Cannabinoid Receptor Agonists Protect Cultured Rat Hippocampal Neurons from Excitotoxicity

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ABSTRACT
Cannabinoid receptor agonists act presynaptically to inhibit the release of glutamate. Because other drugs with this action are known to reduce excitotoxicity, we tested several cannabimimetics in a model of synaptically mediated neuronal death. Reduction of the extracellular Mg2+ concentration to 0.1 mM evoked a repetitive pattern of intracellular Ca2+ spiking that, when maintained for 24 hr, resulted in significant neuronal death. The [Ca2+]i spiking and cell death in this model result from excessive activation of N-methyl-D-aspartate receptors, as indicated by the inhibition of both [Ca2+]i spiking and neuronal death by the N-methyl-D-aspartate receptor antagonist CGS19755 (10 μM). The cannabimimetic drug Win55212–2 (100 nM) completely blocked [Ca2+]i spiking and prevented neuronal death induced by low extracellular Mg2+ concentrations. These effects on [Ca2+]i spiking and viability were stereoselective and were prevented by the CB1 receptor antagonist SR141716 (100 nM). The partial agonist CP55940 (100 nM) also afforded significant protection from excitotoxicity. Cannabimimetic drugs did not protect cells from the direct application of glutamate (30 μM). These data suggest that cannabimimetic drugs may slow the progression of neurodegenerative diseases.

The medicinal use of marijuana is controversial (Annas, 1997). The euphoria produced by Δ9-tetrahydrocannabinol, the principal psychoactive ingredient in marijuana, has led to the widespread recreational use of this drug. Therefore, the potential for abuse must be weighed against the therapeutic benefits of cannabimimetics. These drugs have been reported to be effective in the treatment of chronic pain, glaucoma, epilepsy, and the wasting and emesis associated with acquired immunodeficiency syndrome and cancer chemotherapy (Plasse et al., 1991; Howlett, 1995; Abood and Martin, 1996; Adams and Martin, 1996). The in vitro studies described in this report suggest additional therapeutic applications for cannabimimetic drugs.

Cannabinoid receptors are distributed throughout the central nervous system (Herkenham et al., 1990) and are present at high densities on the presynaptic terminals of glutamatergic synapses (Twitchell et al., 1997). We previously reported that the activation of these receptors attenuates glutamatergic neurotransmission by acting presynaptically to inhibit the release of glutamate (Shen et al., 1996). Presumably, activated CB1 receptors, acting via inhibitory G proteins (Childers et al., 1993), activate K+ channels (Deadwyler et al., 1993; Henry and Chavkin, 1995; Mackie et al., 1995) and inhibit Ca2+ channels (Mackie and Hille, 1992; Twitchell et al., 1997; Shen and Thayer, 1998), resulting in reductions in the release of neurotransmitter. Drugs that attenuate glutamatergic synaptic transmission show promise for the treatment of neurodegenerative disorders (Meldrum, 1993; Choi, 1994; Rothman and Olney, 1995). Several drugs that protect cells from excitotoxic neuronal death have a presynaptic component to their actions, including those that activate adenosine receptors (Abele et al., 1990; Thompson et al., 1992), activate K+ channels (Abele and Miller, 1990), and inhibit voltage-gated Ca2+ channels (Yamada et al., 1994).

Here we evaluate several cannabimimetic agents for their abilities to inhibit an aberrant pattern of glutamatergic synaptic activity and the neurotoxicity that results from it. Cannabinoid receptor agonists, acting via CB1 receptors, act potently and stereoselectively to inhibit [Ca2+]i spiking and neuronal death evoked by excitation of the synaptic network that forms in cultures of rat hippocampal neurons.

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ABBREVIATIONS: [Ca2+]i, intracellular Ca2+ concentration; [Mg2+]o, extracellular Mg2+ concentration; NMDA, N-methyl-D-aspartate; CGS19755, [(-)-2-amino-5-phosphonopentanoic acid; Win55212-2 (R-enantiomer), (+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoazin-6-yl][1-naphthalenyl]methanone monomethanesulfonate; Win55212-3, S-enantiomer of Win55212; SR141716, N-piperidino-5-(4-chloro phenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazolecarboxamide; CP55940, [1α,2β(R),5α]-(-)-5-(1,1-dimethylheptyl)-2-[5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]phenol.
Materials and Methods

