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Activation of the Cannabinoid Type-1 Receptor Mediates the Anticonvulsant Properties of Cannabinoids in the Hippocampal Neuronal Culture Models of Acquired Epilepsy and Status Epilepticus

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ABSTRACT

Cannabinoids have been shown to have anticonvulsant properties, but no studies have evaluated the effects of cannabinoids in the hippocampal neuronal culture models of acquired epilepsy (AE) and status epilepticus (SE). This study investigated the anticonvulsant properties of the cannabinoid receptor agonist *R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolol[1,2,3 *de*]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone (WIN 55,212-2) in primary hippocampal neuronal culture models of both AE and SE. WIN 55,212-2 produced dose-dependent anticonvulsant effects against both spontaneous recurrent epileptiform discharges (SRED) (EC_{50} = 0.85 μM) and SE (EC_{50} = 1.51 μM), with total suppression of seizure activity at 3 μM and of SE activity at 5 μM. The anticonvulsant properties of WIN 55,212-2 in these preparations were both stereospecific and blocked by the cannabinoid type-1 (CB1) receptor antagonist *N*-(piperidin-1-yl-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-*1H*-pyrazole-3-carboxamidehydrochloride (SR141716A; 1 μM), showing a CB1 receptor-dependent pathway. The inhibitory effect of WIN 55,212-2 against low Mg^{2+}-induced SE is the first observation in this model of total suppression of SE by a selective pharmacological agent. The clinically used anticonvulsants phenytoin and phenobarbital were not able to abolish low Mg^{2+}-induced SE at concentrations up to 150 μM. The results from this study show CB1 receptor-mediated anticonvulsant effects of the cannabimimetic WIN 55,212-2 against both SRED and low Mg^{2+}-induced SE in primary hippocampal neuronal cultures and show that these in vitro models of AE and SE may represent powerful tools to investigate the molecular mechanisms mediating the effects of cannabinoids on neuronal excitability.

Since the isolation and purification of the psychotropically active constituent Δ^9-tetrahydrocannabinol from *Cannabis* in the 1960s (reviewed in Mechoulam, 2000), a number of studies have shown the anticonvulsant effects of cannabinoids in a variety of experimentally induced seizure models that include maximal electroshock-induced convulsions, electrical kindling, chemoconvulsants, and audiogenic and photogenic seizures (Corcoran et al., 1973; Karler et al., 1974; Wada et al., 1975; Consroe and Wolkin, 1977; Chiu et al., 1979; Wallace et al., 2001, 2002; Shafaroodi et al., 2004). In addition, several reports have been published on the clinical use of cannabinoids as antiepileptic agents in humans (reviewed in Consroe, 1998). Thus, it is important to elucidate the molecular mechanisms mediating the anticonvulsant effects of cannabinoids.

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ABBREVIATIONS: CB1, cannabinoid type 1; MES, maximal electroshock; WIN 55,212-2, *R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolol[1,2,3 *de*]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone; SRED, spontaneous recurrent epileptiform discharge(s); AE, acquired epilepsy; HNC, hippocampal neuronal culture; SE, status epilepticus; WCC, whole-cell current clamp; WIN 55,212-3, *S*(−)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolol[1,2,3 *de*]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone; SR141716A, *N*-(piperidin-1-yl-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamidehydrochloride; pBRS, physiological bath recording solution; PDS, paroxysmal depolarization shift(s); AEA, arachidonylethanolamine; DSI, depolarization-induced suppression of inhibition; DSE, depolarization-induced suppression of excitation.

A major advance in the field of cannabinoid research came with the discovery and cloning of receptor proteins that bind cannabinoids with high affinity (reviewed in Mechoulam, 2000). Within the central nervous system, the $G_{i/o}$ protein-coupled cannabinoid type-1 (CB1) receptor is widely distributed and is the primary mediator of the physiological and psychotropic effects of cannabinoids in the brain (Devane et al., 1988; Matsuda et al., 1990; Herkenham et al., 1991; Howlett, 1995). In recent years, a better understanding of the endocannabinoid system has led to the development of highly specific synthetic compounds that have been instrumental in the pharmacological evaluation of cannabinoid receptor-mediated regulation of synaptic transmission (Howlett et al., 2004). Utilizing the maximal electroshock (MES)-induced seizure model, studies from this laboratory provided the first evidence that both cannabinoid and endocannabinoid compounds produced anticonvulsant effects through activation of the CB1 receptor (Wallace et al., 2001, 2002). Additionally, the active cannabimimetic compound WIN 55,212-2 was shown to totally suppress spontaneous recurrent epileptiform discharges (SRED; seizures) via CB1 receptor activation in the rat pilocarpine model of acquired epilepsy (AE) (Wallace et al., 2003). This study further showed that endocannabinoids, acting through the CB1 receptor, were essential for maintaining tonic inhibition of seizure frequency and duration in this in vivo model of AE (Wallace et al., 2003). Although these in vivo models of acute seizure and AE are useful for studying the anticonvulsant effects of CB1 receptor activation on intact systems (Wallace et al., 2001, 2002, 2003), they are limited in their ability to carry out sophisticated molecular techniques needed to study underlying cellular mechanisms. Thus, it is important to utilize well established in vitro neuronal preparations that are more amenable to sophisticated electrophysiological and molecular biological procedures to evaluate the cellular mechanisms underlying the anticonvulsant properties of cannabinoids. The hippocampal neuronal culture (HNC) model of AE is a well established model that exhibits SRED for the life of the neurons in culture (Sombati and DeLorenzo, 1995) and has been shown by our laboratory and others to manifest many of the electrophysiological and molecular properties of intact animal models of AE (Kim and Rhim, 2004; Delorenzo et al., 2005). It is also important to evaluate the anticonvulsant effects of cannabinoids against continuous seizure activity, status epilepticus (SE), a major neurological emergency that is often resistant to conventional anticonvulsant treatments (Delorenzo et al., 2005). The well established HNC model of SE (Sombati and DeLorenzo, 1995) has been widely used to evaluate the effects of SE on neuronal cell physiology and molecular changes (Pal et al., 1999; Blair et al., 2004; Mangan and Kapur, 2004; Delorenzo et al., 2005). Thus, the HNC models of AE and SE may serve as valuable tools for elucidating the cellular mechanisms underlying the anticonvulsant properties of cannabinoids because these in vitro models are amenable to experimental manipulation and allow for direct analysis of neurons undergoing SRED and SE in culture (Sombati and DeLorenzo, 1995; Churn et al., 2000; Blair et al., 2004; Delorenzo et al., 2005).

