Epileptiform activity in the CA1 region of the hippocampus becomes refractory to attenuation by cannabinoids in part due to endogenous GABA_B receptor activity

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Abstract

The anticonvulsant properties of marijuana have been known for centuries. The recently characterized endogenous cannabinoid system thus represents a promising target for novel anticonvulsant agents; however, administration of exogenous cannabinoids has shown mixed results in both human epilepsy and in animal models. The ability of cannabinoids to attenuate release of both excitatory and inhibitory neurotransmitters may explain the variable effects of cannabinoids in different models of epilepsy, but this has not been well explored. Using acute mouse brain slices, we monitored field potentials in the CA1 region of the hippocampus to systematically characterize the effects of the cannabinoid agonist WIN55212-2 (WIN) on evoked basal and epileptiform activity. WIN, acting presynaptically, significantly reduced the amplitude and slope of basal field excitatory postsynaptic potentials as well as stimulus-evoked epileptiform responses induced by omission of magnesium from the extracellular solution. In contrast, the combination of omission of magnesium plus elevation of potassium induced an epileptiform response that was refractory to attenuation by WIN. The effect of WIN in this model was partially restored by blocking GABA_B, but not GABA_A, receptors. Subtle differences in models of epileptiform activity can profoundly alter the efficacy of cannabinoids. Endogenous GABA_B receptor activation played a role in the decreased cannabinoid sensitivity observed for epileptiform activity induced by omission of magnesium plus elevation of potassium. These results suggest that interplay between presynaptic G protein-coupled receptors with overlapping downstream targets may underlie the variable efficacy of cannabinoids in different models of epilepsy.

Keywords

GABA; cannabinoids; hippocampus

Introduction

Epilepsy is a neurologic disorder characterized by multiple episodes of unprovoked seizures that affects almost 1% of the world's population. Approximately 30% of affected individuals do not respond to the current therapies; thus, novel pharmacologic agents are needed (French 2007). Anecdotal evidence has long supported the anticonvulsant properties of *Cannabis sativa*, or marijuana, in some forms of epilepsy (Iversen 2008). However, in the last few decades, cannabinoid compounds have shown mixed results in animal studies. For instance, cannabinoid agonists inhibit seizures induced by agents that potentiate excitatory neurotransmission, such as L-glutamate, NMDA, and kainate, but are ineffective or even exacerbate seizures induced by agents that attenuate inhibitory neurotransmission, such as

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the GABA$_A$ receptor antagonist bicuculline or the benzodiazepine receptor antagonist DMCM (Clement et al. 2003; Hayase et al. 2001). Thus, the anticonvulsant properties of cannabinoids may be influenced by the balance of excitation and inhibition during seizure activity.

Indeed, the endogenous cannabinoid (endocannabinoid or eCB) system has the potential to modulate both inhibitory and excitatory synaptic activity (for review see Mackie 2008) The eCB system includes the cannabinoid receptors CB1R and CB2R, the endogenous ligands anandamide and 2-AG and their synthetic enzymes, and the eCB degradation machinery, including fatty acid amid hydrolase (FAAH) and MGL (for review see Kano et al. 2009). In the brain, $G_{i/o}$-coupled CB1Rs inhibit neurotransmitter release via their predominately presynaptic localization and inhibitory effect on calcium influx (Alger 2002; Schlicker and Kathmann 2001). Interestingly, cannabinoid receptors are highly expressed in a subset of inhibitory interneurons (Tsou et al. 1999) and more moderately expressed in glutamatergic terminals (Kawamura et al. 2006). Endocannabinoids are involved in several forms of activity-dependent synaptic plasticity, including depolarization-induced suppression of inhibition (DSI), depolarization-induced suppression of excitation (DSE), and some forms of long-term depression (LTD) (Mackie 2008). Acute application of exogenous cannabinoids can inhibit both glutamate and GABA release in numerous brain regions, including the hippocampus (Ohno-Shosaku et al. 2002). Cannabinoids can attenuate epileptiform activity induced by omission of magnesium in hippocampal slice cultures (Blair et al. 2006; Shen and Thayer 1998) and acute brain slices (Ameri and Simmet 2000; Ameri et al. 1999); however, the conditions that determine the effectiveness of cannabinoids in suppressing epileptiform activity have not been well examined.

In the present study, we monitored extracellular hippocampal field potentials in the CA1 region to characterize the factors that influence the effects of cannabinoids on *in vitro* epileptiform activity. Further, we coadministered GABA receptor agonists and antagonists with cannabinoids to explore the interplay between the GABAergic and cannabinoid systems.

Materials and Methods

Hippocampal slice preparation

P20–30 CD1 mice (Charles River Laboratories, Inc. Boston, MA) were euthanized by rapid decapitation according to protocols approved by the University of Connecticut Health Center Animal Care and Use Committee and in accordance with the guidelines presented in the NIH *Guide for the Care and Use of Laboratory Animals* and the principles outlined in the "Guidelines for the Use of Animals in Neuroscience Research" by the Society for Neuroscience. The brain was rapidly removed and cut into 300 μm coronal sections using a DTK-1000 vibratome (Dosaka; Kyoto, Japan) in ice cold, oxygenated incubation buffer (in mM: 125 NaCl, 25 NaCO$_3$, 15 dextrose, 2.5 KCl, 1.25 NaH$_2$PO$_4$, 3.5 MgCl$_2$, 0.5 CaCl$_2$, 0.4 ascorbic acid, 4 lactic acid, and 2 pyruvic acid; 300 ± 5 mOsm; pH 7.3). Slices were allowed to incubate for 60–90 minutes at room temperature (21–24° Celsius) prior to recording.

Electrophysiological recordings

Brain slices containing the hippocampus were placed in a recording chamber perfused with warmed (32° Celsius) oxygenated artificial cerebrospinal fluid (aCSF, in mM: 125 NaCl, 25 NaCO$_3$, 15 dextrose, 2.5 KCl, 1.25 NaH$_2$PO$_4$, 2 MgCl$_2$, and 2 CaCl$_2$; 300 ± 5 mOsm; pH 7.3) at a flow rate of 1–2 ml/min. A borosilicate glass micropipette (2–6 ΩM) filled with aCSF was placed in the stratum radiatum layer of CA1, and a bipolar tungsten stimulating electrode was placed in the Schaffer collateral pathway approximately 250 μm lateral to the

recording electrode. Extracellular field excitatory postsynaptic potentials (fEPSPs) were evoked every 20 seconds with a Grass S88 Stimulator (Grass Instrument Co., West Warwick, RI) and recorded with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Following a 30 minute baseline period in aCSF, epileptiform responses were induced by either omission of magnesium (zero added magnesium - "No Mg") or omission of magnesium and elevation of potassium (zero added magnesium, 8 mM KCl - "No Mg/High K"). Epileptiform responses were allowed to evolve and stabilize for at least one hour before drug conditions were tested. For clarity, stimulation artifacts have been blanked from all traces.