Materials were obtained from the following companies: NMDA, Win55212–2, Win55212–3, and CP55940 (levorotatory enantiomer), RBI (Natick, MA); SR141716, Sanofi Recherche (Montpellier, France); all other reagents, Sigma Chemical Co. (St. Louis, MO). Rat hippocampal neurons were grown in primary culture as previously described (Shen et al., 1996). Neurons dissociated from hippocampi of embryonic day 17 rats were plated as droplets on microetched coverslips (Belco Biotechnology, Vineland, NJ), at a density of 5 \times 10^4 cells/well (approximately 2.2 \times 10^4 cells/cm^2). Cultures were grown without mitotic inhibitors for a minimum of 12 days before use. For neurotoxicity experiments, at least 100 neurons were counted on each coverslip. Coverslips were then treated with the appropriate control or reduced-[Mg^{2+}]_o solutions, and the same fields of cells were recounted 18–24 hr later. Drugs, when included, were added to the 0.1 mM Mg^{2+} medium before application to the cells. Viable neurons were identified based on morphological criteria; they were phase-bright, had rounded somata, and extended long fine processes. In some experiments, viability was confirmed by demonstrating that cells identified as viable also excluded propidium iodide (2 \mu g/ml). We found that pairing pretreatment and post-treatment cell counts provided more reproducible assessments of the relatively modest degree of cell death resulting from this treatment. Analysis of variance with Bonferoni’s post hoc test was used to determine significance. Data are presented as mean ± standard error. [Ca^{2+}]_i was...

Fig. 1. Cannabinimetics inhibit glutamatergic synaptic activity via CB1 receptors. Excitatory synaptic activity was induced by reducing [Mg^{2+}]_o to 0.1 mM, and the resulting [Ca^{2+}]_i spiking activity was recorded from a single hippocampal neuron using indo-1-based microfluorimetry. The [Mg^{2+}]_o was 0.1 mM throughout the recording, and 100 nM Win55212–2 and 100 nM SR141716 were applied by superfusion at the times indicated (horizontal bars).

Fig. 2. A cannabinoid receptor agonist protects cells against 0.1 mM [Mg^{2+}]_o-induced neurotoxicity. Phase-contrast photomicrographs show the same field of cultured rat hippocampal neurons before treatment (left) and 18–24 hr after treatment (right). Neurons (identified by a light halo around the soma and long fine processes) grew on a bed of non-neuronal cells that formed a mosaic beneath them. The neurons were treated with fresh medium (A), 0.1 mM [Mg^{2+}]_o (B), or 0.1 mM [Mg^{2+}]_o plus 100 nM Win55212–2 (C). Image contrast was enhanced digitally.
measured in single hippocampal neurons using indo-1-based microfluorimetry, as described previously (Shen et al., 1996).

## Results

Reduction of [Mg$$^{2+}$$]$_o$ to 0.1 mM elicits an intense pattern of [Ca$$^{2+}$$] spiking activity that depends on glutamatergic synaptic transmission (Shen et al., 1996). As shown in Fig. 1, the cannabimimetic agonist Win55212–2 was a potent inhibitor of this synaptic activity. The Win55212–2-induced inhibition of low-[Mg$$^{2+}$$]$_o$-induced [Ca$$^{2+}$$] spiking was mediated by the CB1 receptor, as indicated by the complete reversal of the effect by the CB1 receptor antagonist SR141716 (100 nM) (three experiments).

The excitatory synaptic activity elicited by reduction of the [Mg$$^{2+}$$]$_o$ in central nervous system cultures has been shown to produce neurotoxicity, when sustained (Abele et al., 1990; Rose et al., 1990). The efficacy of cannabimimetics in reducing [Ca$$^{2+}$$] spiking activity led us to hypothesize that cannabinoid receptor agonists would protect cells from this form of excitotoxicity. Cell viability was determined by counting the number of viable neurons before and 18–24 hr after treatment, as described in Materials and Methods. In control cultures (medium exchange only), 22 ± 2% of the neurons died (Fig. 2A). This value is in good agreement with the previous observation that, as central nervous system cultures mature in vitro, increased synaptic activity parallels spontaneous cell death (Peterson et al., 1989). Reducing [Mg$$^{2+}$$]$_o$ to 0.1 mM increased neuronal death to 42 ± 3% (19 experiments) (Fig. 2B), which was a significant increase relative to control cultures from the same plating ($p < 0.001$). These levels of spontaneous and low-[Mg$$^{2+}$$]$_o$-induced cell death are in good agreement with findings from previous reports describing the use of this model of synaptically mediated cell death (Abele et al., 1990; Rose et al., 1990). As shown in Figs. 2C and 3, 100 nM Win55212–2 was a very effective neuroprotective agent. In the presence of Win55212–2, only 23 ± 3% of the neurons died; this value is comparable to that seen in the control wells (22 ± 2%) and is significantly less than that seen with 0.1 mM [Mg$$^{2+}$$]$_o$ ($p < 0.001$).

