JPET Fast Forward. Published on January 31, 2007 as DOI: 10.1124/jpet.106.116319

JPET #116319

EFFECTS OF CANNABINOIDS ON SYNAPTIC TRANSMISSION IN THE FROG NEUROMUSCULAR JUNCTION

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Number of text pages: 33

Number of figures: 6

Number of references: 40

Number of words: Abstract (248)

Introduction (539)

Discussion (1206)

List of non-standard abbreviations:

WIN, WIN55,212-2, *R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)]pyrolol[1,2,3*de*]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone; ACPA, Arachidonylcyclopropylamide / *N*-(2-Cyclopropyl)-5Z,8Z,11Z,147-eicosatetraenamide; AM281, 1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-4-morpholinyl-1*H*-pyrazole-3-carboxamide; AM630, 6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl](4-methoxyphenyl)methanone; mEPPS, miniature endplate potentials; CB1, cannabinoid type 1; CB2, cannabinoid type 2; DMSO, dimethyl sulfoxide; PTX, pertussis toxin; ω-CgTX, ω-conotoxin GVIA; nAChR, nicotinic acetylcholine receptor.

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Abstract

This study aimed to investigate the function of the cannabinoid receptor in the neuromuscular junction of the frog (*Rana pipiens*). Miniature end-plate potentials were recorded using the intracellular electrode recording technique in the *cutaneous pectoris* muscle in the presence of the cannabinoid agonists WIN and ACPA and the cannabinoid antagonists AM281 and AM630. Adding WIN to the external medium decreased the frequency and amplitude of the MEPPs; the WIN EC_{50} value was 5.8 ± 1.0 μM. Application of ACPA, a selective agonist of CB_1, also decreased the frequency of the MEPPs; the ACPA EC_{50} value was 115.5 ± 6.5 nM. The CB_2 antagonist AM630 did not inhibit the effects of WIN, indicating that its action is not mediated through the CB_2 receptor. However, the CB_1 antagonist AM281 inhibited the effects of WIN and ACPA, suggesting that their actions are mediated through the CB_1 receptor. Pre-treatment with the *Pertussis* toxin inhibited the effects of WIN and ACPA, suggesting that their effects are mediated through $G_{i/o}$-protein activation. The N-type Ca^{2+}-channel blocker ω-CgTX diminished the frequency of the MEPPs, being the ω-CgTX EC_{50} value of 2.5 ± 0.40 μM. Blocking the N-type Ca^{2+}-channels with ω-CgTX (5 μM) before addition of ACPA to the bath had no additional inhibitory effect on the MEPPs, while in the presence of ω-CgTX (1 μM), ACPA whether had additional inhibition effect. These results suggest that cannabinoids modulate transmitter release in the endplate of the frog neuromuscular junction by activating CB_1 cannabinoid receptors in the nerve ending.

Introduction

Cannabinoids, the active components of *Cannabis sativa* (marijuana), cause psychoactive and motor effects when the plant is consumed. These effects are produced by the interaction of these compounds with the membrane receptors named CB_1 and CB_2 (Howlett *et al.,* 2002). Both subtypes of cannabinoid receptors alter cellular activity through activation of G proteins of the $G_{i/o}$ subtype, which are sensitive to *Pertussis* toxin (Soderstrom *et al.,* 2000), although some effects are thought to occur through G_s (Bayewitch *et al.,* 1995; Glass and Felder 1997). Activation of $G_{i/o}$ proteins by CB_1 and CB_2 receptors decreases adenylate cyclase activity, leading to decreased levels of intracellular cyclic AMP and protein phosphorylation. G protein activation by both cannabinoid receptors has been implicated in the modulation of the mitogen-activated protein kinase signaling pathway and Krox-24 (zenk) induction (Bouaboula *et al.,*1995). However, activation of CB_1, but not CB_2, is negatively coupled through $G_{i/o}$ proteins to N-type and P/Q-type calcium channels and is positively coupled to the inward-rectifying potassium channels (Mackie *et al.,* 1995; Twitchel *et al.,* 1997; Vásquez *et al*., 2003).