Pharmacologic agents

WIN55212-2 (WIN; Sigma-Aldrich, St. Louis, MO), SR141716A (SR; NIH, Bethesda, MD), 6,7-Dinitroquinoxaline-2,3-dione (DNQX; Tocris Cookson Inc., St. Louis, MO), AM404 (Tocris Cookson), URB597 (Sigma-Aldrich), and CGP55845 (CGP; Sigma-Aldrich) were solubilized with 0.05–0.1% DMSO. (R)-Baclofen (Tocris Cookson), (RS)-3-(2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP; Tocris Cookson), and 4-[6-imino-3-(4-methoxyphenyl)pyridazin-1-yl] butanoic acid hydrobromide (GABAzine; Sigma-Aldrich) were dissolved in aCSF.

Statistical analysis

The maximum amplitude (hereafter referred to as amplitude), rise slope, and area of the evoked field potentials were measured off-line using Clampfit software. The paired pulse ratio (PPR) was calculated as the average second pulse amplitude/average first pulse amplitude for 5-minute bins (PPR = Avg P2/Avg P1). Data were compared using Student's t-tests unless otherwise indicated. Values are reported as mean ± standard error of the mean (S.E.M.).

Results

Field excitatory postsynaptic potentials (fEPSPs) are attenuated by CB1 receptor activation

We first characterized the effects of the cannabinoid agonist WIN on basal fEPSPs evoked in aCSF containing standard concentrations of magnesium (2 mM) and potassium (2.5 mM). After a stable 30-minute baseline period, WIN (5 µM) significantly decreased fEPSP slope and amplitude within 25 minutes of exposure and had a maximal effect at 50–60 minutes. A representative example of this effect is shown in Figure 1A (traces) and Figure 1B (black squares). Group data (n=6) shown in Figure 1C and Table 1 demonstrate that WIN significantly inhibited the slope and amplitude of fEPSPs to a similar degree. The CB1R-selective antagonist SR141716A (SR; 6 µM) applied 30 minutes prior and throughout the 60-min WIN application completely blocked the effect of WIN (Figure 1B & C; 114.9 ± 15.7% of BL slope and 97.4 ± 5.7% of BL amplitude, n=4), indicating that the actions of WIN were CB1R-mediated.

Cannabinoids typically modulate synaptic transmission via inhibition of presynaptic neurotransmitter release. We sought to confirm this by examining the effects of WIN on the paired pulse ratio (PPR). WIN significantly increased the PPR (Figure 1D, baseline = 1.48 ± 0.06 vs. WIN = 1.63 ± 0.09, n=5, p<0.05), suggesting that WIN attenuates fEPSPs by decreasing presynaptic release probability. The AMPA/kainate receptor antagonist DNQX (10 µM) combined with the NMDA receptor antagonist CPP (3 µM) completely abolished fEPSPs (to 0.1 ± 4.0% of baseline), indicating that these responses were mediated by ionotropic glutamate receptor activation. Taken together, these data are consistent with WIN attenuation of fEPSPs occurring via inhibition of glutamate release.

"No Mg" epileptiform responses are also attenuated by CB1R activation

Stimulus-evoked epileptiform responses induced by omission of magnesium from the extracellular bath solution ("No Mg" model) have been shown to be inhibited by cannabinoid agonists (Ameri and Simmet 2000; Ameri et al. 1999). We found that "No Mg" evoked responses were characterized by a maximal, rapid first peak, followed by additional, smaller and slower peaks that reflect recurrent activity occurring in the slice (see Figures 2A and 5A for examples). DNQX and CPP completely abolished these responses. Figures 2A and 2B show traces and the time course of a representative recording of 5 μM WIN on "No Mg" responses. WIN decreased the slope and amplitude of "No Mg" responses to a similar degree (n=8, Figure 2C and Table 1). In addition, WIN reduced the overall area of the "No Mg" responses, a measure of the level of recurrent activity (Figure 2C and Table 1). The WIN concentration-response relationships for basal and "No Mg" fEPSPs were essentially identical (Figure 2D). Pretreatment with 6 μM SR for 30 minutes completely blocked the antiepileptiform effect of WIN (Figure 2E), confirming that it was mediated by CB1R activation.

We next asked whether epileptiform activity induced the release of endogenous cannabinoids (eCBs), which could inhibit the magnitude of these responses and limit the effectiveness of exogenous cannabinoids. As shown in Figure 2E, however, a 60 minute application of the CB1R antagonist SR alone had no effect on fEPSPS in the "No Mg" model, suggesting a lack of eCB tone. We then considered whether the actions of released eCBs could be limited by rapid reuptake and breakdown. In the presence of the eCB reuptake blocker AM404 combined with the fatty acid amide hydrolase (FAAH) inhibitor URB574, we found no attenuation of "No Mg" responses (Figure 2E). Taken together, these results suggest that epileptiform activity induced by omission of magnesium can be inhibited by CB1R activation but does not induce release of eCBs.

"No Mg/High K" epileptiform responses are less sensitive to WIN

Previous studies had shown that omitting magnesium and increasing the extracellular potassium concentration induced spontaneous epileptiform activity in the CA3 region of the hippocampus that was attenuated by cannabinoid agonists (Ameri and Simmet 2000; Ameri et al. 1999). We evoked field potentials in the CA1 region using a similar extracellular solution (omission of magnesium plus elevation of potassium to 8 mM - the "No Mg/High K" model), We did not observe spontaneous synchronous activity, consistent with the less epileptogenic nature of CA1; however, we did note that evoked fEPSPs in the "No Mg/High K" model were epileptiform in nature, with additional peaks representing recurrent activity (see Figures 3B and 5B for example traces), although the average amplitude and slope were smaller than those of the "No Mg" model (see Table 1). These responses were completely abolished by application of DNQX and CPP.

In contrast to our findings with the "No Mg" model, 5 μM WIN had no effect on "No Mg/High K" responses. Interestingly, higher concentrations of WIN attenuated "No Mg/High K" responses. Example time courses of individual recordings utilizing 5 or 30 μM WIN are shown in Figure 3A. Group data (n=7) revealed that 5 μM WIN had no significant effect on slope, amplitude, or area (Table 1). Figure 3B illustrates example traces before and during 30 μM WIN application from the same recording as Fig 3A (see Table 1 for group data, n=5). Although 30 μM WIN was able to significantly suppress "No Mg/High K" responses, the overall concentration-response curve of the effects of WIN on amplitude (Figure 3C) was shifted to the right and never reached the maximal effect seen in the "No Mg" models (n=4–8 for each concentration; p < 0.01; non-linear regression analysis). The effect of 30 μM WIN was completely blocked by pretreatment with 15 μM SR (Fig. 3D), confirming that it was mediated by CB1R activation. These results suggest that the "No Mg/High K"

model is less sensitive to the attenuating effects of cannabinoids. To address the possibility that the "No Mg/High K" model induced release of eCBs which occluded the effects of WIN, we examined the effects of SR alone (6 µM). Although we found a small but statistically significant potentiation of amplitude compared to baseline, SR did not significantly alter "No Mg/High K" responses compared to DMSO (Fig 3D, SR $117.0 \pm 4.7\%$ of baseline, n=6; DMSO $110.9 \pm 4.9\%$ of baseline, n=7) or no treatment (Fig 3D, 60 mins after baseline period $113.6 \pm 5.4\%$ of baseline, n=5), suggesting that the amplitude of "No Mg/High K" fEPSPs simply increased modestly over time. Together, these data indicate that the decreased efficacy of WIN in the "No Mg/High K" model is not due to occlusion by endocannabinoids.

