Cannabinoid CB1 receptor antagonists cause status epilepticus-like activity in the hippocampal neuronal culture model of acquired epilepsy

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Abstract

Status epilepticus is a major medical emergency associated with a significant morbidity and mortality. Little is known about the mechanisms that terminate seizure activity and prevent the development of status epilepticus. Cannabinoids possess anticonvulsant properties and the endocannabinoid system has been implicated in regulating seizure duration and frequency. Endocannabinoids regulate synaptic transmission and dampen seizure activity via activation of the presynaptic cannabinoid receptor 1 (CB1). This study was initiated to evaluate the role of CB1 receptor-dependent endocannabinoid synaptic transmission towards preventing the development of status epilepticus-like activity in the well-characterized hippocampal neuronal culture model of acquired epilepsy using patch clamp electrophysiology. Application of the CB1 receptor antagonists SR141716A (1 μM) or AM251 (1 μM) to "epileptic" neurons caused the development of continuous epileptiform activity, resembling electrographic status epilepticus. The induction of status epilepticus-like activity by CB1 receptor antagonists was reversible and could be overcome by maximal concentrations of CB1 agonists. Similar treatment of control neurons with CB1 receptor antagonists did not produce status epilepticus or hyperexcitability. These findings suggest that CB1 receptor-dependent endocannabinoid endogenous tone plays an important role in modulating seizure frequency and duration and preventing the development of status epilepticus-like activity in populations of epileptic neurons. The regulation of seizure activity and prevention of status epilepticus by the endocannabinoid system offers an important insight into understanding the basic mechanisms that control the development of continuous epileptiform discharges.

Keywords

CB1 receptor; status epilepticus; cannabinoid; epilepsy; endocannabinoid tone

Epilepsy is one of the most common neurological disorders affecting approximately 1–2% of the world population [5]. It is characterized by the occurrence of spontaneous recurrent epileptiform discharges (SREDs) or seizures [9,16]. Status epilepticus (SE) is a major

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neurological emergency characterized by prolonged seizures [3] and is associated with significant morbidity and mortality [17]. Seizure initiation and termination are governed by complex synaptic regulation of neuronal excitability [4,9]. However, little is known about the molecular mechanisms that mediate seizure termination and prevent development of SE in populations of epileptic neurons.

Cannabinoids, such as marijuana and other derivatives, have been used since ancient times for the treatment of seizures [1] and have also been shown to possess anticonvulsant properties [6]. The endocannabinoid system consists of at least two cannabinoid receptors (CB1 and CB2), its endogenous ligands (endocannabinoids: anandamide and 2-AG) and the protein machinery for their synthesis, transport and degradation [11]. It is well documented that in response to physiological (and pathological) synaptic stimulation, endocannabinoids are synthesized and released "on demand" and travel in a retrograde manner to activate the presynaptic CB1 receptors to inhibit neurotransmitter release [15]. It is thought that endocannabinods tonically activate CB1 receptors to generate an "endocannabinoid tone" that modulates neuronal excitability [18]. Recent studies in animal models and neuronal cultures have demonstrated that both cannabinoids and the endocannabinoid system may act to regulate seizure duration and termination [2,12,18,19]. The CB1 receptor has been shown to mediate many of the anticonvulsant effects of cannabinoids [20] and to play an important role in regulating synaptic transmission [15]. Thus in the epileptic phenotype, the endocannabinoid system may provide an intrinsic mechanism for terminating seizure activity and preventing the development of SE.

This study was initiated to evaluate the role of the CB1 receptor-dependent endocannabinoid tone in preventing the development of SE in populations of epileptic neurons. The endocannabinoid tone was disrupted by utilizing the CB1 receptor antagonists, N-(piperidin-1-yl-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamidehydrochloride (SR141716A) and N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251), in the well characterized hippocampal neuronal culture (HNC) model of acquired epilepsy using patch clamp electrophysiology [16]. The results indicate that application of CB1 receptor antagonists caused "epileptic" neurons to develop SE-like activity, characterized by essentially continuous epileptiform discharges. Our findings provide the first direct evidence that CB1 receptor-dependent endocannabinoid tone is essential for maintaining tonic inhibition of continuous seizure activity and prevention of SE.