In the current study, we set out to investigate the effects of the cannabimimetic WIN 55,212-2 on seizure activity in the in vitro HNC models of AE and SE (Sombati and DeLorenzo, 1995). Whole-cell current-clamp (WCC) analysis was utilized to directly evaluate the effect of WIN 55,212-2 on SRED and SE and to determine whether a CB1 receptor-dependent pathway was involved. The results show that WIN 55,212-2 was effective in terminating SRED and SE in a stereoselective manner. In addition, these anticonvulsant effects of WIN 55,212-2 were mediated through activation of the CB1 receptor. This study shows that the HNC models of AE and SE provide powerful tools to further elucidate the cellular mechanisms underlying the effect of cannabinoids on seizure activity and neuronal excitability.

Materials and Methods

Materials. WIN 55,212-2, WIN 55,212-3, phenytoin (5,5-diphenyl-2,4-imidazolidinedione), and phenobarbital (5-ethyl-5-phenyl-2,4,6-trioxohexahydropyrimidine) were purchased from Sigma Chemical (St. Louis, MO). SR141716A was supplied through the National Institute on Drug Abuse Chemical Synthesis and Drug Supply Program. Stocks of WIN 55,212-3, WIN 55,212-2, and SR141716A were made up in a vehicle stock solution of absolute ethanol, Emulphor-620 (Rhone-Poulenc, Inc., Princeton, NJ), and 0.9% saline at a ratio of 1:1:18 that was then diluted at a minimum of 1:500 to a final working concentration in the physiological bath recording solution.

Hippocampal Neuronal Culture. Primary mixed hippocampal cultures were prepared as described previously by our laboratory with slight modifications (Sombati and DeLorenzo, 1995). In brief, hippocampal cells were prepared from 2-day postnatal Sprague-Dawley rats (Harlan, Frederick, MD) and plated at a density of 2.5×10^4 cells/cm^2 onto a glial support layer previously plated onto poly-L-lysine–coated (0.05 mg/ml), 35-mm grid cell culture dishes (Nunc, Naperville, IL). Cultures were maintained at 37°C in a 5% CO_2/95% air atmosphere and fed twice weekly with NeuroBasal-A medium supplemented with B-27 (Invitrogen Corp., San Diego, CA) containing 0.5 mM L-glutamine. Unless otherwise noted, reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO).

Induction of SE and SRED by Low Mg^{2+} Treatment of Hippocampal Neuronal Cultures. After 2 weeks, cultures were utilized for experimentation. Maintenance medium was replaced with physiological bath recording solution (pBRS) with or without $MgCl_2$ containing 145 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 2 mM $CaCl_2$, 10 mM glucose, and 0.002 mM glycine, pH 7.3, and osmolarity adjusted to 325 mOsm with sucrose. Thus, low Mg^{2+} treatment was carried out with pBRS without added $MgCl_2$, whereas sham controls were treated with pBRS containing 1 mM $MgCl_2$. Unless indicated as low Mg^{2+} treatment, experimental protocols in this study utilized pBRS containing 1 mM $MgCl_2$.

HNC Model of SE. Continuous epileptiform high-frequency bursts (SE) were induced by exposing neuronal cultures to pBRS without added $MgCl_2$ (low Mg^{2+}). The SE continued until pBRS containing 1 mM $MgCl_2$ was added back to the cultures. This represents the HNC model of SE used in this study that has been well characterized as an in vitro model of SE manifesting essentially identical electrographic feature of SE observed with in vivo animal models and in human SE (Sombati and DeLorenzo, 1995; Mangan and Kapur, 2004; Delorenzo et al., 2005). Thus, the in vitro HNC model of SE is well suited to study the effects of cannabinoids on SE.