The endogenous cannabinoid system, represented by cannabinoid receptors, appeared early in evolution and conservation of CB1 receptor appear to be restricted to vertebrata species. Therefore, in amphibians, only the CB1 receptor ortholog has been reported to date (Soderstrom, 2000; McPartland et al., 2006). The endocannabinoids (endogenous cannabis-like substances) are fatty acids derived from arachidonic acid, and notably include anandamide, and 2-arachidonylglycerol. Once anandamide and 2-AG are formed, in some cases they

target the CB_1 receptors in the same cell where they are formed, via diffusion within the plasmalemma, or they can be released from the extracellular fluid, where they reach presynaptic terminals (Piomelli, 2003; Rodriguez de Fonseca et al., 2005).

In the last three decades, the actions of cannabinoids have been shown to be related to the inhibition of transmitter release in different synapses of the central nervous system and in smooth muscle (Ishac *et al.,* 1996; Engler *et al.,* 2006). The mechanism by which cannabinoids affect transmitter release is thought to work through the activation of potassium channels (I_A) or by blocking voltage-dependent Ca^{2+} channels or both (Elphick and Egertová 2001). The inhibition of N-type calcium channels decreases neurotransmitter release in several tissues (Liang *et al.,* 2004; Szabo *et al.,* 2004). With ∆9-tetrahydrocannabinol present in the neuromuscular junction, Kumbaraci and Nastuk (1980) reported a diminution in the frequency and an increase in the amplitude of the MEPPs, while Turkanis and Karler in 1986 reported contradictory data — an increase in the frequency of the MEPPs and a reduction in their amplitude. These studies were performed before the cannabinoid receptors were identified and before the development of specific agonists and antagonists for each type of cannabinoid receptor.

On the other hand, although cannabinoids reduce motor activity at the central level in humans and other mammals, few studies exist about the effects of cannabinoids on skeletal muscle. Therefore, we explored whether part of the effects of cannabinoids (e.g., -reduced motor activity- Dewey 1986; Sañudo-Peña

et al., 2000) occurs directly at the level of the skeletal muscle. The aim of this study was to investigate the function of cannabinoid receptors in the neuromuscular junction of the frog (*Rana pipiens*) and the effects of cannabinoids on synaptic transmission.

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Methods

The experiments were performed on the neuromuscular junction of the *cutaneous pectoris* muscle of *Rana pipiens*. Frogs were used in accordance with the Institute for Laboratory Animal Research (ILAR) Guide for the Care and Use of Laboratory Animals and were sacrificed by decapitation and demedulated. The nerve and muscle were dissected, pinned on a Sylgard-coated dish (World Precision Instruments Inc., Sarasota, FL, USA), and bathed in normal frog Ringer's solution (NFR) consisting of 117 mM of NaCl, 2.5 mM of KCl, 1.8 mM of $CaCl_2$, and 2 mM of imidazole-Cl, pH 7.4.

Electrophysiology. We used conventional methods for single-electrode intracellular recording in skeletal muscle. Glass microelectrodes (A-M Systems, Inc., Sequim, WA, USA) filled with 3 M of KCl gave 10 to 20 MΩ of resistance. Stock solutions for each drug (see Drug preparations) were added to the bath solution by a three-way changer and allowed to achieve the specific concentration by diffusion. The resting membrane potential was monitored continuously, and an experiment was rejected when more than a 10 mV change in membrane potential remained after the drug administration. The frog *cutaneous pectoris* muscles were always bathed by the normal solution. All recordings of MEPPs were performed at room temperature (22 to 24°C). Data were acquired through an Axoclamp 2B (Axon Instruments, Foster City, CA, USA), digitized with a Digidata 1322A (Axon Instruments) using Clampex, a subroutine of pClamp 9.0 software (Axon Instruments), in 70-second periods, and identified and analyzed by the Clampfit subroutine of pClamp 9.0. This program summarizes the amplitudes of MEPPs and

a mean is obtained. In addition, we obtained normalized cumulative amplitude histograms, which show the differences more clearly, in order to compare the event amplitude distributions (Clements and Bekkers, 1997).

To quantify the effect of each drug, MEPPs were recorded continuously from the same neuromuscular junctions every 5 minutes during the 15 minutes before the application of each drug (control conditions) and for 30 minutes in the presence of each drug, similar to protocols established in previous investigations (Turkanis and Karler, 1986). After 30 minutes, drugs were removed from the bathing solution, excepting nifedipine and ω-CgTX, unless another condition is specified.