R(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolol[1,2,3 de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone (WIN55,212–2) was purchased from Sigma Chemical (St. Louis, MO). AM251 was purchased from Tocris Cookson Inc (Ellisville, MO). SR141716A was supplied through the NIDA Chemical Synthesis and Drug Supply Program. Stocks of WIN55,212–2 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 (pBRS). Stock solutions of AM251 were prepared in DMSO and stored aliquoted at −20°C. The final working concentration of DMSO was 0.01%. The pBRS consisted of (in mM): 145 NaCl, 2.5 KCl, 10 HEPES, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 0.002 glycine, pH 7.3, and osmolarity adjusted to 325 ± 5 mOsm with sucrose. All the drugs were bath-applied using a multi valve perfusion assembly (Warner Instrument Corp., CT, USA).

All animal use procedures were in strict accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by Virginia Commonwealth University's Institutional Animal Care and Use Committee. Studies were conducted on primary mixed hippocampal neuronal cultures prepared as described previously with slight modifications [16]. In brief, hippocampal cells were obtained 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. All other reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO) unless otherwise noted.

After 2 weeks, cultures were utilized for experimentation. Neuronal cultures were rendered "epileptic" by exposing them for 3-h to a solution containing no added $MgCl_2$ (low Mg^{2+}) [2,16]. Briefly, after the removal of maintenance media, neurons were gently washed with 3 x 1.5 ml of pBRS (±1 mM $MgCl_2$) and then allowed to incubate in this solution at 37oC under 5% CO_2/95% air atmosphere. At the end of the 3-h period, cultures were restored to the physiological concentration (1 mM) of $MgCl_2$ by gently washing with 3 x 1.5 ml of minimum essential medium, returned to the maintenance medium and incubated at 37°C under 5% CO_2/95% air atmosphere. Thus, low Mg2+ treatment was carried out with pBRS without added $MgCl_2$, whereas sham controls were treated with pBRS containing 1 mM $MgCl_2$.

Whole cell current clamp recordings were performed using previously established procedures in our laboratory [2,16]. Briefly, cell culture medium was replaced with pBRS, mounted on the stage of an inverted microscope (Nikon Diaphot, Tokyo, Japan), continuously perfused with pBRS. 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, filled with a solution containing (in mM): 140 K$^+$ gluconate, 1 $MgCl_2$, and 10 Na-HEPES, pH 7.2, and the osmolarity was adjusted to 290 ± 10 mOsm with sucrose. Intracellular recordings were carried out using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) in current clamp mode. Data were digitized and transferred to videotape using a PCM device (Neurocorder, New York, NY) and played back on a DC-500 Hz chart recorder (Astro-Med Dash II, Warwick, RI).

Employing the HNC model of acquired epilepsy, we evaluated the effects of CB1 receptor antagonists on seizure activity. Fig. 1A is a representative current clamp recording from a control neuron showing occasional spikes. The induction of acquired epilepsy by the low Mg^{2+} treatment is shown in Fig. 1B. Recordings from neurons in cultures 1-day after a 3-h, low-Mg^{2+} treatment demonstrated SREDs, a characteristic of acquired epilepsy (Fig. 1B). These SREDs or "seizure episodes" occurred for the life of the neurons in culture and demonstrated paroxysmal depolarization shifts, a pathophysiological characteristic of epilepsy (Fig. 1B) [9,16]. Two SREDs lasting between 1–1.5 min are observed in this epileptic neuron in a continuous 30-min recording (Fig. 1B). Each SRED started and stopped spontaneously. Multiple recordings (n= 6) from adjacent neurons demonstrate that these SREDs were synchronous events occurring in populations of neurons. SREDs were never observed in control neurons (n=45). There were no significant differences in membrane potential and input resistance between control and epileptic neurons. Control neurons exhibited a mean membrane potential of -60.2 ± 1.5 mV and a mean input resistance of 119.6 ± 7.4 MΩ, whereas the epileptic neurons demonstrated a mean membrane potential of -59.4 ± 1.1 mV and a mean input resistance of 109.8 ± 8.9 MΩ. The HNC model of acquired epilepsy represents an ideal preparation to evaluate the effects of CB1 receptor antagonists in a controlled environment on seizure discharge and the development of SE.