Elevation of potassium does not account for the decrease in WIN efficacy in the "No Mg/High K" model

The efficacy of signaling via G protein-coupled receptors, including CB1Rs, can be impaired in the presence of elevated extracellular potassium (Brody and Yue 2000; Wilson and Nicoll 2001); therefore, we next examined whether the loss of WIN sensitivity in the "No Mg/High K" model was due solely to the increased potassium concentration. We assayed the effects of WIN on fEPSPs evoked in normal magnesium (2 mM) plus elevated potassium (8 mM). This "High K" response was also epileptiform in nature, with additional peaks appearing due to recurrent activity. An example of the effect of WIN on the "High K" response is shown in the traces in Figure 4A and the time course from the same experiment is shown in Fig. 4B. WIN significantly inhibited the slope, area, and amplitude of "High K" epileptiform responses to a similar degree as basal and "No Mg" responses (Fig. 4C and Table 1). Thus, the elevation of extracellular potassium concentration does not solely account for the decreased WIN sensititivy of the "No Mg/High K" model.

GABA$_A$ activity recruited in the "No Mg/High K" model does not account for the decreased WIN efficacy

Cannabinoid receptors are expressed at both excitatory and inhibitory terminals in the hippocampus (Alger 2002). The reduced efficacy of cannabinoids in the "No Mg/High K" model may result from increased inhibitory tone, which could counteract the actions of cannabinoids at excitatory terminals. We utilized the GABA$_A$ receptor antagonist GABAzine (10 µM) to examine the presence of inhibitory tone and its interaction with WIN in both models of epileptiform activity. Figure 5A illustrates "No Mg" example traces before and during GABAzine application. Overall, we found that GABAzine did not significantly alter the slope (Figure 5C, $102.1 \pm 4.5\%$ of BL, n=4) or maximum amplitude ($105.5 \pm 3.2\%$ of BL) but did enhance the area of the epileptiform response (Figure 5D, $121.7 \pm 2.5\%$ of BL, p<0.05). Subsequent application of 5 µM WIN significantly suppressed the slope (Figure 5C, $77.6 \pm 3.8\%$ of GABAzine period, p<0.05), amplitude ($65.4 \pm 2.9\%$, p<0.05) and area (Figure 5D, $68.3 \pm 3.9\%$, p<0.05) in a manner similar to that seen in recordings performed without GABAzine (see Figure 2), suggesting that the effect of WIN was not influenced by GABAergic activity in the "No Mg" model.

We hypothesized that the "No Mg/High K" model recruited significant inhibitory tone which would account for the decreased cannabinoid sensitivity. Figure 5B illustrates "No Mg/High K" example traces before and during GABAzine application. GABAzine failed to modify the slope (Figure 5C, $120.6 \pm 6.3\%$ of BL, n=4) or maximum amplitude of "No Mg/High K" responses ($104.7 \pm 2.3\%$ of BL). However, we found a dramatic enhancement of the area (Figure 5D, $167.5 \pm 8.8\%$ of BL, p<0.05), indicating a high level of inhibitory activity in the "No Mg/High K" model. Nonetheless, in the presence of GABAzine, 5 µM WIN failed to significantly attenuate the slope (Figure 5C, $94.9 \pm 10.2\%$ of GABAzine period), amplitude ($90.4 \pm 2.8\%$, n=4), or area (Figure 5D, $96.2 \pm 4.4\%$). Therefore,

offsetting cannabinoid actions at $GABA_A$-expressing inhibitory synapses did not explain the decreased efficacy of WIN in the "No Mg/High K" model.

Blockade of endogenous $GABA_B$ activity partially unmasks the effects of WIN in the "No Mg/High K" model

The striking effect of GABAzine on recurrent activity in the "No Mg/High K" model led us to consider the possibility that the increased GABAergic tone could result in activation of presynaptic $GABA_B$ receptors. $GABA_B$ receptors are $G_{i/o}$ protein-coupled receptors whose activation results in decreased neurotransmitter release, similar to CB1 receptors (Nicoll 2004). Increased $GABA_B$ activity in the "No Mg/High K" model could occlude the effects of WIN. We first asked whether the $GABA_B$ receptor agonist baclofen could mimic and occlude the effects of WIN in the "No Mg" model. Baclofen (2.5, 10, and 25 µM) decreased the amplitude of "No Mg" evoked responses in a dose-dependent manner (Figure 6A and 6B; $73.1 \pm 4.2\%$, $58.2 \pm 2.4\%$, and $41.3 \pm 4.4\%$ of respective BL period, n=5, p<0.05 for each concentration). In the presence of either 10 or 25 µM baclofen, subsequent coapplication of 5 µM WIN failed to significantly attenuate "No Mg" responses (Figure 6A; $107.3 \pm 7.5\%$ and $85.7 \pm 3.2\%$ of the baclofen period respectively, n=5 for each concentration), whereas in the presence of a submaximal concentration of baclofen (2.5 µM), 5 µM WIN inhibited the "No Mg" response in a manner similar to recordings performed without baclofen (Figure 6A; $57.3 \pm 14.8\%$ of the baclofen period, n=5, p<0.05). Hence, baclofen occluded the effects of 5 µM WIN on "No Mg" responses in a concentration-dependent manner.

Since baclofen significantly suppressed "No Mg" responses (Figure 6B, n=5–6 per concentration, p<0.05 compared to baseline for all concentrations), a possible "floor" effect may be occurring whereby 5 µM WIN may have been unable to further suppress the amplitude below a certain level. To rule this out, we first established a baseline "No Mg" response and then lowered the stimulation intensity until the evoked response was approximately 50% of the original amplitude ($50.6 \pm 5.2\%$ of BL, n=6, p<0.05 compared to BL). We then applied 5 µM WIN, which inhibited the "No Mg" response in a similar fashion to recordings done at the normal stimulation intensity ($57.3 \pm 6.3\%$ of the lowered stimulation intensity period, p<0.05 compared to low stimulation period), ruling out a "floor" effect.

Interestingly, the $GABA_B$ antagonist CGP55845 (CGP, 10 µM) had no effect on "No Mg" responses (CGP alone: $95.6 \pm 4.7\%$ of BL, n=5) and did not modify the effects of WIN (CGP+WIN: $70.3 \pm 5.0\%$ of CGP period, n=5, p<0.05), indicating that the "No Mg" model exhibited no significant endogenous $GABA_B$ activity. However, 10 µM CGP completely reversed the effects of 25 µM baclofen in the "No Mg" model (baclofen alone: $44.1 \pm 8.9\%$ of BL, n=4, p<0.05; baclofen+CGP: $99.0 \pm 10.2\%$ of BL n=4, p<0.05), demonstrating the specificity of this antagonist at the $GABA_B$ receptor. In contrast, we speculated that the "No Mg/High K" model would have a high level of endogenous $GABA_B$ activity, which would be expected to make exogenous $GABA_B$ activation less efficacious. Indeed, we found that the effects of 2.5, 10, and 25 µM baclofen on amplitude were diminished in the "No Mg/High K" model (Figure 6B, n=5–6 per concentration, p<0.05 compared to baseline for 10 and 25 µM) compared to the "No Mg" model.