The neuroprotection afforded by 100 nM Win55212–2 was blocked by 100 nM SR141716, consistent with a CB1-mediated process (Fig. 3A). The antagonist alone had no effect on cell viability. The inactive enantiomer Win55212–3 was also without effect. We previously showed that CP55940 acts as a partial agonist to inhibit low-[Mg$$^{2+}$$]$_o$-induced [Ca$$^{2+}$$] spiking activity in these hippocampal cultures (Shen et al., 1996). This compound was highly effective in preventing low-[Mg$$^{2+}$$]$_o$-induced neurotoxicity, as shown in Fig. 3. Consistent with the glutamatergic mechanism of this excitotoxic model, the NMDA receptor antagonist CGS19755 (10 μM) not only prevented low-[Mg$$^{2+}$$]$_o$-induced cell death but also improved viability, relative to the control cultures.

In a previous study, we found that CP55940 and Win55212–2 inhibited glutamatergic synaptic transmission, in identically prepared hippocampal cultures, by acting presynaptically to inhibit the release of glutamate (Shen et al., 1996). But if the neuroprotection we describe here resulted from inhibition of glutamate release, then cannabimimetics would not be predicted to protect cells from the direct application of glutamate. This was indeed the case, as shown in Fig. 3B. Treatment of the cultures with 30 μM glutamate in medium with 0.1 mM [Mg$$^{2+}$$]$_o$ resulted in 62 ± 8% cell death after a 18–24-hr exposure. This toxicity was completely prevented by including the NMDA receptor antagonist CGS19755 (10 μM) in the medium. However, neither Win55212–2 (100 nM) nor CP55940 (100 nM) affected the survival of the cultures. Thus, cannabimimetics protect cells from synaptically mediated excitotoxicity but not from exposure to exogenous glutamate.

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**Fig. 3.** Cannabimimetics are neuroprotective. Excitotoxicity was elicited by treatment with 0.1 mM [Mg$$^{2+}$$]$_o$ (Fig. 2) and was quantified as described in Materials and Methods. The limited size of each culture plating precluded the testing of all treatments on a single plating. Therefore, each treatment has separate bars for control (□) and for 0.1 mM [Mg$$^{2+}$$]$_o$-induced death (□). In all experiments, 0.1 mM [Mg$$^{2+}$$]$_o$-induced significantly greater cell death, relative to control wells. Drugs were applied at the following concentrations: Win55212–2, 100 nM; SR141716, 100 nM; Win55212–3, 100 nM; CP55940, 100 nM; CGS19755, 10 μM. A, Drugs were applied with 0.1 mM [Mg$$^{2+}$$]$_o$ (□), as indicated below the histogram. B, Cells were treated for 18–24 hr with 0.1 mM [Mg$$^{2+}$$]$_o$, 30 μM glutamate (Glu), and drug, as indicated (horizontal bars) above the histogram. The specific drugs added are indicated below the histogram. *, $p < 0.05$; **, $p < 0.001$, 0.1 mM [Mg$$^{2+}$$]$_o$ versus control; +, $p < 0.05$; ++, $p < 0.01$; ++++, $p < 0.001$, drug plus 0.1 mM [Mg$$^{2+}$$]$_o$ versus 0.1 mM [Mg$$^{2+}$$]$_o$; #, $p < 0.001$, glutamate plus 0.1 mM [Mg$$^{2+}$$]$_o$ plus CGS19755 versus glutamate plus 0.1 mM [Mg$$^{2+}$$]$_o$ (analysis of variance with Bonferroni’s post hoc test).
In this report, we show that cannabinoid receptor agonists inhibit the [Ca\(^{2+}\)]\(_i\) spiking and neurotoxicity induced in hippocampal cultures by the reduction of [Mg\(^{2+}\)]\(_o\), to 0.1 mm. This action was stereoselective and mediated by the CB1 receptor. Excitotoxicity, such as that produced in this model system, is thought to underlie a number of neurodegenerative disorders, suggesting that cannabinimetics may prove useful in slowing neurodegenerative processes.

Cannabinimetic compounds with neuroprotective effects have been described previously, although this is the first description of neuroprotective effects mediated by the CB1 receptor. HU-211 was shown to protect neurons from NMDA-mediated neurotoxicity by acting as an antagonist at the NMDA receptor (Nadler et al., 1993). The endogenous lipid palmitoylethanolamide, acting on CB2 receptors via an unknown mechanism, protected cultured cerebellar granule cells from the direct application of NMDA (Skaper et al., 1996). In this report, we show that cannabinoids, acting through presynaptic CB1 receptors, protected cells from excitotoxicity. Cannabinimetics protected cells from the synaptic release of glutamate but not from the direct application of glutamate.

Excitotoxicity mediated by glutamate contributes to neuronal loss after acute insults, such as hypoxia-hypoglycemia, seizures, and head trauma (Meldrum, 1993; Choi, 1994; Rothman and Olney, 1995). A sensitization to glutamatergic seizures, and head trauma (Meldrum, 1993; Choi, 1994; Beal MF, Hyman BT, and Koroshetz W (1993) Do defects in mitochondrial energy metabolism underlie the pathology of neurodegenerative diseases? Trends Neurosci 16:125–131.


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