SR141716A is a well-characterized inhibitor of CB1 receptors. Application of SR141716A (1 μM) to "epileptic" neurons enhanced seizure activity and produced essentially continuous epileptiform discharges, a characteristic of SE (Fig. 1C). Multiple spikes overlaying a depolarization shift characterized the epileptiform discharges (Fig. 1a, b, c inset). In epilepsy, the seizure discharge or SREDs occurred intermittently and self terminated with a seizure free period in between two SREDs throughout the life of the culture (Fig. 1B). However, upon CB1

receptor antagonism with SR141716A, these epileptiform discharges were dramatically altered and were not terminated, thus producing continuous epileptiform discharges. Treatment of control neurons with SR141716A (1 μM) alone did not produce epileptiform discharges or hyperexcitability (data not shown). In addition, no spontaneous SE-like activity was observed in epileptic neurons.

Recently a newer CB1 antagonist AM251 that is structurally similar to SR141716A has been developed and is reported to be more selective towards CB1 receptors [8]. We also tested the effects of AM251 in the HNC model of acquired epilepsy (Fig. 2). Similar to SR141716A, AM251 (1 μM) produced essentially continuous epileptiform discharges in the epileptic neurons (Fig. 2C), causing the development of SE-like activity and a break down in the ability of the neurons to terminate seizure activity. AM251 (1 μM) alone did not produce any hyperexcitability in control neurons (Fig. 3A). Further, no significant changes were observed in the membrane potential and input resistance with SE neurons. SE neurons had a mean membrane potential of -58.7 ± 2.1 mV and a mean input resistance of 121.1 ± 5.2 MΩ.

The continuous epileptiform discharges produced by both CB1 receptor antagonists are identical in frequency and pattern to the electrographic activity observed in SE, with spike discharges occurring at a frequency greater than 3 Hz and lasting essentially indefinitely (Figs. 1C and 2C, insets). To our knowledge these results represent the first demonstration that CB1 receptor antagonists can cause electrographic SE-like activity in a model of acquired epilepsy. The high CB1 receptor selectivity of AM251 provides strong evidence that CB1 receptor activation plays an important role in preventing the transition from SREDs to SE in epileptic neurons.

It is important to determine if the SE-like activity caused by the CB1 receptor antagonists was reversible and not caused by some permanent damage to the neurons. Removing the CB1 receptor antagonists from the recording medium abolished the continuous epileptiform discharges and the neurons were restored to the SREDs phenotype (Fig. 3B). Another way of overcoming an antagonist block is to displace it with an agonist. The specific CB1 receptor agonist WIN55,212–2 is a potent agonist and is effective in reversing CB1 antagonist in a concentration dependent manner [2]. In the presence of AM251 (1 μM), a low dose of WIN55,212–2 (up to 2 μM) could not block SE. However, treatment with higher concentrations of WIN55,212–2 (5 μM) completely blocked the effects of AM251 and abolished SE-like high frequency activity. WIN55,212–2 (5 μM) not only blocked the continuous epileptiform discharges but it also abolished the SREDs further underscoring the powerful anticonvulsant effects of CB1 receptor activation (Fig. 3C). At concentrations below the maximal effect of 5 μM WIN55,212–2, the SE-like activity induced by AM-251 was decreased to a condition with epileptiform discharges analogous to the epileptic state (data not shown). In addition, DMSO and ethanol vehicle had no effects on control neurons. These results demonstrate that the development of continuous epileptiform discharges produced by CB1 antagonists could be selectively reversed by a CB1 agonist thereby supporting that CB1 receptor-dependent endocannabinoid tone contributes to the prevention of the development of SE-like activity in epileptic neurons.

The brain is in a continuous state of plasticity allowing it to adapt to both physiological and pathological events from the external environment. Upon an excitotoxic insult to neuronal systems, it is this adaptation that allows the brain to initiate intrinsic compensatory mechanisms that suppress neuronal excitotoxicity and limits further damage. Understanding the molecular mechanisms that underlie these compensatory mechanisms is a major question in the field of neuroscience.