HNC Model of AE. A 3-h exposure of SE was used to induce AE with SRED in the HNC model using established procedures (Sombati and DeLorenzo, 1995). In this model of AE, it has been shown that the brief 3-h exposure and injury from SE produces SRED for the life of the neurons in culture (Sombati and DeLorenzo, 1995; DeLorenzo et al., 1998; Kim and Rhim, 2004). We used neurons 1 day after exposure to SE in this study. The 1 day after SE time point represents the chronic phase of AE in this model where the neurons manifest SRED with the same frequency as neurons sampled from cultures at days 3, 5, and 7 after SE injury. Thus, the HNC model of

AE is ideally suited for the pharmacological studies on the effects of cannabinoids on SRED. Briefly, after the removal of maintenance media, cells were washed gently with 3 × 1.5 ml of pBRS (±1 mM $MgCl_2$) and then allowed to incubate in this solution at 37°C under 5% CO_2/95% air. For the HNC model of AE, at the end of treatment with either sham control (1 mM $MgCl_2$) or low Mg^{2+} (without added $MgCl_2$) conditions for 3 h, cultures were restored to the physiological concentration (1 mM) of $MgCl_2$ by washing gently with 3 × 1.5 ml of minimum essential medium at 37°C, returned to maintenance feed, and incubated at 37°C under 5% CO_2/95% air. The neurons exposed to this 3-h treatment with low Mg^{2+} pBRS manifested SRED for the life of the neurons in culture.

WCC Recordings in Low Mg^{2+}-Treated Hippocampal Neuronal Cultures. WCC recordings were performed using previously established procedures in our laboratory (Sombati and DeLorenzo, 1995; Blair et al., 2004). In brief, cell culture medium was replaced with pBRS, mounted on the stage of an inverted microscope (Nikon Diaphot, Tokyo, Japan), continuously perfused with pBRS, and then studied using the WCC recording procedure. Patch electrodes with a resistance of 2 to 4 MΩ were pulled on a Brown-Flaming P-80C electrode puller (Sutter Instruments, Novato, CA), fire-polished, and filled with a solution containing 140 mM K^+ gluconate, 1 mM $MgCl_2$, and 10 mM Na-HEPES, pH 7.2, with osmolarity adjusted to 310 ± 5 mOsm with sucrose. Data were digitized and transferred to videotape using a PCM device (Neurocorder, New York, NY) and then played back on a DC-500 Hz chart recorder (Astro-Med Dash II, Warwick, RI). Intracellular recordings were carried out using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) in WCC mode.

Data Analysis. For concentration-response analysis, suppression of SRED and SE was determined as a percentage decrease in frequency over increasing concentrations of WIN 55,212-2. Analysis of SRED frequency for each recorded neuron was carried out over 60 min and determined by counting individual epileptiform events that had discreet onset and termination and consisted of multiple individual paroxysmal depolarization shifts (PDS). SE frequency was determined by counting individual epileptiform bursts over a recording duration of 5 min for each neuron analyzed. For both SRED and SE, WCC frequency analysis was carried out on multiple hippocampal cultured neurons at each concentration of WIN 55,212-2. Mean frequencies at each concentration of WIN 55,212-2 were then represented as a percentage inhibition from control frequency (SRED or SE frequency in the absence of WIN 55,212-2). Least-square linear regression analysis was used to calculate the EC_{50} (effective concentration that produced 50% of maximal effect) of WIN 55,212-2 for suppression of both SRED and SE burst discharges. Data were plotted using SigmaPlot analysis software 8.02 (SPSS Inc., Chicago, IL).

Results

Anticonvulsant Effects of WIN 55,212-2 Are Stereospecific in the HNC Model of AE. To evaluate the anticonvulsant activity of the optically active cannabimimetic compound WIN 55,212-2 against AE, this study used the in vitro HNC model of AE previously developed in our laboratory (Sombati and DeLorenzo, 1995). This in vitro model of AE has been established as a model of AE with many of the electrographic and biochemical features of in vivo models of AE (Sombati and DeLorenzo, 1995; DeLorenzo et al., 1998; Kim and Rhim, 2004). To induce AE in cultured neurons, 2-week-old hippocampal neuronal cultures were exposed to Mg^{2+}-free media for 3 h and then returned to a maintenance medium containing Mg^{2+}. Hippocampal neurons develop SRED for the life of the culture following this treatment (Sombati and DeLorenzo, 1995). WCC recordings of age-matched control neurons revealed "normal" baseline recordings, displaying spontaneously occurring action potentials and not manifesting SRED (Fig. 1A). One day after 3 h of low Mg^{2+} treatment, WCC recordings of a neuron showed an "epileptic" phenotype as evident by the presence of SRED or "seizure" events (Fig. 1B). Expansion of one of the SRED (Fig. 1Ba) revealed the presence of individual PDS, a pathophysiological characteristic similar to those observed in epilepsy (Lothman et al., 1991). The SRED comprised multiple PDS and occurred intermittently for the life of the neurons in culture (Sombati and DeLorenzo, 1995).