We then determined whether antagonism of endogenous $GABA_B$ activity with CGP (10 µM) would unmask the effects of 5 µM WIN in the "No Mg/High K" model. CGP alone did not significantly alter the amplitude of "No Mg/High K" responses ($120.4 \pm 10.3\%$ of baseline, n=5). However, in the presence of CGP, WIN attenuated "No Mg/High K" responses (for individual time course, see Figure 6C; for group data see Figure 6D, slope: $84.4 \pm 2.3\%$ of CGP period n=5, p<0.05 and amplitude: $83.0 \pm 3.8\%$ of CGP period,

p<0.05). The presence of vehicle (0.1% total DMSO) did not account for the improved WIN efficacy (Figure 6D, n=5). Taken together, these data indicate that endogenous $GABA_B$ activity partially occluded the effects of WIN in the "No Mg/High K" model.

Discussion

Aberrations in both glutamatergic and GABAergic signaling are thought to contribute to the pathogenesis of epilepsy. The hippocampus is uniquely predisposed to developing seizure activity due to the extensive reciprocal innervations between excitatory pyramidal cells as well as the powerful synchronizing influence of GABAergic interneuron inputs (McCormick and Contreras 2001). Cannabinoids, by virtue of their presynaptic localizations at both GABAergic and glutamatergic terminals, have the potential to reduce both excitatory and inhibitory synaptic transmission and can, theoretically exert anticonvulsant or proconvulsant properties. Using models of epileptiform activity which differentially impact glutamatergic and GABAergic activity, we have evaluated the factors that influence the antiepileptiform actions of cannabinoids in the CA1 region of the hippocampus. In doing so, we have identified recruitment of endogenous $GABA_B$ receptor activity as a contributor to the reduced efficacy of cannabinoids in the "No Mg/High K" model. This phenomenon which could potentially mediate the conflicting results of cannabinoids in animal models of epilepsy.

We began by demonstrating that WIN inhibited field potentials evoked in standard extracellular media by approximately 55%, consistent with previous studies (Domenici et al. 2006; Takahashi and Castillo 2006). We determined that WIN increased the paired pulse ratio, as expected, indicating decreased glutamate release probability as the mechanism of action of CB1R activation. We further demonstrated that basal and "No Mg" responses have identical sensitivities to the attenuating effects of WIN. Our data suggest that at a fundamental level, cannabinoid agonists are capable of suppressing epileptiform activity to the same degree as normal synaptic transmission.

Multiple lines of evidence suggest that endocannabinoids are released during epileptiform activity. For instance, spontaneous epileptiform activity induced by omission of magnesium results in an eCB-mediated DSI-like suppression of inhibitory activity in the hippocampus (Beau and Alger 1998). Further, both *in vitro* and *in vivo* animal models of epilepsy are often potentiated by CB1R antagonism, potentially due to the blockade of endocannabinoid activity (Ameri et al. 1999; Deshpande et al. 2007; Lutz 2004). We evaluated the possibility that eCBs are produced during "No Mg" epileptiform responses and determined that the "No Mg" model was not significantly potentiated by CB1R antagonism. Because eCBs are rapidly taken into cells by a selective reuptake process (Hillard et al. 1997) and subsequently broken down by the intracellular enzyme FAAH (Cravatt et al. 1996), eCBs may have been neutralized before they were able to have any appreciable effect on the field response. *In vivo* FAAH inhibition by URB597 has been shown to reduce hippocampal hyperexcitability induced by kainate (Coomber et al. 2008). However, in our studies, continual reuptake blockade and FAAH inhibition failed to attenuate the "No Mg" responses. This does not completely rule out a role for endocannabinoid tone in this model, as other degradative enzymes such as monoglyceride lipase (MGL) also contribute to the rapid clearance and inactivation of endocannabinoids (Dinh et al. 2002). Future studies may explore this possibility.

Given that CB1R is highly expressed at a subset of GABAergic interneurons in the CA1 region of the hippocampus (Tsou et al. 1999) and cannabinoids are thought to inhibit GABAergic transmission more potently than glutamatergic transmission in the hippocampus (Ohno-Shosaku et al. 2002), CB1R activation at GABAergic terminals could result in

disinhibition that would undermine anti-epileptiform effects at glutamatergic terminals. We first asked if the "No Mg" model recruited significant inhibitory activity. Indeed, a moderate GABA component is present in the later portion of the "No Mg" field potential, indicated by the increase in area without a corresponding increase in amplitude or slope in response to $GABA_A$ receptor blockade. Nonetheless, the magnitude of the WIN effect is not altered by blockade of the inhibitory component with GABAzine. This suggests that the anti-epileptiform actions of WIN are not significantly impacted by the disinhibitory actions of WIN. Alternatively, WIN may not exert significant anti-GABAergic actions in the "No Mg" model, perhaps because the CB1-expressing interneurons do not comprise a significant portion of the recruited inhibitory activity. Previous work from our lab has shown that the net effect of cannabinoids on neuronal excitability can vary, such that in layer 2/3 of the neocortex, the overall effect of WIN is disinhibitory, resulting in an increase in pyramidal cell excitability, whereas in layer 5, eCB release causes a transient decrease in pyramidal neuron excitability (Fortin and Levine 2007). The data presented here indicate that in the CA1 region of the hippocampus, the overall effect of WIN in the context of omission of magnesium is to decrease neuronal excitability and is, therefore, antiepileptiform.

In the context of elevated potassium plus omission of magnesium, however, WIN lost both potency and efficacy, as indicated by the rightward shift and lower maximal effect in the WIN concentration-response curve. We examined a number of possibilities to explain this. Endocannabinoid competition with WIN was ruled out by the lack of potentiating effect of the CB1R antagonist, SR141617A. Depolarization-induced by elevated external potassium has been shown to decrease the efficacy of G protein-coupled receptor mediated inhibition of calcium channels (Brody and Yue 2000), and specifically, the magnitude of depolarization-induced suppression of inhibition (DSI) is reduced by increased extracellular potassium concentrations (Wilson and Nicoll 2001), indicating that the CB1R is sensitive to the attenuating effects of depolarization. However, fEPSPs evoked in the presence of normal magnesium and elevated potassium ("High K" responses) were inhibited by 5 μM WIN to a similar extent as basal and "No Mg" responses. Thus, the combination of elevated potassium and omission of magnesium yielded an evoked epileptiform response that was uniquely less sensitive to WIN.