The CB1 receptor is coupled to a $G_{i/o}$ protein, and upon activation results in downstream cellular events that include decreased cAMP production, decreased Ca^{2+} conductance, increased K^+ conductance and MAP-kinase activation [11]. Depolarization-induced "on-demand" synthesis of endocannabinoids from the postsynaptic membrane are thought to act at CB1 receptors in a retrograde manner to inhibit presynaptic neurotransmitter release; this neuronal process has been termed either depolarization-induced suppression of excitation (DSE) [7] or depolarization-induced suppression of inhibition (DSI) [13] by acting at glutamatergic or GABAergic synapses respectively. Previous findings have shown that repetitive neuronal stimulation, characteristic of neuronal epileptic seizure discharge, results in a shift from DSI to DSE bringing about an overall suppression of neuronal excitation [14]. It is speculated that epileptiform seizure activity elicits an increase in the "on-demand" synthesis of endocannabinoids resulting in increased activation of presynaptic CB1 receptors with subsequent regulation of neuronal hyperexcitability and seizure termination [10]. Indeed, several in vivo studies have shown that intense synaptic activity, as a result of seizure discharges, increases synthesis of the endocannabinoids 2-AG [18] and anandamide [12]. Furthermore, previous studies from our laboratory demonstrated that the CB1 receptor agonist WIN 55,212–2 produced an anticonvulsant effect in both an in vivo [20] and in vitro [2] model of seizure. In addition, disruption of endocannabinoid tone by genetic ablation of the CB1 receptor rendered knockout mice more susceptible to kainic acid-induced seizures than their wild-type littermates [12]. Thus, in the epileptic phenotype, CB1 receptors may provide a negative feedback mechanism to guard against persistent epileptic neuroexcitation.

Antagonizing the CB1 receptor would prevent activation by its endogenous ligands, thereby disrupting the endocannabinoid tone and subsequent regulation of neuronal excitability. In the present study, antagonism of CB1 receptor-dependent regulation of neuronal excitability in epileptic cells resulted in not only increased seizure frequency and duration, but also it caused continuous epileptiform discharges that meet the criteria for electrographic SE. The components of endocannabinoid system have been implicated as potential therapeutic targets for the treatment of various disorders including pain, obesity, glaucoma, migraine and epilepsy [11]. Further investigations into the role of endocannabinoids in the prevention of SE may offer important therapeutic insights into pharmacological measures to prevent this neurological emergency.