Drug preparations. Each of the cannabinoids — WIN, ACPA, AM630, and AM281 (Tocris Bioscience, Ellisville, MO, USA) — was prepared as a 10 mM stock solution in DMSO or ethanol (Sigma Co., St. Louis, MO, USA; diluent concentration was < 0.01 percent). Stock solutions were stored at –20°C. The L-type Ca^{2+}-channel blocker nifedipine (Sigma) was dissolved in ethanol to make a 100 mM stock solution. The N-type Ca^{2+}-channel blocker ω-CgTX (Sigma) was prepared as a 0.5-mg/ml stock solution in water. Experiments using ACPA and nifedipine were performed in the dark.

Pre-treatment with PTX. PTX (Sigma) was reconstituted as a stock solution (50 μg/ml) with 5 mg/ml of bovine serum albumin (Sigma) in water. The muscle was incubated with 2 μg/ml of PTX in NFR for 22 to 24 hours at 4°C (Sugiura and Ko, 1997).

Statistical methods. Differences between the means of the frequencies were evaluated using a *t*-test; $P < 0.05$ was considered significant. To evaluate the differences in the amplitude of the MEPPs, the Kolmogorov–Smirnov test for cumulative probability curves was used; $P < 0.05$ was considered significant (Liang *et al.,* 2004; Szabo *et al.,* 2004). The dose-effect graphs were elaborated using Origin 6.0, and the data were adjusted to a Hill equation of the type $I = Imax/(1+(EC_{50}/x)^{h})$.

Results

Effects of WIN on spontaneous transmitter release in the frog neuromuscular junction

To examine whether cannabinoids modulate transmitter release in the neuromuscular junction of the frog *cutaneous pectoris* muscle, we first used the cannabinoid agonist WIN, which binds to both types of cannabinoid receptors. Adding this agonist (0.1 to 50 μM) to the external media decreased both the frequency and amplitude of the MEPPs compared with control conditions. Figure 1 shows that adding WIN (10 μM) to the bath reduced the amplitude of the MEPPs by 10.6 percent ± 2.9 percent and their frequency by 25.3 percent ± 3.2 percent in all five cells tested (see Fig. 1 A and B). Maximum inhibition by WIN was reached 25 to 30 minutes after its application (Figure 1), and this effect was sustained for at least 15 minutes after washout (data not shown).

To test whether the effects of WIN involve G-protein-mediated inhibition of transmitter release at the neuromuscular junction, we used PTX, which inhibits certain types of G-proteins (G_i and G_o; Reisine and Law, 1992). We incubated cells overnight in PTX to allow internalization of PTX and inactivation of G-proteins (Reisine and Law, 1992). Figure 1 (A and B, compare the first versus the second columns) shows that PTX pre-treatment (2 μg/ml, 22 to 24 hours) prevented inhibition of the MEPPs by WIN. However, the averaged amplitude and frequency of the MEPPs did not differ significantly before and after WIN treatment. These

results suggest that inactivation of PTX-sensitive G-proteins prevents WIN from inhibiting MEPPs.

We next tested whether the receptor antagonists of CB_1 or CB_2 could reverse the effect of WIN. Adding the CB_1 receptor antagonist AM281 (1 μM) reversed the inhibitory effect of WIN on the MEPPs (Figure 1 A and B, fourth column), suggesting that the effect of WIN occurs through the activation of CB_1 receptors. When superfused alone for 30 minutes, AM281 (1 μM) did not change the frequency or amplitude of the MEPPs (data not shown). Adding the CB_2 receptor antagonist AM630 (1 μM) to the bath 5 minutes before the addition of WIN produced a similar effect to that observed in the absence of AM630 (Figure 1 A and B, fifth column), suggesting that the effect of WIN is not mediated by CB_2 receptors.

Figure 2 shows the dose–response curve for the inhibitory effect of WIN over the concentration range from 0.1 to 50 μM. The threshold concentration for the effect of WIN on the frequency of the MEPPs was 1 μM, and the maximum effect was reached with 50 μM of WIN. Each point in the curve (Figure 2) corresponds to the frequency averaged from five experiments. Data were adjusted to a Hill equation, and the EC_{50} for the inhibition of the MEPPs frequency by WIN was 5.8 ± 1.0 μM. The Imax value calculated by the Hill equation was 52.68 percent ± 3.26 percent, and the Hill coefficient was 0.96 ± 0.11.