Cannabinoid actions at inhibitory terminals are not responsible for the decreased efficacy of WIN in the "No Mg/High K" model, since coadministration of the ionotropic $GABA_A$ receptor antagonist GABAzine failed to augment or unmask the effects of WIN in the "No Mg/High K" model, despite revealing a substantial GABA component in the responses. These data suggest that either significant CB1R-expressing interneuron activity was not recruited or the same process that diminished the effect of WIN at glutamatergic terminals also decreased the effect of WIN at GABAergic terminals. At this point, we cannot discern between these two possibilities. However, given the dramatic effect of GABAzine on the area of "No Mg/High K" responses, we can conclude that substantial interneuron activity is recruited in this model. Repetitive stimulation of GABAergic terminals and subsequent GABA pooling are necessary to activate presynaptic $G_{i/o}$ coupled $GABA_B$ receptors on glutamatergic terminals (Nicoll 2004), which act to decrease neurotransmitter release via calcium channel inhibition, much like the CB1R. The "No Mg/High K" model likely provided these conditions. Indeed, the decreased efficacy of baclofen in the "No Mg/High K" model is consistent with a high level of endogenous $GABA_B$ tone. Interestingly, $GABA_B$ antagonism has been shown to exacerbate spontaneous epileptiform activity induced by removal of magnesium in the CA3 region of the hippocampus (Brown et al. 2003); however, the $GABA_B$ antagonist CGP55845 did not potentiate epileptiform responses in either model. This was not surprising in the "No Mg" model, which did not recruit substantial $GABA_B$ activity, but the lack of effect in the "No Mg/High K" model was unexpected. We speculate

that CGP may have blocked presynaptic GABA$_B$ receptors expressed at both inhibitory and excitatory terminals, resulting in no net change in the epileptiform response.

Immunoelectron microscopy has demonstrated that both CB1R and the GABA$_B$ receptors are expressed at presynaptic glutamatergic terminals in the CA1 region of the hippocampus (Katona et al. 2006; Kawamura et al. 2006; Kulik et al. 2003), and these receptors share the same specificity for G protein alpha subunits (Straiker et al. 2002). Therefore, we reasoned that the diminished WIN sensitivity could be explained by endogenous activity at GABA$_B$ receptors competing with CB1Rs for overlapping downstream targets. Indeed, coadministration of WIN and the GABA$_B$ receptor antagonist CGP55845 demonstrated that when GABA$_B$ receptors were blocked, the efficacy of WIN was increased, though not to the extent seen for basal fEPSPs and "No Mg" epileptiform responses. Numerous studies have examined interactions between cannabinoid and GABA systems. Hippocampal membrane preparations demonstrate reciprocal inhibition between CB1R and GABA$_B$ receptors, such that a low concentration of a GABA$_B$ antagonist prevents the actions of a cannabinoid agonist, and vice versa, supporting the intriguing possibility that CB1R and GABA$_B$ receptor subunits form hetero-oligomers (Cinar et al. 2008). Although we cannot fully rule out a role for hetero-oligomers, the potentiation of the effects of WIN in the "No Mg/High K" model that we found with a high dose of CGP is more consistent with relief of competition for signaling pathways between CB1R and GABA$_B$ receptors. Another interesting finding is that both exogenous and endogenous cannabinoids are unable to attenuate evoked GABA$_B$ responses in the hippocampus (Hoffman and Lupica 2000; Lafourcade and Alger 2008), suggesting that the population of interneurons responsible for postsynaptic GABA$_B$ receptor activation does not express CB1Rs. These same interneurons may be the source of the endogenous GABA tone seen in the "No Mg/High K" model, which could explain why WIN could not decrease GABA release enough to attenuate presynaptic GABA$_B$ receptor activation. Interestingly, elevated extracellular potassium alone has been shown to trigger increased release of GABA from hippocampal interneurons (Shin et al. 2011) which could result in GABA$_B$ receptor activation and decreased WIN sensitivity. Our finding that the "High K" model was equally sensitive to WIN suggests that only the combination of elevated potassium and omission of magnesium generates sufficient GABA$_B$ activity to interfere with the actions of WIN.

Our findings suggest that endogenous GABA$_B$ tone partially accounted for the loss of WIN sensitivity in the "No Mg/High K" model, but additional factors also contributed. The most likely candidates are other G$_{i/o}$ protein-coupled receptors expressed at presynaptic glutamatergic terminals in the CA1 region. The metabotropic mGluR7 glutamate receptor is one possibility (Millan et al. 2002). The loss of WIN efficacy in the "No Mg/High K" model is in contrast to previous studies in which 5 μM WIN attenuated the frequency of spontaneous epileptiform activity induced by identical extracellular magnesium and potassium concentrations (Ameri and Simmet 2000; Ameri et al. 1999; Blair et al. 2006). However, those studies were performed in hippocampal neuronal cultures or in the CA3 region of hippocampal slices and likely involved mossy fiber synapses, which do not express mGluR7 receptors (Shigemoto et al. 1997). A second potential candidate for occluding the actions of WIN is the adenosine A1 receptor, which was recently shown to be responsible for the inability of WIN to attenuate hippocampal fEPSPs in C57BL/6J mice (Hoffman et al. 2010). Another study found that A1 receptor activation impairs cannabinoid-mediated inhibition of glutamate release in hippocampal synaptosomes (Sousa et al. 2011), further supporting a negative interaction between the A1 receptor and CB1R. Future studies will examine the promising roles of mGluR7 and A1 receptors in the differential effects of cannabinoids in the "No Mg" and "No Mg/High K" models.

In summary, the present study demonstrates that minor differences in models of epileptiform activity can lead to dramatic alterations in the efficacy of cannabinoids due to interactions between the CB1R and GABA$_B$ receptors. These findings allow a glimpse into the mechanisms underlying the variable efficacy of cannabinoids in different *in vitro* and *in vivo* models and pave the way toward a better understanding of the utility of cannabinoid agents as anticonvulsants.