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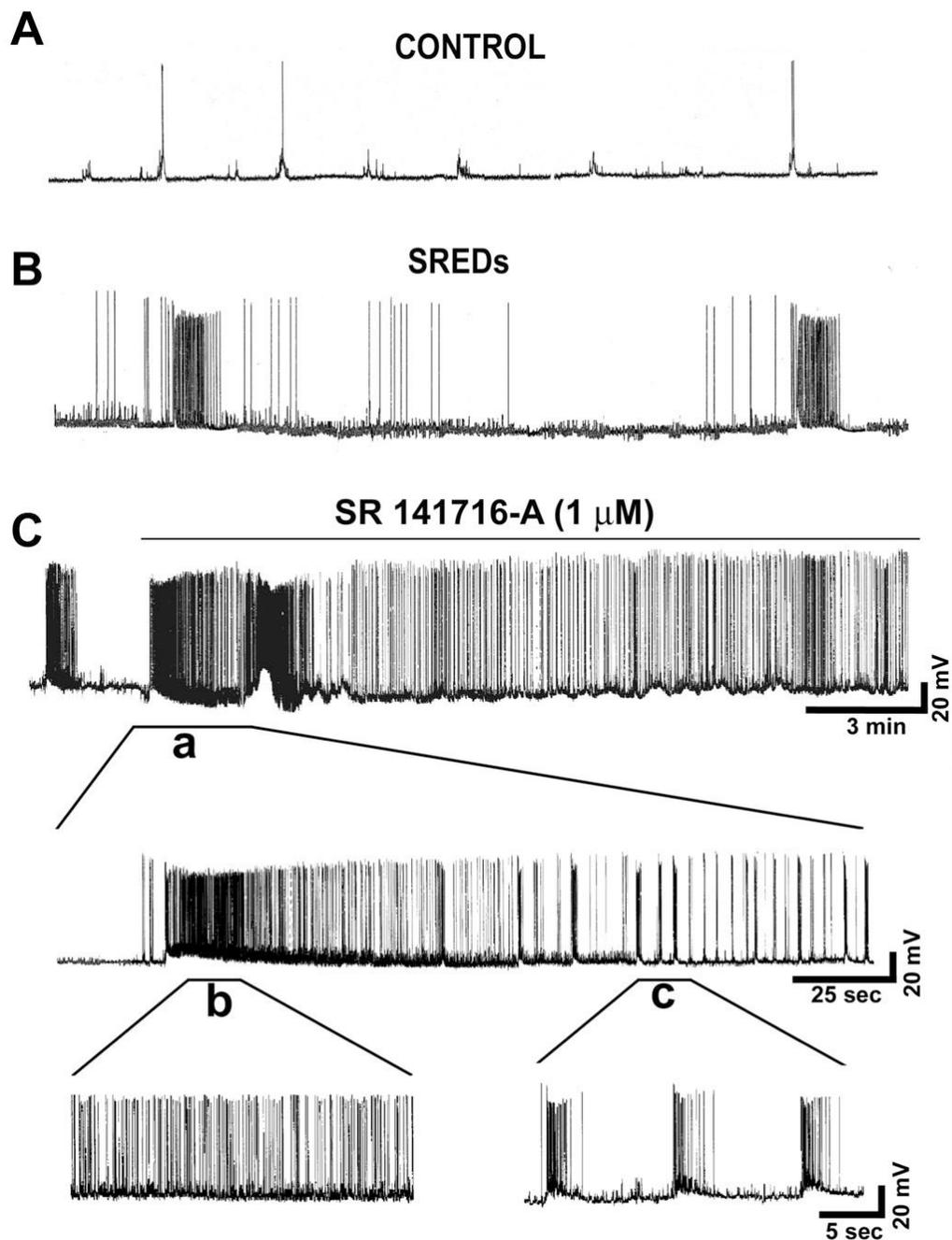
Figure 1.
Antagonism of CB1 receptors by SR141716A causes SE-like activity in "epileptic" hippocampal neurons in cultures. Whole-cell current clamp recordings were obtained from hippocampal pyramidal neurons in cultures before and 1-day after a 3-h exposure to low Mg^{2+} solution. **A**, recording from a representative control neuron displaying intrinsic baseline activity consisting of intermittent spontaneous action potentials. **B**, a continuous 30-min recording from a representative "epileptic" neuron 1-day following a 3-h exposure to low Mg^{2+} solution. Two SREDs or spontaneous seizure episodes lasting ~60–80s can be seen in this time frame. These SREDs occurred throughout the life of the cultures and are indicative of the pathophysiological "epileptic" phenotype. **C**, Representative current-clamp recording