WIN 55,212 inhibited SRED stereoselectively in the HNC model of AE. WIN 55,212-2 (3 μM), the active enantiomer at the CB1 receptor, fully inhibited the expression of SRED (Fig. 2C) compared with the nontreated "epileptic" cultures (Fig. 2A). Conversely, the inactive stereoisomer WIN 55,212-3 (3 μM) had no effect on the expression or frequency of SRED in "epileptic" cultures (Fig. 2B). These results show that only the active enantiomer of the cannabimimetic WIN 55,212-2 had anticonvulsant effects.

We carried out a concentration-response analysis of inhibition of SRED with WIN 55,212-2 and found that it inhibited SRED in a concentration-dependent manner with an EC_{50} = 0.85 μM as determined by least-square linear regression analysis (Fig. 3A). The anticonvulsant effects of WIN 55,212-2 were observed with concentrations as low as 500 nM and were maximal at 3 μM (Fig. 3A). The total inhibition of SRED by WIN 55,212-2 in a concentration-dependent and stereospecific manner indicates that this cannabimimetic compound is functioning as a full CB1 receptor agonist.

Anticonvulsant Effects of WIN 55,212-2 Are Mediated by the CB1 Receptor in the HNC Model of AE. Because CB1 receptor antagonists have been shown to be effective in

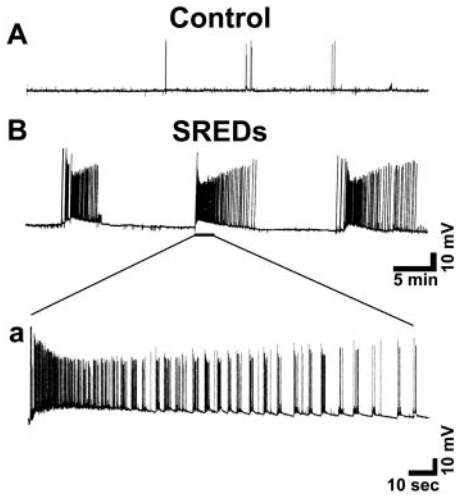

Fig. 1. Induction of SRED following a 3-h exposure of hippocampal neuronal cultures to low Mg^{2+}. WCC recordings were obtained from hippocampal pyramidal neurons before and 1 day after a 3-h exposure to Mg^{2+}-free solution. A, a recording from a representative control neuron displaying intermittent spontaneous action potentials that are consistently observed during basal activity in this hippocampal culture preparation. B, a continuous 45-min recording from a representative "epileptic" neuron 1 day following exposure to 3 h of Mg^{2+}-free media. The pathophysiological state of SRED in this in vitro preparation is evident by the presence of three independent spontaneous seizure episodes. The lower panel recording (a) is an expansion of the corresponding segment in the trace above (B) to discriminate the high-frequency burst discharges that overlie individual PDS, a pathophysiological characteristic observed in epilepsy, and the numerous spikes associated with each SRED.

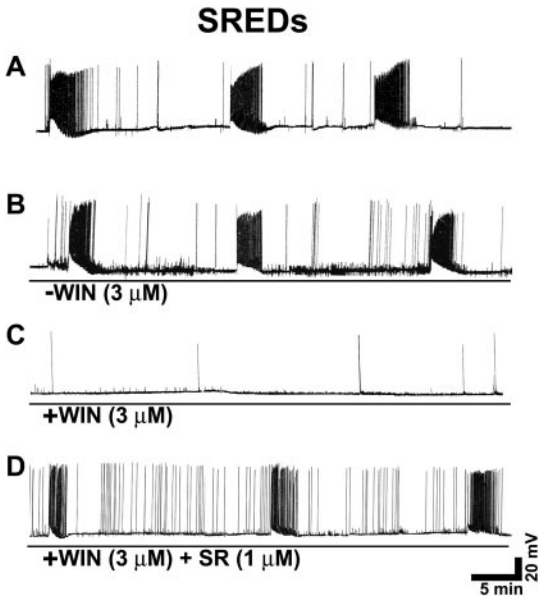

Fig. 2. CB1-dependent suppression of SRED activity. A, WCC recordings were obtained from hippocampal pyramidal neurons displaying SRED activity 1 day following a 3-h exposure to Mg^{2+}-free solution. B, addition of the inactive stereoisomer WIN 55,212-3 (−WIN) (3 μM) had no effect on the expression of SRED compared with A. C, the active cannabimimetic WIN 55,212-2 (+WIN) (3 μM) fully inhibited the expression of SRED in "epileptic" hippocampal cultures. This anticonvulsant effect was prolonged and was observed as far out as 60 min following the addition of WIN 55,212-2 (data not shown). D, the anticonvulsant effect of WIN 55,212-2 (3 μM) in suppressing the expression of SRED was completely blocked by addition of the CB1 receptor antagonist SR141716A (SR) (1 μM).