Acknowledgments

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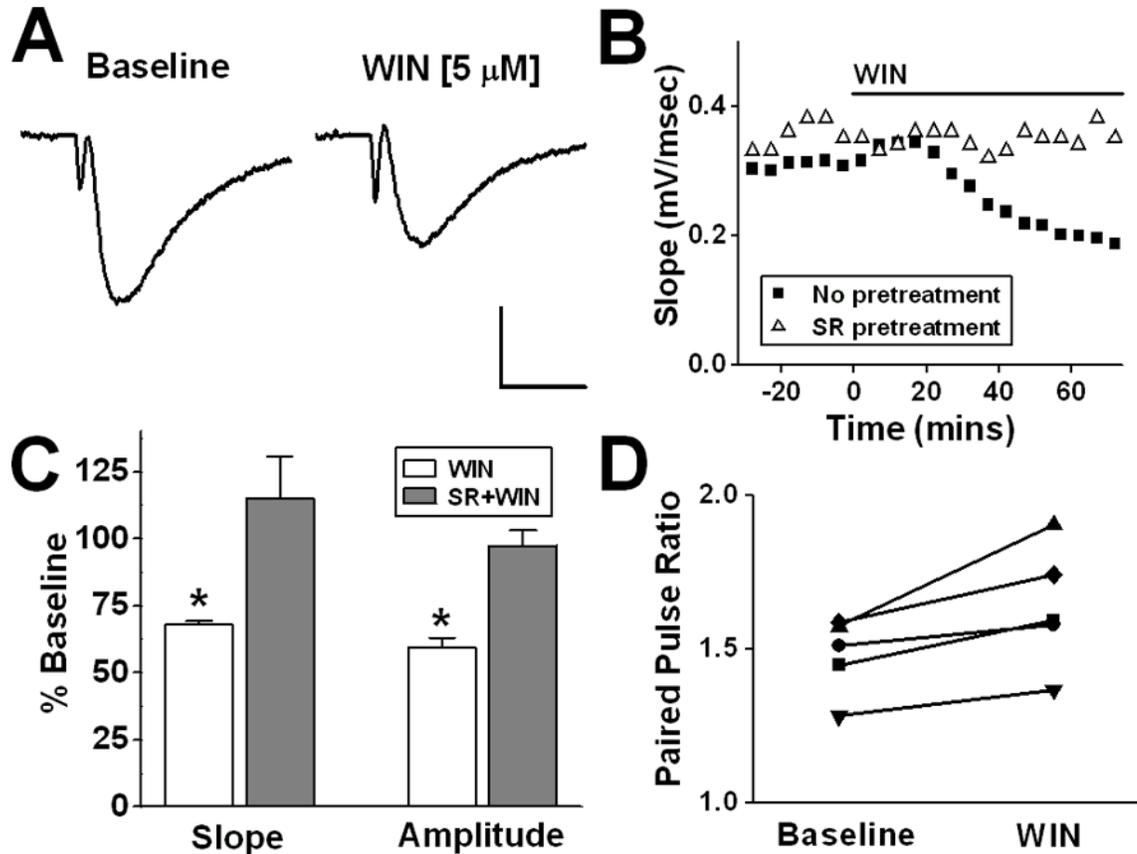
Figure 1.
The cannabinoid agonist WIN55212-2 (WIN) attenuates fEPSPs and increases the paired pulse ratio (PPR). **A.** Average traces from 15 sweeps from a representative individual recording before and during bath application of 5 µM WIN. Scale bar = 0.3 mV, 10 msec. **B.** Time course of WIN effect on fEPSP amplitude from the same recording shown in A (black squares) and from a recording in which the CB1R antagonist SR141716A (SR; 6 µM) was applied prior to and throughout the WIN period (white triangles). **C.** Group data for the effect of WIN (n=6) and SR+WIN (n=4) on fEPSP amplitude and slope. * denotes p<0.05 compared to baseline. **D.** PPR values at baseline and after 60 min of WIN from 5 different recordings.
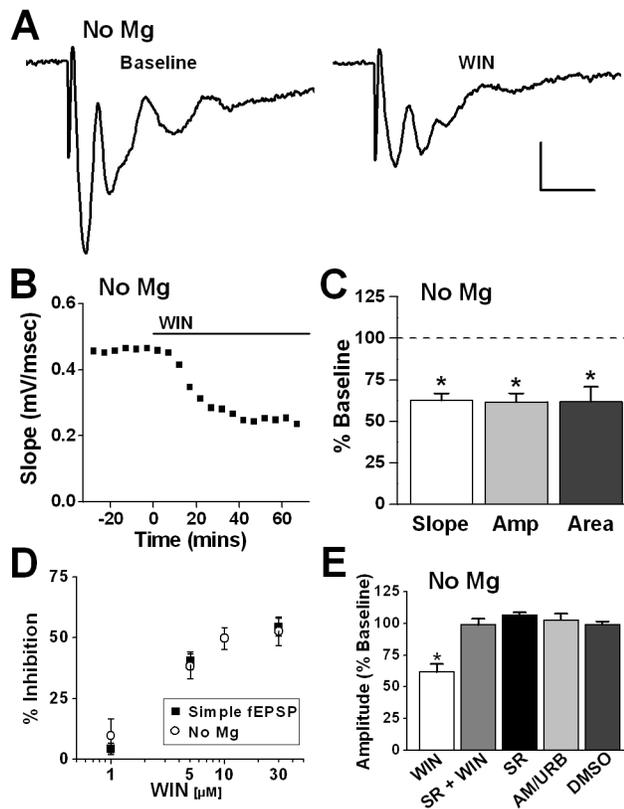
Figure 2.
Epileptiform responses induced by omission of magnesium are inhibited by the cannabinoid agonist WIN. **A.** Average traces from 15 sweeps from a representative individual "No Mg" recording before and during bath application of 5 μM WIN. Scale bar = 0.2 mV, 10 msec. **B.** Time course of the effect of WIN on the slope of an individual "No Mg" recording. **C.** Group data for the effects of 5 μM WIN on the slope, amplitude, and area of "No Mg" responses (n=8). **D.** Concentration-response curves for basal fEPSPs (black squares) and "No Mg" responses (white circles), as represented by the percent inhibition of amplitude (the reciprocal of percent baseline, n=4–8 for each concentration). Data points for 5 μM WIN are identical to those from Figure 1C (basal fEPSPs) and Figure 2C ("No Mg"). **E.** Group data for the effects of various cannabinoid agents on "No Mg" responses: WIN (5 μM; n=8, data from Fig 2C); SR (6 μM) + WIN (n=4); SR alone (n=6); AM404 (25 μM) + URB597 (10 μM; n=4); DMSO (n=5). * denotes p<0.05 compared to baseline.
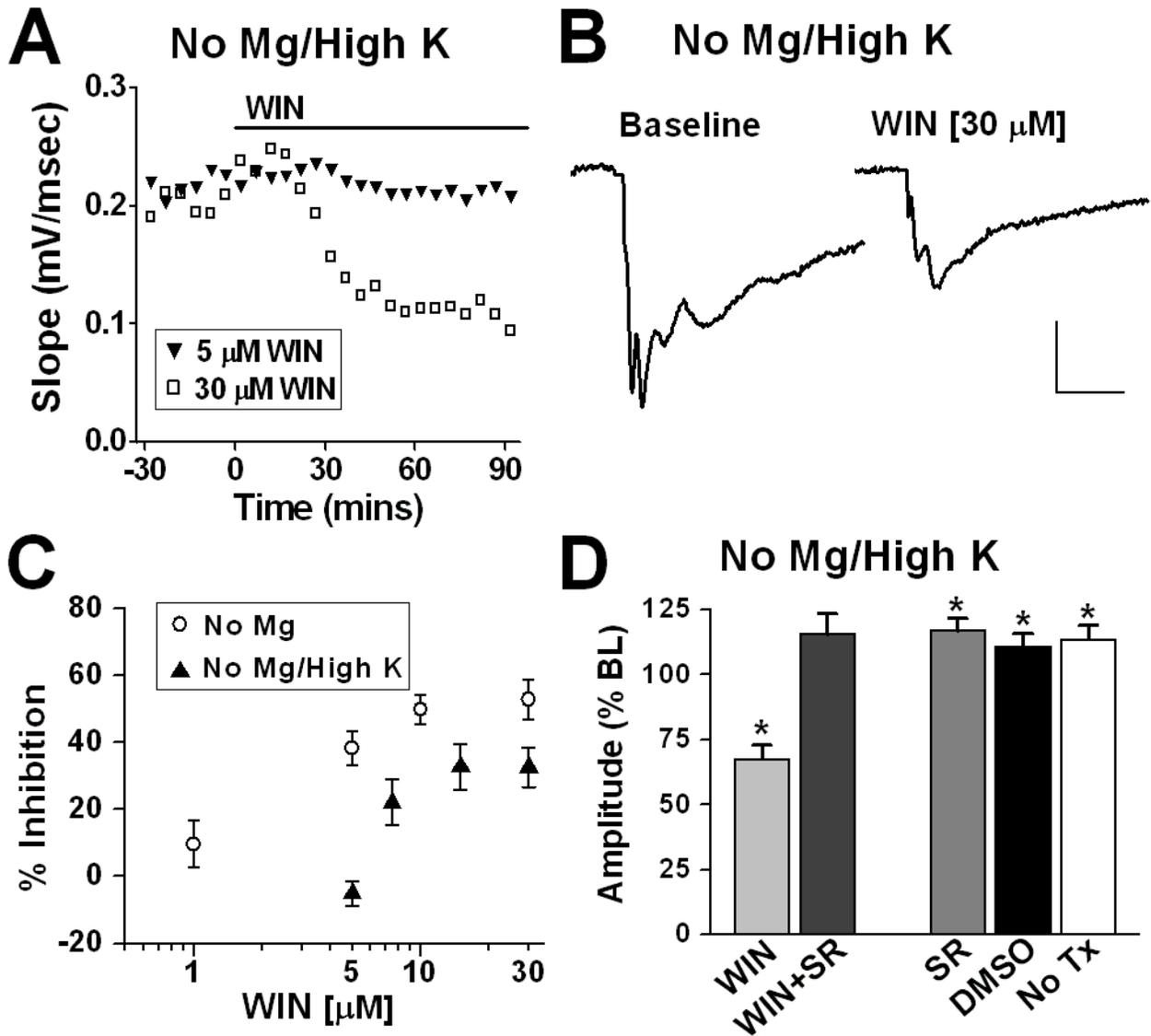
Figure 3.