showing transition of an epileptic neuron from SREDs to continuous epileptiform discharges (SE) after the addition of the CB1 receptor antagonist SR141716A (1 μM). After the appearance of first seizure event SR141716A was applied to neuron and within a minute after application the neuron manifested a response of essentially continuous epileptiform activity, a characteristic of SE. Also note the increased seizure frequency and duration. The patterns shown in C demonstrate the transitions from very rapid (10–15 Hz) to slower (3–5 Hz) spike discharges that occur during the continuous epileptiform discharges. **a**: is an expansion of a segment from C showing the transition to SE-like activity on a faster time scale. **b** and **c**: are further expansions of segments from "a" showing spike pattern in the fast and slow spike region respectively. Spike frequencies ranged from 3–5 Hz in slow area (c) to up to 10–15 Hz in fast area (b). Individual epileptiform bursts; each consisting of depolarization shifts overlaid with multiple spike activity can be seen. This data was representative of 6 experiments.
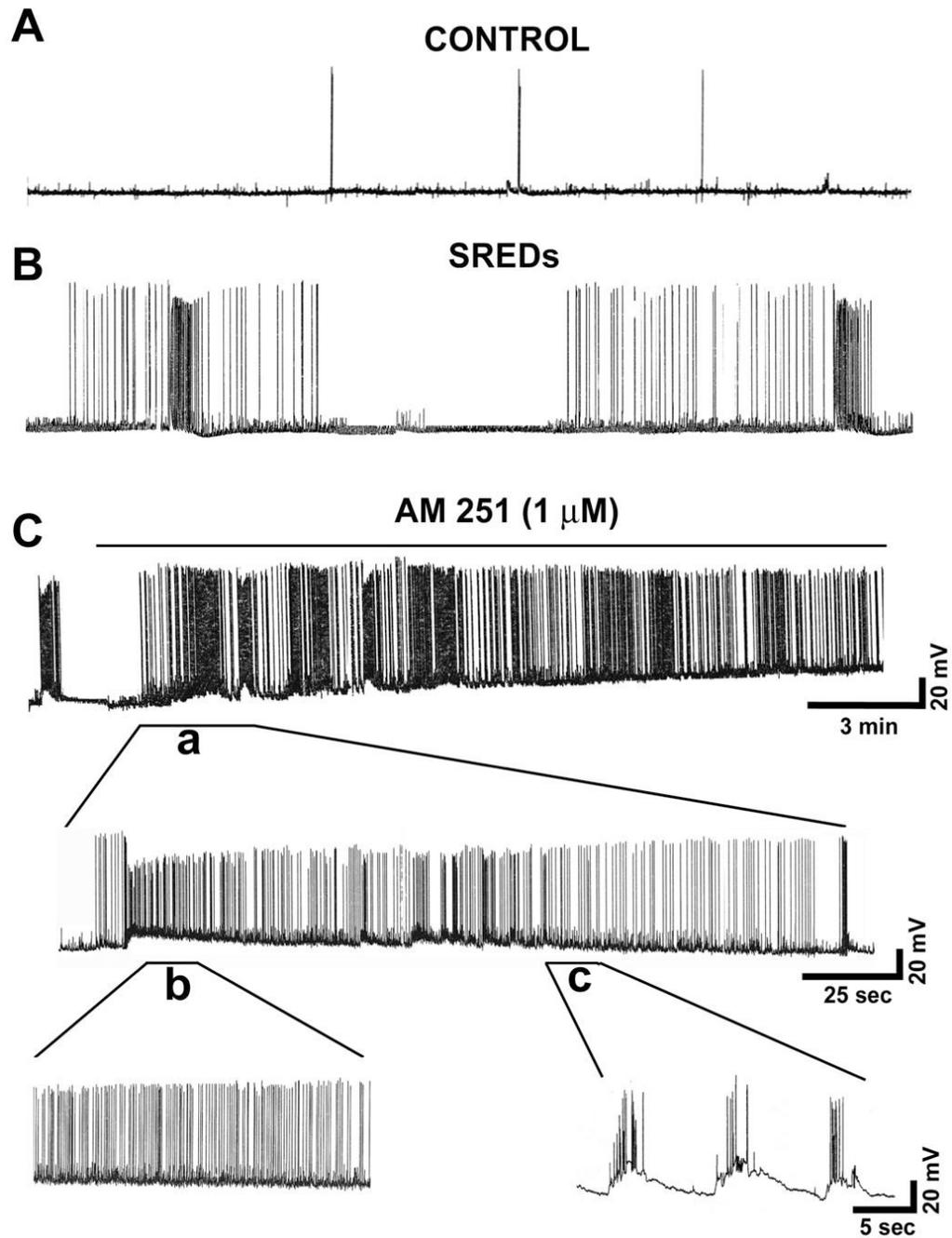
Figure 2.
Antagonism of CB1 receptors by AM251 causes SE-like activity in "epileptic" neurons in cultures. **A**, representative recording from a control neuron showing occasional spontaneous action potentials. **B**, Whole cell current clamp recording from a neuron displaying SREDs 1-day following a 3-h