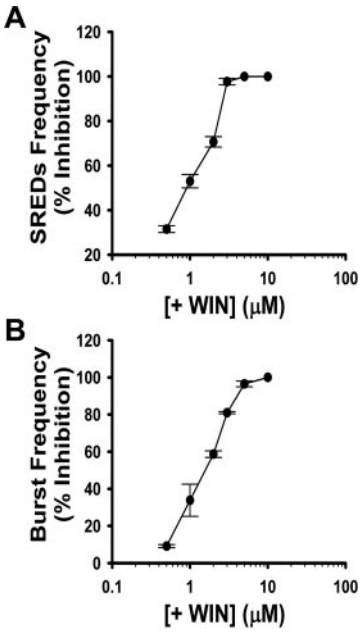

Fig. 3. Log concentration-response curves for WIN 55,212-2 in the HNC models of low Mg^{2+}-induced AE and SE. A, concentration-dependent inhibition of SRED frequency by WIN 55,212-2 (EC_{50} = 0.85 μM as determined by least-square linear regression analysis, under *Materials and Methods*). Frequency of individual SRED events (see Fig. 1B) in the presence of varying concentrations of WIN 55,212-2 were measured over a 60-min period and then plotted as a percentage change (inhibition) from control (absence of WIN 55,212-2) SRED frequencies. B, concentration-dependent inhibition of high-frequency epileptiform burst (SE) frequency by WIN 55,212-2 (EC_{50} = 1.51 μM as determined by least-square linear regression analysis, under *Materials and Methods*). Frequency of epileptiform bursts (see Fig. 4B, b and c) in the presence of varying concentrations of WIN 55,212-2 were measured over a 5-min period and then plotted as a percentage change (inhibition) from control (absence of WIN 55,212-2) SE frequencies. Each data point represents percentage change from control ± S.E.M. (n = 3–7 neurons/concentration).

demonstrating the role of CB1 receptor activation in mediating anticonvulsant effects (Wallace et al., 2001, 2002, 2003), it is important to determine whether the anticonvulsant effects of WIN 55,212-2 in the HNC model of AE, described above, are also mediated by CB1 receptor activation. To evaluate the role of CB1 receptor activation, we used the CB1 receptor antagonist SR141617A to evaluate the effect of CB1 receptor blockade on the ability of WIN 55,212-2 to inhibit SRED in the HNC model of AE. SR141617A (1 μM) totally blocked the ability of WIN 55,212-2 to suppress SRED in the HNC model of AE (Fig. 2D). Thus, both the stereospecificity of WIN 55,212-2 to fully inhibit SRED and the antagonism of this effect by SR141617A confirm that this anticonvulsant effect of WIN 55,212-2 occurs via a CB1 receptor-mediated mechanism.

Anticonvulsant Effects of WIN 55,212-2 against SE Are Stereospecific in the HNC Model of SE. The anticonvulsant properties of the cannabimimetic WIN 55,212-2 were further evaluated in the in vitro HNC model of electrographic SE to determine its effectiveness in suppressing continuous epileptiform high-frequency bursts induced by low Mg^{2+} treatment. The HNC model of SE shares many of the same electrophysiological and biochemical changes observed in the in vivo models of SE and in human SE (Sombati and DeLorenzo, 1995; Mangan and Kapur, 2004; Delorenzo et al., 2005). The continuous epileptiform activity in this HNC model of SE is characterized by 3- to 20-Hz high-frequency epileptiform bursts during the low Mg^{2+} exposure and has been shown to be dependent on interneuronal networking (Sombati and DeLorenzo, 1995). WCC recordings from age-matched control neurons showed baseline activity with the occasional spontaneous action potential (Fig. 4A). Removal of Mg^{2+} (low Mg^{2+}) from the recording solution resulted in continuous tonic high-frequency epileptiform bursts (Fig. 4B). This hyperexcitable state consisted of repetitive individual burst discharges (Fig. 4Ba), of which each burst comprises multiple spikes that overlay a depolarization shift (Fig. 4B, b and c).

WIN 55,212-2 inhibited the continuous tonic high-frequency epileptiform bursts stereospecifically in the HNC model of SE. The addition of the active enantiomer WIN 55,212-2 (5 μM) during the low Mg^{2+} treatment totally inhibited expression of high-frequency epileptiform bursts (Fig. 5C) compared with low Mg^{2+} treatment alone (Fig. 5A). The inactive stereoisomer WIN 55,212-3 (5 μM) had no effect on low Mg^{2+}-induced SE (Fig. 5B).

To evaluate the effectiveness of WIN 55,212-2 at suppressing SE, inhibition of burst frequency was determined over a concentration-response curve (see *Materials and Methods*). WIN 55,212-2 inhibited low Mg^{2+}-induced SE in a concentration-dependent manner with an EC_{50} = 1.51 μM, as determined by least-square linear regression analysis (see *Materials and Methods*). Suppression of SE was observed at concentrations of WIN 55,212-2 ranging from partial inhibition at 500 nM to full inhibition at 5 μM, showing that it is functioning as a full agonist (Fig. 3B).