Inhibitory effects of ACPA on spontaneous transmitter release

We next examined whether the synthetic CB_1 selective agonist ACPA, which is analogous to the endocannabinoids anandamide (Hillard *et al*. 1999) has similar effects to WIN on the frequency and amplitude of the MEPPs. Figure 3 shows the dose-response curve obtained using ACPA over a concentration range of 0.01 to 50 μM. ACPA produced a pattern similar to that induced by WIN; each point in the curve corresponds to the frequency averaged from five experiments. Data were adjusted by the Hill equation, and the EC_{50} for the inhibition by ACPA of the frequency of the MEPPs was 115.5 ± 6.5 nM. The Imax value calculated by the Hill equation was 67.94 percent ± 0.78 percent, and the Hill coefficient was 1.40 ± 0.10.

Adding ACPA (1 μM) to the bath diminished both the mean frequency and amplitude of the MEPPs compared with control conditions (Figures 4A and 4B). Maximum inhibition was reached 30 minutes after its application, when the mean inhibition of MEPP frequency was 63.0 percent ± 2.9 percent. ACPA decreased mean MEPP amplitude 35.2 percent ± 10.1 percent. Figure 4 shows that ACPA significantly decreased the cumulative amplitude distribution compared with the control condition (Figure 4B).

To test whether the effects of ACPA are mediated through $G_{i/o}$ proteins (i.e., similar to the action of WIN), we incubated the *cutaneous pectoris* muscles with PTX (2 μg/ml, 22 to 24 hours). ACPA (1 μM) had no effect on the frequency or amplitude of the MEPPs. The mean frequency of MEPPs after 30 minutes

exposure to the presence of ACPA was 100.8 percent ± 3.6 percent compared with the control (n = 4). The cumulative probability plots indicated no significant difference in MEPP amplitude (*P* = 0.92, Kolmogorov–Smirnov test, data not shown). These data suggest that ACPA acts through a mechanism that involves $G_{i/o}$ proteins.

Adding the CB_1 receptor antagonist AM281 (1 μM) 5 minutes before adding ACPA did not reduce MEPP frequency or amplitude. Mean MEPP frequency was 95.9 percent ± 4.1 percent, and mean MEPP amplitude was 95.2 percent ± 3.3 percent; these results were not statistically significant. Similarly, the cumulative amplitude did not differ significantly from control after 30 minutes in the presence of ACPA. These results suggest that ACPA activates pre- and postsynaptic CB_1 receptors and that this activation decreases the transmitter release and/or probably acetylcholine receptor sensitivity.

The N-type Ca^{2+}-channel blocker ω-CgTX inhibits the miniature endplate potentials

To explore whether Ca^{2+}-channels are involved in spontaneous transmitter release in the frog neuromuscular junction, we blocked the L- and N-type Ca^{2+}-channels, both of which are found in the neuromuscular junction of the frog (Meir *et al.,* 1999). After 30 minutes in the presence of nifedipine (10 μM), mean MEPP frequency was 103.7 percent ± 5.8 percent and mean MEPP amplitude was 98.6 percent ± 5.1 percent. These data indicate that L-type Ca^{2+}-channels do not

participate in spontaneous transmitter release and do not affect acetylcholine receptors in the postsynaptic membrane. On the other hand, Figure 5A, shows the dose-response curve for the inhibitory effect of ω-CgTX, which blocks N-type Ca^{2+}-channels, on the frequencies of the MEPPs, over the concentration range from 0.1 to 10 μM. Each point in the curve, corresponds to the frequencies averaged from four experiments. The threshold concentration for the effect of ω-CgTX on the frequencies of the MEPPs was 0.1 μM, and the mean of the maximum effect was reached with 10 μM of ω-CgTX. Data were adjusted to the Hill equation, and the EC_{50} for the inhibition of the MEPPs frequency with ω-CgTX was 2.5 ± 0.48 μM. The I_{max} value calculated by the Hill equation was 102 percent ± 4.72 percent and the Hill coefficient was 1.02 ± 0.16. These results indicate that the Ca^{2+} current through the N-type Ca^{2+}-channels is involved in spontaneous transmitter release in our preparation. On the other hand, ω-CgTX had no significant effect on the cumulative amplitude, Figure 5B shows an example when we used 5 μM ω-CgTX.