exposure to low Mg^{2+} solution. **C**, application of AM251 (1 μM) to an epileptic neuron caused continuous epileptiform discharges, characteristic of SE. **a**, is an expansion of a 3-min segment from C showing the transition to SE-like activity. **b and c**, are further expansions of segments from "a" showing spike pattern and frequency in the fast and slow spike areas respectively. This data was representative of 6 experiments.
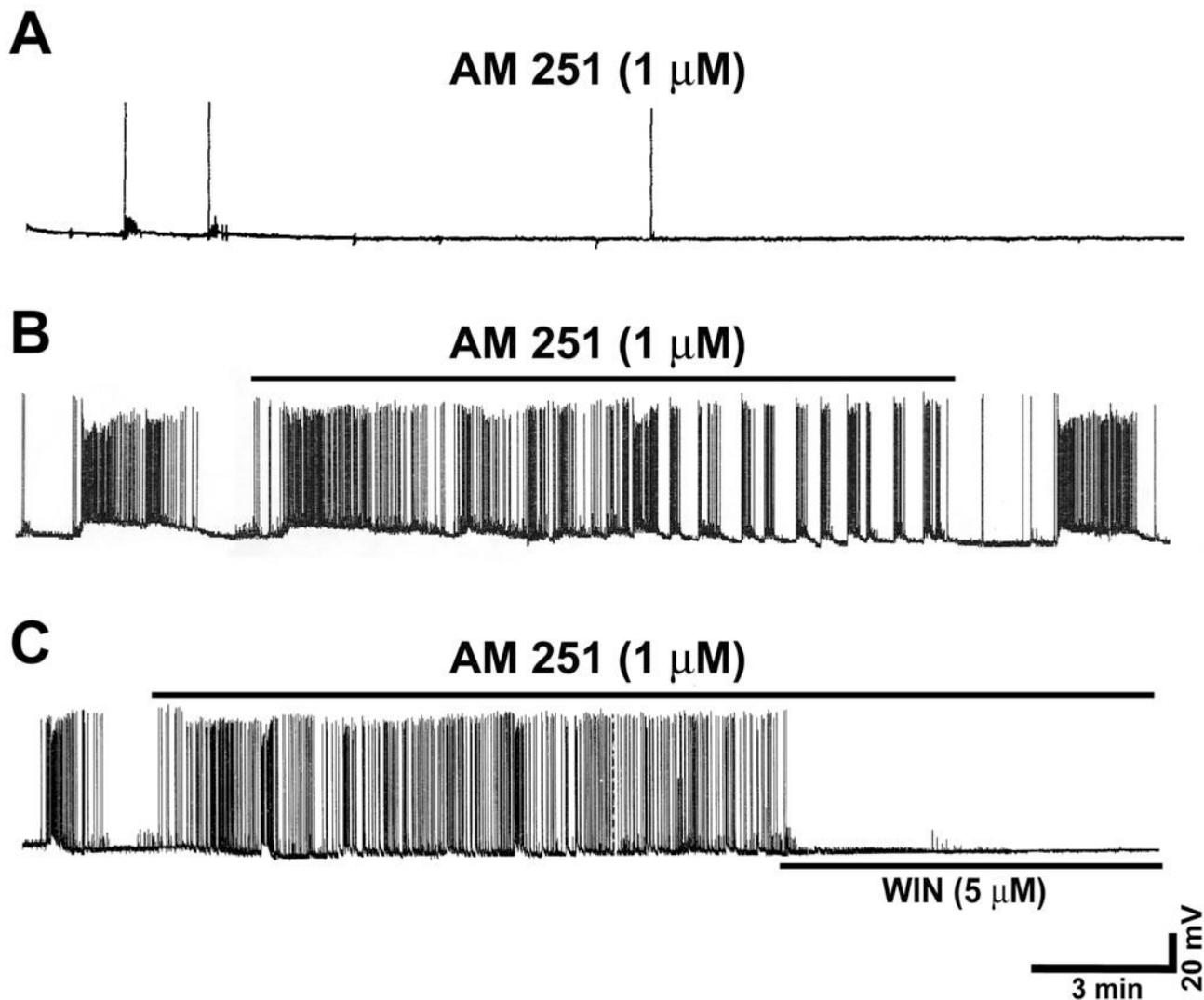
Figure 3.
Restoration of endocannabinoid tone prevents SE-like activity in epileptic neurons. **A**, representative control neuron displaying occasional action potentials when treated with AM-251 (1 μM) alone. **B**, removal of AM251 stops the continuous epileptiform discharges and restores the neuron to SREDs/seizure phenotype. After the first seizure event AM251 (1 μM) is applied to neuron. This causes disruption of endocannabinoid tone resulting in progression of neuron to SE-like activity. Upon washout of AM251, SE-like activity stops and the neuron revert back to the original electrophysiological pattern manifesting SREDs. Thus, the production of SE-like activity by AM251 was reversible. **C**, WIN55,212–2 (5 μM) overcomes the AM251 mediated block of CB1 receptors and completely suppresses the spontaneous epileptiform discharges. Using WIN55,212–2 (5 μM) not only blocked SE-like activity, but also suppressed the SREDs. This data was representative of 5 experiments.