Anticonvulsant Effects of WIN 55,212-2 on SE Are Mediated by the CB1 Receptor. To determine whether

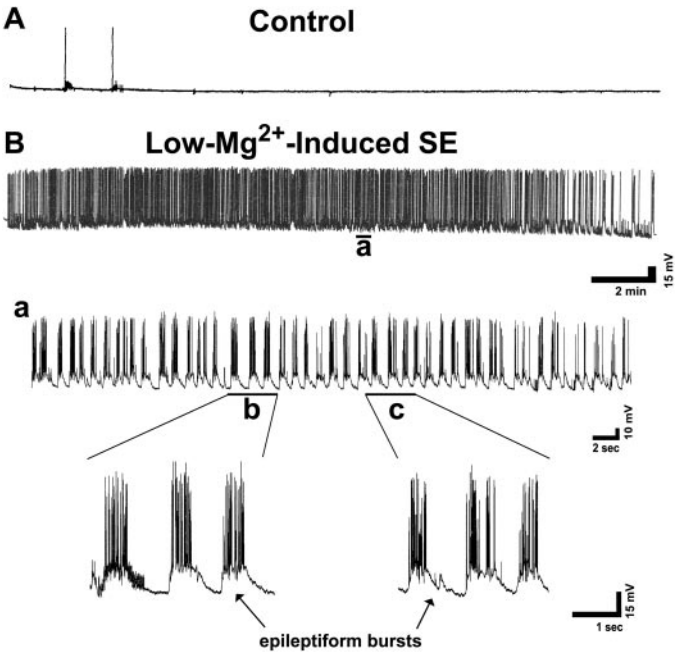

Fig. 4. Induction of tonic high-frequency epileptiform bursts (SE) in cultured hippocampal neurons during exposure to low Mg^{2+}. A, representative intracellular recording from a control neuron showing occasional spontaneous action potentials. The resting potential of individual neurons ranged from 65 to 50 mV. B, representative intracellular recordings during low Mg^{2+} treatment showing continuous seizure activity (SE) with burst discharge frequencies of 3 to 20 Hz. a, expansion of a segment from B shows the presence of continuous burst discharges. b and c, further expansion of two segments from a, revealing the individual epileptiform bursts, each consisting of depolarization shifts overlaid with multiple spike activity. Response data in Fig. 3B were determined by frequency analysis of epileptiform bursts.

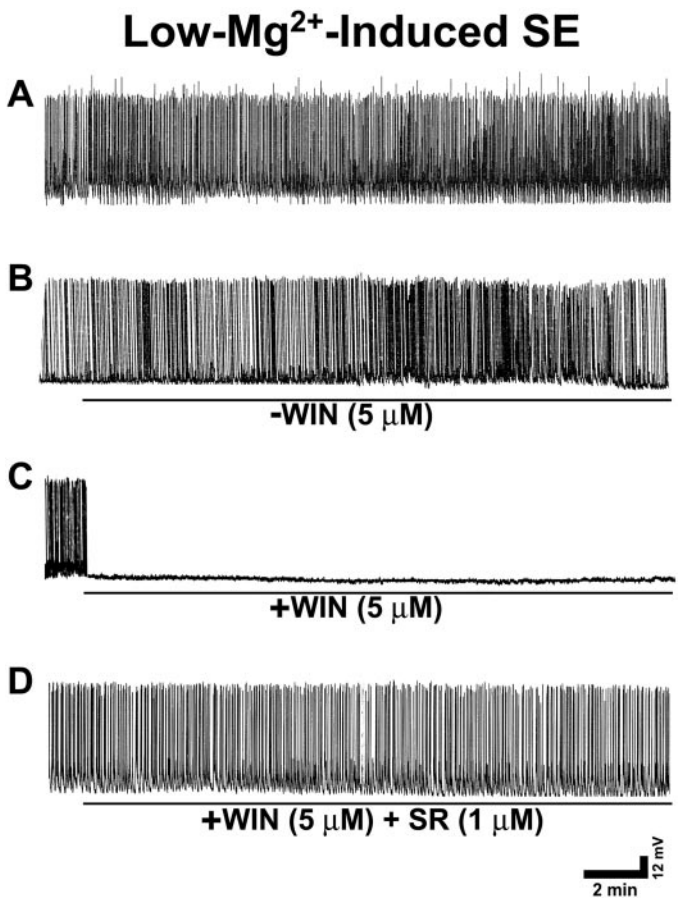

Fig. 5. CB1 receptor-dependent suppression of low Mg^{2+}-induced SE activity. A, exposure of hippocampal cultures to Mg^{2+}-free media (low Mg^{2+}) induces tonic high-frequency epileptiform bursts (SE). B, the presence of the inactive isomer WIN 55,212-3 (−WIN) (5 μM) during exposure to low Mg^{2+} had no effect on high-frequency epileptiform burst activity. C, WIN 55,212-2 (+WIN) (5 μM), the active cannabimimetic, totally suppressed low Mg^{2+}-induced high-frequency epileptiform bursts (SE). D, the addition of the CB1 receptor antagonist SR141716A (1 μM) completely blocked the anticonvulsant effects of WIN 55,212-2 (5 μM) against low Mg^{2+}-induced SE.

WIN 55,212-2 is anticonvulsant via a CB1 receptor-dependent mechanism, analysis was carried out in the presence of the CB1 receptor antagonist SR141716A. The total suppression of low Mg^{2+}-induced SE by 5 μM WIN 55,212-2 was blocked by the addition of 1 μM SR141716A (Fig. 5D). Thus, the anticonvulsant properties of WIN 55,212-2 with low Mg^{2+}-induced SE in hippocampal cultures were both stereospecific and blocked by SR141716A, showing a CB1 receptor-dependent mechanism of SE suppression.