N-type Ca^{2+}-channels are involved in the ACPA-induced synaptic inhibition

To determine whether ACPA decreases transmitter release by a mechanism involving inhibition of the presynaptic N-type Ca^{2+}-channels, we explored the effects of adding 1 μM of ACPA, which reduced MEPPs frequency by 63.1 % ± 2.9 %, after blocking the N-type Ca^{2+}-channels with ω-CgTX with maximal sub-threshold concentrations (1 and 5 μM). Thirty minutes after the addition of ω-CgTX, we recorded MEPPs for 15 minutes, then added the ACPA to the bath and

recorded MEPPs for 30 minutes more. With the N-type Ca^{2+}-channels blocked (ω-CgTX 5 μM), which reduced MEPPs frequency by 67.9 % ± 5.2 %, ACPA did not additionally decrease MEPP frequency (99.6 percent ± 2.05 percent). However, MEPP amplitude was still diminished (25.4 percent ± 3.5 percent) by ACPA (Figure 6 B, C), suggesting a postsynaptic site of action for this cannabinoid agonist. When we used 1 μM ω-CgTX, which reduced MEPPs frequency by 29.6 % ± 1.0 %, an additional decrease in the MEPPs frequency by 60 % in the presence of ACPA (1 μM) was observed (Fig. 6A). These results suggest that ACPA decreased spontaneous transmitter release by blocking the presynaptic N-type Ca^{2+} channels. However we do not discard that ACPA has a presynaptic inhibition of amount of Ach released.

Discussion

The present results show that the cannabinoid WIN over a concentration range of 1 to 50 μM (the WIN EC_{50} value was 5.8 ± 1.0 μM), decreased neurotransmitter release in a concentration-dependent manner at the neuromuscular junction of the frog *cutaneous pectoris* muscle by decreasing the frequency of the MEPPs. Activated cannabinoid receptors interact with $G_{i/o}$ proteins, which can be blocked by PTX (Bokoch *et al.,* 1983). Pre-treating the muscles with PTX (2 μg/ml) before adding WIN caused WIN to have no significant effect on the MEPPs, indicating that WIN acts through $G_{i/o}$-protein-coupled receptors. In contrast, because WIN has similar affinity for CB_1 and CB_2 receptors (Howlett *et al.,* 2002), to determine which of these receptors are activated by WIN, we used selective antagonists for each type of cannabinoid receptor. In the presence of the CB_2 antagonist AM630 (1 μM) (Ross *et al.,* 1999), WIN had a similar effect to that observed in the absence of AM630, indicating that the CB_2 receptors are not involved in the effects observed after addition of WIN. However, the presence of the CB_1-selective antagonist AM281 (1 μM) (Howlett *et al.,* 2002) blocked the effects of WIN on the frequency and amplitude of the MEPPs, indicating that WIN acts through activation of the CB_1 receptors. These results are accord with only the CB_1 receptor ortholog having reported in amphibians (Soderstrom, 2000; McPartland *et al.,* 2006).

To confirm that cannabinoids inhibit neurotransmitter release in the frog neuromuscular junction by their interaction with CB_1 receptors, we also used the

synthetic CB_1-selective agonist ACPA (the ACPA EC_{50} value was 115.5 ± 6.5 nM), analogous to the endocannabinoid anandamide (Hillard *et al.,* 1999), which diminished both the amplitude and frequency of the MEPPs in a concentration-dependent manner. WIN and ACPA reached the peak effect within 15 min. Similar results have been reported (Turkanis and Karler, 1986). However, more time-effects studies are necessary to know this mechanism. These effects were more pronounced than those observed in the presence of WIN, confirming that ACPA is a more potent and efficient cannabinoid receptor agonist than WIN. A possible cause of the lower potency of WIN versus ACPA could be that: 1) the amphibian CB_1 receptor lacks some amino acid residues necessary for the binding of WIN and 2) for its efficacious functional coupling of the receptor to the G protein. Furthermore, it has been suggested that 3) WIN binds to an uncharacterized non-CB_1 cannabinoid receptor (Breivogel *et al.,* 2001).

Moreover, it has been reported that the different efficacies and potencies for anandamide (ACPA is analogous to anandamide) and WIN, depend on sensitivity to differences in receptor density, cell background and/or receptor/G-protein stoichiometry (Huang *et al.* 2005). The ACPA binding site is at residue 192 and the WIN binding site will be next to this site, it would be in the third transmembrane domain of CB1 (Song and Bonner, 1996). With respect to the WIN concentration used in this study, similar concentrations (10 μM) have been used in studies of synaptic transmission in the cerebellar cortex of rats (Szabo *et al.,* 2004) and an

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IC_{50} 4.6 μM has been used in studies of glutamatergic transmission in rat brain stems (Liang *et al.,* 2004).

