. Because transmitter release is dependent on the voltage-gated influx of Ca<sup>2+</sup> and CB1R activation has been shown to reduce Ca<sup>2+</sup>-conductance through N and P/Q-type Ca<sup>2+</sup> channels (Caulfield and Brown 1992; Twitchell et al. 1997), we tested the hypothesis that WIN55,212-2 reduced mIPSC frequency by retarding presynaptic Ca<sup>2+</sup>-influx. When 100 μM Cd<sup>2+</sup> was added to the extracellular solution to block voltage-gated Ca<sup>2+</sup>-influx, mIPSC frequency was reduced to 68.1 ± 6.1% of BL (Fig. 2E; n = 4, P < 0.03). The addition of WIN55,212-2 to the Cd<sup>2+</sup>-containing bath, however, did not cause a further reduction in the frequency of the remaining fraction of mIPSCs (Fig. 2E; 107 ± 6.7% of Cd<sup>2+</sup> BL, n = 4). Similarly in four experiments, removing Ca<sup>2+</sup> from the medium (see METHODS) reduced mIPSC frequency (Fig. 2E; 60 ± 6.6% of BL; P < 0.05) and occluded the effect of WIN55,212-2 (89.8 ± 8.2% of Ca<sup>2+</sup>-free BL). WIN55,212-2 had no effect on mIPSC amplitude (P > 0.5, K-S) or kinetics (P > 0.1, Student’s t-test) in either Ca<sup>2+</sup> or Ca<sup>2+</sup>-free conditions (data not shown).

To further test the presynaptic locus of the CB1R-mediated suppression of GABAergic transmission, we repeated eIPSC experiments by pairing two stimuli at a 75-ms interstimulus interval and determined the PPR as mIPSC<sub>2</sub>/mIPSC<sub>1</sub> (see Fig. 3A). As shown in the example in Fig. 3B, exposure to WIN55,212-2 increased the PPR, switching the response from depression to facilitation. By comparison, in the same cell decreasing [Ca<sup>2+</sup>]<sub>i</sub> from 2.0 to 0.5 mM mimicked the effect of
WIN55,212-2 on the PPR, albeit with different temporal characteristics. In five cells, the mean PPR was increased from 0.65 ± 0.05 to 1.11 ± 0.15 following WIN55,212-2 treatment (Fig. 3C; \( P < 0.05 \)), indicating a reduction in the probability of transmitter release.

**Discussion**

The primary finding of this report is that application of the synthetic cannabinoid WIN55,212-2 depresses inhibitory synaptic transmission at GABAergic synapses received by layer 2/3 pyramidal cells in the mouse neocortex. The suppression, measured as a decrease in the amplitude of evoked IPSCs, was reliable (i.e., 15/15 cells) and was mediated by CB1R. This observation is in accord with the recent in vivo observations that WIN55,212-2 leads to decreased levels of extracellular GABA in the frontal cortex of the awake rat (Ferraro et al. 2001) and increased firing rates of prefrontal pyramidal neurons in anesthetized rats (Pistis et al. 2001). These latter results may also be partly attributable to a decrease in the spontaneous and stimulus-evoked firing rate of the interneurons. In the frontal cortex cannabinoid receptors have previously been shown to suppress glutamate release at layer 5 synapses received by pyramidal cells (Auclair et al. 2000), which could reduce the excitatory drive on interneurons. Nonetheless, we have demonstrated that WIN55,212-2 increases the excitability of layer 2/3 pyramidal neurons in response to extracellular, intralaminal field stimulation without altering pyramidal cell membrane potential (Trettel and Levine 2001), further supporting the idea that cannabinoids depress cortical inhibition. Taken together, it appears that activation of the CB1R in the neocortex results in a suppression of GABAergic inhibition from local circuit interneurons onto pyramidal neurons.

The suppression of cortical inhibition involves a presynaptic mechanism. Two primary observations reported here, 1) a reduction in the frequency of spontaneous, action potential–independent neurotransmitter release events from GABAergic terminals and 2) an increase in paired-pulse facilitation of evoked GABA \(_A\) currents, suggest that cannabinoids depress GABA release from presynaptic terminals. Evidence against a postsynaptic mechanism of action stems from our observation that WIN55,212-2 does not alter the kinetic properties of evoked or mIPSCs. The localization of CB1R also supports a presynaptic locus. Within layer 2/3 of the neocortex, CB1R mRNA is mostly restricted to a subset of GABAergic interneurons (Marsicano and Lutz 1999). Furthermore, CB1R-immunoreactive fibers have been identified surrounding the soma of layer 2/3 pyramidal cells, which themselves do not express CB1R (Egerova and Elphick 2000). Cannabinoids have also been shown to inhibit serotonin (5-HT) (Nakazi et al. 2000) and acetylcholine release (Kathmann et al. 2001), raising the possibility that some CB1R-immunoreactive fibers may not originate from GABAergic interneurons. In the hippocampus of mice lacking CB1R, cannabinoids fail to suppress GABAergic transmission (Hajos et al. 2001; Wilson et al. 2001), further suggesting that the effect of cannabinoids on GABA release would occur via presynaptic CB1R receptors.