Comparison of the Cannabinoid Anticonvulsant Effects against SE with Other Anticonvulsant Agents. Both antiepileptic agents phenobarbital and phenytoin, used at very high concentrations up to 150 μM, had no effect at reducing low Mg^{2+}-induced high-frequency epileptiform bursts in the HNC model of SE (Fig. 6). Thus, whereas agents known to be effective anticonvulsants had no effects on SE expression in this preparation, the cannabimimetic compound WIN 55,212-2 (5 μM) fully suppressed low Mg^{2+}-induced SE in a stereospecific manner (Fig. 5). This is the first evidence of a selective pharmacological agent to fully suppress high-frequency epileptiform burst activity in the HNC model of SE.

Discussion

In the current study, the synthetic cannabinoid WIN 55,212-2 was anticonvulsant in a stereospecific and concentration-dependent manner against SRED and acute low Mg^{2+}-induced high-frequency epileptiform bursts (SE) in the HNC models of AE and SE (Sombati and DeLorenzo, 1995). The anticonvulsant properties of WIN 55,212-2 for SE and SRED were both stereospecific and blocked by the CB1 receptor antagonist SR141716A (1 μM), showing CB1 receptor-dependent mechanisms. The antiepileptic effect of WIN 55,212-2, showing total suppression of SRED at 3 μM, was as efficacious as the clinically established agents phenytoin (10 μM) and phenobarbital (100 μM) previously characterized in this HNC model of AE (Sombati and DeLorenzo, 1995). WIN 55,212-2 (5 μM) produced a total suppression of high-frequency epileptiform bursts (SE) during low Mg^{2+} treatment. Previous studies have shown that SE in this model was not significantly blocked by inhibiting *N*-methyl-D-aspartate, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, kainic acid, and metabotropic glutamate receptors or L-type Ca^{2+} channels (DeLorenzo et al., 1998). Furthermore, total suppression of SE in this model was not produced by the clinically established anticonvulsants phenobarbital or phenytoin, even at very high concentrations of these anticonvulsants up to 150 μM. SE is often refractory to treatment, and the ability of cannabinoids to abolish SE discharges in this model is an important obser-

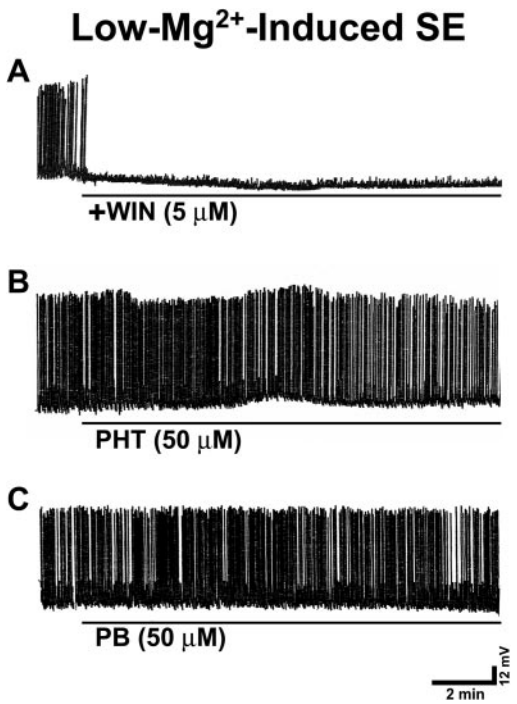

Fig. 6. Evaluation of anticonvulsant agents on low Mg^{2+}-induced SE activity in hippocampal neuronal cultures. A, following the addition of the cannabimimetic WIN 55,212-2 (+WIN) (5 μM), the low Mg^{2+}-induced high-frequency epileptiform burst activity was fully suppressed. The addition of the established anticonvulsant drugs phenytoin (PHT) (50 μM) (B) or phenobarbital (PB) (50 μM) (C) during low Mg^{2+} treatment at high therapeutic concentrations had no inhibitory effect on SE activity in this preparation. In addition, PHT and PB up to concentrations as high as 150 μM were not able to abolish SE as shown in A for WIN 55,212-2 at 5 μM.

vation that may have clinical implications for considering the use of cannabinoids in the treatment of refractory SE. In addition, the demonstration that cannabinoids are effective in totally preventing SRED and SE in these HNC in vitro models offers a major advance over in vivo models in the ability to investigate the molecular mechanisms involved in the CB1 receptor-dependent regulation of seizure frequency and duration. It is hoped that the use of these models to study the effects of cannabinoids on seizure activity and SE in vitro will lead to new advances in understanding the molecular mechanisms regulating the actions of cannabinoids in modulating neuronal excitability.