Epileptiform responses induced by omission of magnesium/elevation of potassium are less sensitive to WIN. **A.** Time course of the effects of 5 μM (black triangles) and 30 μM (white squares) WIN on the slope of representative recordings of "No Mg/High K" responses. **B.** Sample traces (average of 15 sweeps) before and during application of WIN (30 μM). Scale bar = 0.2 mV, 20 msec. **C.** Concentration-response curve for the effect of WIN on the amplitude of "No Mg/High K" responses (black triangles, n=4–8 per group) versus "No Mg" responses (open circles, data from Fig 2C, shown for comparison purposes). **D.** Group data for the effects of various cannabinoid agents on "No Mg/High K" responses: WIN (30 μM, data from Fig 3C, n=5); WIN+SR (30 μM WIN and 15 μM SR, n=4); SR (6 μM n=6); DMSO (0.05%, n=7); No Tx (no treatment for 60 minutes, n=5). * denotes p<0.05 compared to baseline.
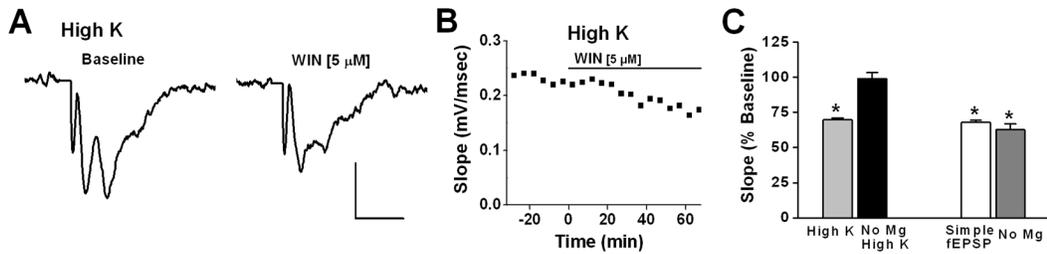
Figure 4.
Elevated potassium alone does not alter cannabinoid sensitivity. **A.** Sample traces (average of 15 sweeps) from an individual recording of the effects of 5 µM WIN on epileptiform activity evoked in the presence of elevated potassium ("High K" responses). Scale bar = 0.15 mV, 10 msec. **B.** Time course for the effects of WIN from the same recording shown in A. **C.** Summary of the effects of 5 µM WIN in the various models: "High K" (2 mM Mg, 8 mM K, n=5); "No Mg/High K" (0 mM Mg, 8 mM K, data from Fig 3C); Simple fEPSPs (2 mM Mg, 2.5 mM K, data from Fig 1C); "No Mg" (0 mM Mg, 2.5 mM K, data from Fig 2C). * denotes p<0.05 compared to baseline.
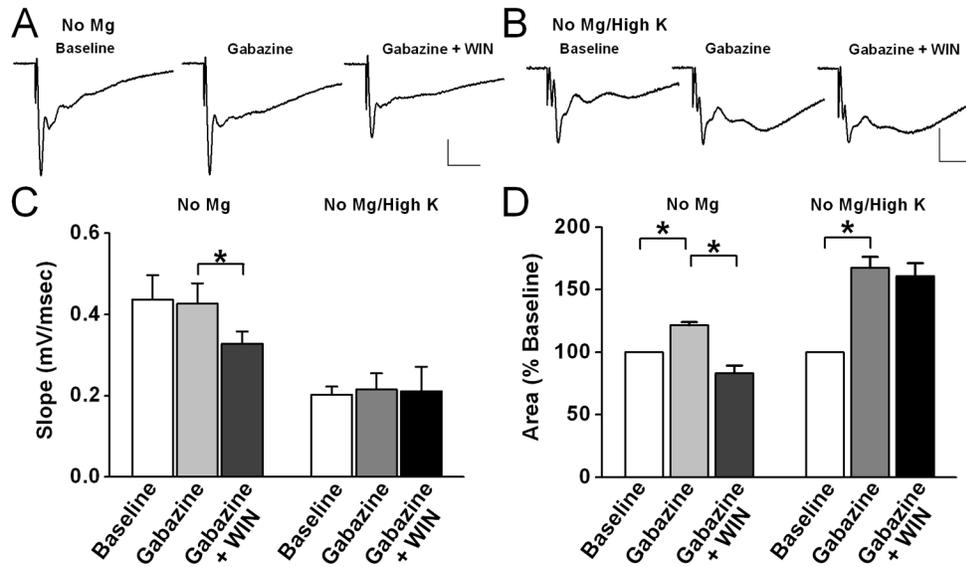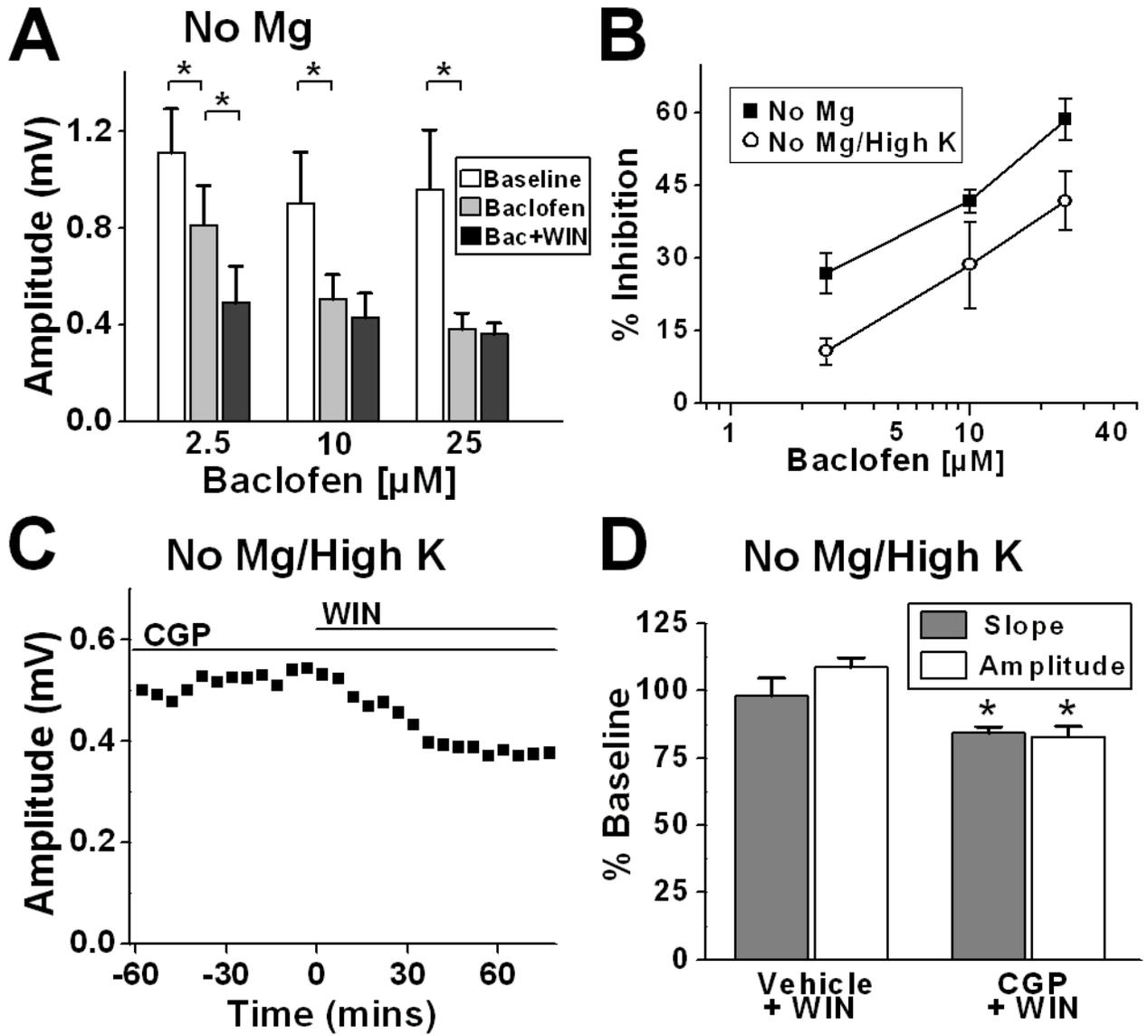
Figure 5.
Blocking inhibitory activity does not alter cannabinoid sensitivity. **A.** Example traces (average of 15 sweeps) from an individual "No Mg" recording in the presence of the GABA$_A$ receptor antagonist GABAzine (10 µM) and subsequent coapplication of 5 µM WIN. Scale bar = 0.2 mV, 20 msec. **B.** Example traces (average of 15 sweeps) from an individual "No Mg/High K" recording with 10 µM GABAzine and subsequent coapplication of 5 µM WIN. Scale bar = 0.2 mV, 20 msec. **C.** Group data for the effects of GABAzine and WIN on slope in the two models (n=4 for all). **D.** Group data for the effects of GABAzine and WIN on area in the two models (n=4 for all). * denotes p<0.05 between conditions.