The mechanis(s) involved in the CB1R-mediated suppression of GABA release have not been resolved but may include modulation of presynaptic voltage-gated Ca\(^{2+}\) and K\(^+\) channels, leading to changes in Ca\(^{2+}\) influx, as well as direct effects on vesicle release processes downstream of Ca\(^{2+}\) entry. In the hippocampus, cannabinoids suppress GABA release through a direct G protein interaction with N-type Ca\(^{2+}\) channels (Wilson et al. 2001), resulting in an inhibition of presynaptic Ca\(^{2+}\) influx (Hoffman and Lupica 2000; Wilson and Nicoll 2001). Moreover, activation of CB1R has been shown to inhibit whole cell N- and P/Q-type Ca\(^{2+}\) currents in cultured neurons (Caulfield and Brown 1992; Twitchell et al. 1997). CB1R activation can also modulate voltage-gated K\(^+\) channels (Deadwyler et al. 1995; Mu et al. 2000), thereby indirectly altering Ca\(^{2+}\)-dependent transmitter release. In the substantia nigra pars reticulata, Cd\(^{2+}\) has been shown to block the actions of WIN55,212-2 on GABA release (Chen and Yung 1998), further supporting the idea that the inhibition of release is ultimately mediated at the level of Ca\(^{2+}\) entry. There is also evidence for direct modulation of vesicle release, independent of Ca\(^{2+}\) influx. For example, in the cerebellum (Takahashi and Linden 2000) and peri-
aqueductal gray (Vaughan et al. 2000) cannabinoids reduced the frequency of Ca$^{2+}$-independent mIPSCs, suggesting that release processes downstream of Ca$^{2+}$ entry can be regulated by CB1R signaling. In the neocortex, we observed that a fraction of mIPSCs depend on Ca$^{2+}$ influx through voltage-gated Ca$^{2+}$ channels and that the frequency of these events is strongly depressed by WIN55,212-2. The remaining pool of Ca$^{2+}$-independent mIPSCs did not demonstrate WIN55,212-2 sensitivity, consistent with the idea that in the neocortex cannabinoids inhibit transmitter release at the point of Ca$^{2+}$ entry, similar to the hippocampus. It is unclear from the present studies, however, whether CB1R activation modulates Ca$^{2+}$ channels directly or if changes in Ca$^{2+}$ influx are secondary to modulation of presynaptic K$^{+}$ channels (e.g., Daniel and Crepel 2001). It is also possible that the population of terminals expressing CB1R generate only Ca$^{2+}$-dependent mIPSCs, in which case the reduction in mIPSC frequency that we observed in response to WIN55,212-2 may still reflect inhibition of vesicle release downstream of Ca$^{2+}$ influx. At the present time, this interpretation is difficult to exclude.

The endogenous cannabinoids anandamide and 2-arachidonylglycerol are synthesized and released from cortical neurons in an activity-dependent manner (Di Marzo et al. 1994; Stella et al. 1997). The recent demonstration that endocannabinoids act retrogradely to inhibit transmitter release in the cerebellum (Kreitzer and Regehr 2001a,b) and hippocampus (Wilson et al. 2001; Wilson and Nicoll 2001) raises the possibility that these compounds have a similar function in the cortex. A reduction in inhibition caused by CB1R activation in layer 2/3 of the neocortex could provide a mechanism whereby pyramidal cells transiently increase their responsiveness to associative inputs and switch from tonic firing to bursting. It is clear that the generation of bursts in regular spiking layer 5 pyramidal neurons is highly sensitive to apical (i.e., layer 2/3) inhibition and occurs when excitatory inputs from basal and apical dendrites are temporally correlated (Larkum et al. 1999, 2001). The release of endogenous cannabinoids from the apical dendrites of pyramidal neurons may suppress inhibition to a degree that would promote burst firing. Furthermore, the finding that endocannabinoid release from cortical neurons is enhanced by acetylcholine (Stella and Piomelli 2001) suggests that ascending inputs may gate the action of these intrinsic neuromodulators.

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CANNABINOIDS DEPRESS CORTICAL INHIBITION

539