Since the discovery and cloning of the CB1 receptor, a number of synthetic compounds have been developed allowing for the study of this receptor's role in modulating seizure activity. Earlier studies from this laboratory have shown that the cannabimimetic compound WIN 55,212-2 had CB1 receptor-dependent anticonvulsant activity in both the MES model of acute seizures and in the rat pilocarpine model of AE (Wallace et al., 2001, 2003). Furthermore, work from our laboratory showed that blockade of CB1 receptor function with the selective antagonist SR141716A reduced seizure threshold in the MES model and increased epileptic seizure frequency and duration in the pilocarpine model of temporal lobe epilepsy (Wallace et al., 2002, 2003). These earlier studies suggested a role for the endocannabinoid system in regulating neuronal seizure discharge in a CB1 receptor-dependent manner. Other studies have shown a protective role of CB1 receptor activation in different models of neuronal excitotoxicity, which include glutamate receptor-dependent–induced neuronal cell death (Abood et al., 2001), kainic acid-induced seizures in conditional mutant mice null for CB1 receptor on principal forebrain neurons (Marsicano et al., 2003), low Mg^{2+}-induced epileptiform activity in hippocampal slice (Ameri and Simmet, 2000), and repetitive Ca^{2+} spiking-dependent neuronal death in hippocampal cultures (Shen and Thayer, 1998). The current findings that the anticonvulsant effects of cannabinoids in the HNC models of AE and SE are mediated by CB1 receptor activation underscore the potential utilization of these in vitro models as powerful tools for future studies on the molecular mechanisms underlying CB1 receptor-mediated regulation of seizure activity.

To date, two endogenous ligands for the CB1 receptor in brain have been isolated and termed arachidonylethanolamine (AEA) and 2-arachidonylglycerol (Devane et al., 1992; Mechoulam et al., 1995), and the highest amount of AEA has been found in the hippocampus (Felder et al., 1996). These endocannabinoids are synthesized "on demand" in response to neuronal depolarization and a subsequent increase in intracellular calcium (Stella et al., 1997). The CB1 receptor is primarily localized presynaptically on nerve terminals and, on activation, has been shown to suppress neurotransmitter release. Thus, cannabinoids can act to significantly modulate neuronal synaptic transmission through their actions at the CB1 receptor.

Considerable research has explored the mechanisms mediating the effects of endogenous CB1 receptor activation. Activation of presynaptic CB1 receptors result in decreased N- and P/Q-type voltage-gated Ca^{2+} currents and increased type-A K^+ conductance via a direct interaction with the receptor-coupled $G_{i/o}$ proteins (reviewed in Howlett et al., 2004). A physiological outcome of presynaptic CB1 receptor activation following postsynaptic depolarization-induced synthesis and release of the endocannabinoids AEA or 2-arachidonylglycerol is the attenuation of Ca^{2+}-dependent neurotransmitter release from nerve terminals. This phenomenon has been termed either depolarization-induced suppression of inhibition (DSI) or excitation (DSE), dependent on its action at GABAergic or glutamatergic neuronal synaptic terminals, respectively (Kreitzer and Regehr, 2001; Wilson et al., 2001). Although the presence of CB1 receptor-dependent DSI and DSE has been observed in vitro, studies have shown that DSE is less likely to take place under physiological conditions (Ohno-Shosaku et al., 2002). In such a scenario, DSI would predominate and would be expected to confer a proconvulsant effect of CB1 receptor activation. However, hippocampal DSE has been shown to be induced by prolonged postsynaptic membrane depolarization (Ohno-Shosaku et al., 2002), a characteristic event that occurs during seizure discharge. Thus, the total suppression of both SE and SRED by WIN 55,212-2 in the current study may result from increasing CB1 receptor-dependent suppression of excitation, a mechanism that may predominate during the seizure discharge in this hippocampal preparation. Such a hypothesis has been recently posed in the literature (Hajos and Freund, 2002).

It has been suggested that the anticonvulsant effects of antiepileptic agents result in the attenuation of hyperexcitability that results from excessive glutamatergic transmission, a phenomenon that is a characteristic occurrence with

seizure discharge (Lothman et al., 1991). Utilizing a hippocampal neuronal culture preparation, Thayer and colleagues observed a CB1 receptor-dependent suppression of glutamatergic transmission as shown by WIN 55,212-2 inhibition of both a low Mg^{2+}-induced increase in intracellular Ca^{2+} concentration spikes and the amplitude of presynaptically evoked excitatory postsynaptic potentials (Shen and Thayer, 1999), while having no effect on GABA-mediated inhibitory postsynaptic potential amplitudes (Shen et al., 1996). Additionally, in a hippocampal brain slice preparation, WIN 55,212-2 blocked stimulus-evoked field excitatory postsynaptic potentials in CA1 pyramidal neurons and decreased low Mg^{2+}/high K^{+}-induced epileptiform burst discharge frequency in hippocampal CA3 neurons (Ameri and Simmet, 2000). In light of these studies, a plausible mechanism for the anticonvulsant properties of WIN 55,212-2 in our model may involve a CB1 receptor-dependent suppression of glutamatergic transmission.

The results of this study show for the first time that the cannabimimetic compound WIN 55,212-2 is anticonvulsant against SE and AE via a CB1 receptor-dependent mechanism using the in vitro hippocampal neuronal culture models of AE and SE. These in vitro hippocampal culture models of electrographic seizure activity are well suited to carry out biochemical (Blair et al., 2004; Delorenzo et al., 2005), electrophysiological (Sombati and DeLorenzo, 1995; DeLorenzo et al., 1998), and molecular investigations (Churn et al., 2000). Future research efforts using the HNC models of AE and SE toward elucidating both presynaptic and postsynaptic neuronal mechanisms involved in the anticonvulsant effects of CB1 receptor activation may allow for the development of more efficacious treatment strategies for epilepsy and SE.

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