The same results were obtained in the experiments with WIN: the treatment of *cutaneous pectoris* muscles with PTX to block $G_{i/o}$ proteins inhibited the effects of ACPA on MEPP frequency and amplitude, indicating that ACPA acts by activating G-protein-coupled receptors. However, adding AM281 before adding ACPA blocked the agonist effects of ACPA, suggesting the functional presence of CB_1 cannabinoid receptors in the neuromuscular junction of the frog. These data indicate that ACPA diminish spontaneous transmitter release in the frog skeletal muscle neuromuscular junction by activating presynaptic CB_1 cannabinoid receptors. A previous study has reported the presynaptic location of cannabinoid receptors (Schlicker and Kathmann, 2001). This diminution in transmitter release could generate, as a consequence, a subliminal plate potential as was reported by Kumbaraci and Nastuk (1980) using Δ^9-tetrahydrocannabinol (0.3 to 30 μM). In this way, action potential cannot be generated.

On the other hand, it is known that the Ca^{2+} entry through voltage-dependent Ca^{2+} channels is essential for transmitter release at the presynaptic end. To address this issue, we have used blockers for L- and N- type Ca^{2+}-channels, which are present in the frog motor end nerve (Kerr and Yoshikami, 1984; Robitaille *et al.,* 1993). The L-type Ca^{2+}-channel blocker nifedipine (10 μM) had no effect on the frequency or amplitude of the MEPPs, indicating that this type

of Ca^{2+} channel does not participate in the entry of Ca^{2+} into the presynaptic cell, which is needed for spontaneous transmitter release. However, the N-type Ca^{2+}-channel blocker ω-CgTX caused a rapid diminution of MEPP frequency in a concentration-dependent manner, suggesting that Ca^{2+} entry occurs through this type of Ca^{2+}-channel and that these channels are involved in spontaneous transmitter release in the frog neuromuscular junction. These results are agree with previous data reported by Grinnell and Pawson (1989). The amplitude of MEPPs was not affected by ω-CgTX in all concentrations, although the frequency decreased by blocking the N-type of Ca^{2+} channels with ω-CgTX 5 μM before addition of ACPA, it had not additional inhibition effect on the frequency of the MEPPs. However, MEPPs amplitude was diminished; while, in the presence of ω-CgTX 1 μM, ACPA whether had additional inhibition effect.

These data suggest that 1) ACPA and WIN decreased transmitter release by blocking the presynaptic N-type Ca^{2+} channels. The effect of ACPA on MEPP amplitude is a postsynaptic effect, suggesting the presence of postsynaptic CB_1 cannabinoid receptors. We have reported the presence of mRNA for CB_1 in frog skeletal muscle fibers (Sanchez-Pastor *et al.,* 2004). Cannabinoid receptors are frequently located presynaptically, but also occur postsynaptically, *e.g.* in the hippocampus (Schweitzer, 2000). Another possibility is that ACPA acts presynaptically inhibiting the Ach release as diminishing the quantal content of vesicles, therefore the amplitude of MEPPs should be diminished. However, reduction in the amplitude occurs within a few minutes, motor end nerve was not

electrically stimulated. Therefore, the turnover of the neurotransmitter is minimal; thus, the presynaptic effect of the drug on the neurotransmitter synthesis is unlikely (Hubbard, Llinás and Quastel, 1969).

To explain these postsynaptic effects, one possibility is that cannabinoids might act in form similarly to how 5-hydroxytryptamine acts on the nicotinic acetylcholine (ACh) response diminution in the currents (mediated by acetylcholine receptors) via a mechanism involving one or more G-proteins (Butt and Pitman, 2002). Recently, the direct action of anandamide, but not WIN, on neuron nAChR were reported in *Xenopus* oocytes expressing α_7-ACh receptors (Oz, 2006). However, Nojima (2000), using arachidonic acid and prostaglandin D2, cooperatively accelerated desensitization of the nAChR channel in mouse skeletal muscle. Those effects were inhibited by staurosporine, an inhibitor of the protein kinase C. In this study, the experiments were performed on intact fast muscle fibers, which permit maintaining all cannabinoid signaling systems in the intracellular media.