Figure 6.
Endogenous $GABA_B$ receptor activity partially occludes the effects of WIN on "No Mg/
High K" responses. **A.** Group data for the effects of the $GABA_B$ agonist baclofen and WIN
(5 μM) on "No Mg" response amplitude (n=5 for each concentration). **B.** Concentration-
response curves of baclofen on "No Mg" (n=5, $p<0.05$ for all concentrations) and "No Mg/
High K" fEPSPs (n=5–6 for each concentration, $p<0.05$ for 10 and 25 μM baclofen). **C.**
Time course of an individual "No Mg/High K" recording with coapplication of the $GABA_B$
receptor antagonist CGP (10 μM) and WIN (5 μM). **D.** Group data for the effects of
coapplication of CGP (10 μM) and WIN (5 μM, n=5) versus coapplication of DMSO
(0.1%) and WIN (5 μM, n=5) on the slope and amplitude of "No Mg/High K" responses.

Table I

Effect of WIN under various conditions

Effect of WIN on amplitude (mV), slope (mV/ms), and area (mV * ms) of fEPSPs under various conditions. WIN concentration 5 μM except where noted.

Condition		Baseline	WIN	% Change
Basal (n=6)	Amplitude	0.39 ± 0.07	0.23 ± 0.05	59.4 ± 3.7 *
	Slope	0.24 ± 0.05	0.16 ± 0.03	67.9 ± 1.5 *
No Mg (n=8)	Amplitude	0.94 ± 0.16	0.62 ± 0.15	61.8 ± 5.2 *
	Slope	0.62 ± 0.1	0.4 ± 0.09	62.6 ± 4.3 *
	Area	20.9 ± 2.63	13.85 ± 3.41	61.9 ± 8.9 *
No Mg/High K (n=7)	Amplitude	0.47 ± 0.09	0.48 ± 0.08	105.0 ± 3.6
	Slope	0.2 ± 0.04	0.19 ± 0.03	99.0 ± 4.3
	Area	18.72 ± 3.82	20.27 ± 3.47	115.5 ± 10.3
No Mg/High K WIN 30 μM (n=5)WIN 30 M (n = 5)	Amplitude	0.39 ± 0.09	0.25 ± 0.03	67.5 ± 5.4 *
	Slope	0.2 ± 0.04	0.12 ± 0.02	64.2 ± 6.4 *
	Area	15.54 ± 3.29	9.39 ± 1.72	65.3 ± 9.1 *
High K (n=5)	Amplitude	0.34 ± 0.05	0.19 ± 0.03	58.1 ± 5.1 *
	Slope	0.2 ± 0.04	0.14 ± 0.03	69.9 ± 1.1 *

*, p < 0.05. Area data for High K model not included because of the presence of contaminating positive-going peaks.