Another possibility is that the cannabinoids modified the membrane input resistance and membrane time-constant at the junctional region. However, more work is needed to elucidate the mechanism responsible for the effects of the cannabinoid agonists on the amplitude of the MEPPs. Finally, the functional significance of the cannabinoid receptors in muscle could be the modulation of tension.

Acknowledgments

We wish to thank to Mr. Ezequiel Viera for technical assistance.

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Footnotes

This work was supported, in part by grant Consejo Nacional de Ciencia y Tecnología (CONACyT-México 43446-M) to MH and grant FRABA-03 (Ramón Álvarez Buylla-México) to MH and XT. ESP is in receipt of a CONACyT-México fellowship.

Legends for Figures

Figure 1. (A) The effect of WIN (10 μM) on the amplitude of the MEPPs. WIN attenuated MEPP amplitude by 10.6% ± 2.9% compared with control conditions (compare first *vs*. second columns). PTX pre-treatment (2 μg/ml, 22–24 hours) and the CB_1 antagonist AM281 (1 μM) reversed the effect of WIN (third column). The CB_2 antagonist AM630 (1 μM) had no additional effect on the inhibition by WIN on MEPPs (see fifth column). (B) Effect of WIN (10 μM) on the frequency of MEPPs. WIN decreased MEPP frequency of compared with control conditions (first *vs.* second columns). Pre-treatment with PTX and the CB_1 antagonist AM281 (1 μM) reversed the effect of WIN (third and fourth columns). The CB_2 antagonist AM630 (1 μM) had no effect on the inhibition of MEPPs by WIN (fifth column). Data are presented as mean ± S. E. M. Asterisks indicate $P < 0.05$ compared with the control condition.

Figure 2. Dose-response curve for the synthetic cannabinoid WIN. The EC_{50} for the inhibition of MEPP frequency by WIN was 5.8 ± 1.0 μM.

Figure 3. Dose-response curve for the selective agonist of CB_1 receptor ACPA. The EC_{50} for inhibition of MEPP frequency by ACPA was 115.5 ± 6.5 nM.

Figure 4. (A) Effects of the CB_1 agonist ACPA (1 μM) on MEPP frequency. ACPA decreased MEPP frequency. Data are presented as means ± S.E.M. Asterisks indicate $P < 0.05$ compared with the control condition (before ACPA administration) (paired Student´s t-test). (B) Cumulative probability plots of the MEPPs before (continuous line) and during (dashed line) application of ACPA (1 μM) is shown. ACPA attenuated MEPP amplitude by 35.2% ± 10.1% 30 minutes after application (n = 5, $P < 0.001$, Kolmogorov–Smirnov test). The inset shows original MEPPs recorded at time 0 and 30 minutes after the addition of ACPA.

Figure 5. (A) Dose-response curve for the N-type Ca^{2+}-channel blocker ω-CgTX. The EC_{50} for the inhibition of MEPPs frequency by ω-CgTX was 2.5 ± 0.48 μM. (B) Cumulative probability plots of the MEPPs before and during application of ω-CgTX (5 μM). ω-CgTX caused no significant changes in MEPP amplitude (dashed line) (Kolmogorov-Smirnov test). The inset shows original MEPPs recorded at time 0 and 30 minutes after the addition of ω-CgTX.

Figure 6. (A) Blocking the N-type Ca^{2+}-channels with ω-CgTX (1 μM) for 45 minutes before adding ACPA (1 μM) did not inhibit additional the effect of ACPA on MEPPs frequency. (B) Blocking the N-type Ca^{2+}-channels with ω-CgTX (5 μM) for 45 minutes before adding ACPA (1 μM) did not inhibit the effect of ACPA on MEPP amplitude. In the presence of ACPA (1 μM), MEPP amplitude was reduced by 25.4% ± 3.5% after 30 minutes ($P = 0.002$, n = 4). (C) Cumulative probability plots

JPET Fast Forward. Published on January 31, 2007 as DOI: 10.1124/jpet.106.116319
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of the MEPPs before and during application of ω-CgTX (5 μM). ω-CgTX caused not significant change in MEPP amplitude (dashed line) (P > 0.05, Kolmogorov-Smirnov test). The inset shows original MEPPs recorded at time 0 and 30 minutes after the addition of ACPA, being the N-type Ca^{2+}-channels blocked by ω-CgTX.

Figure 1

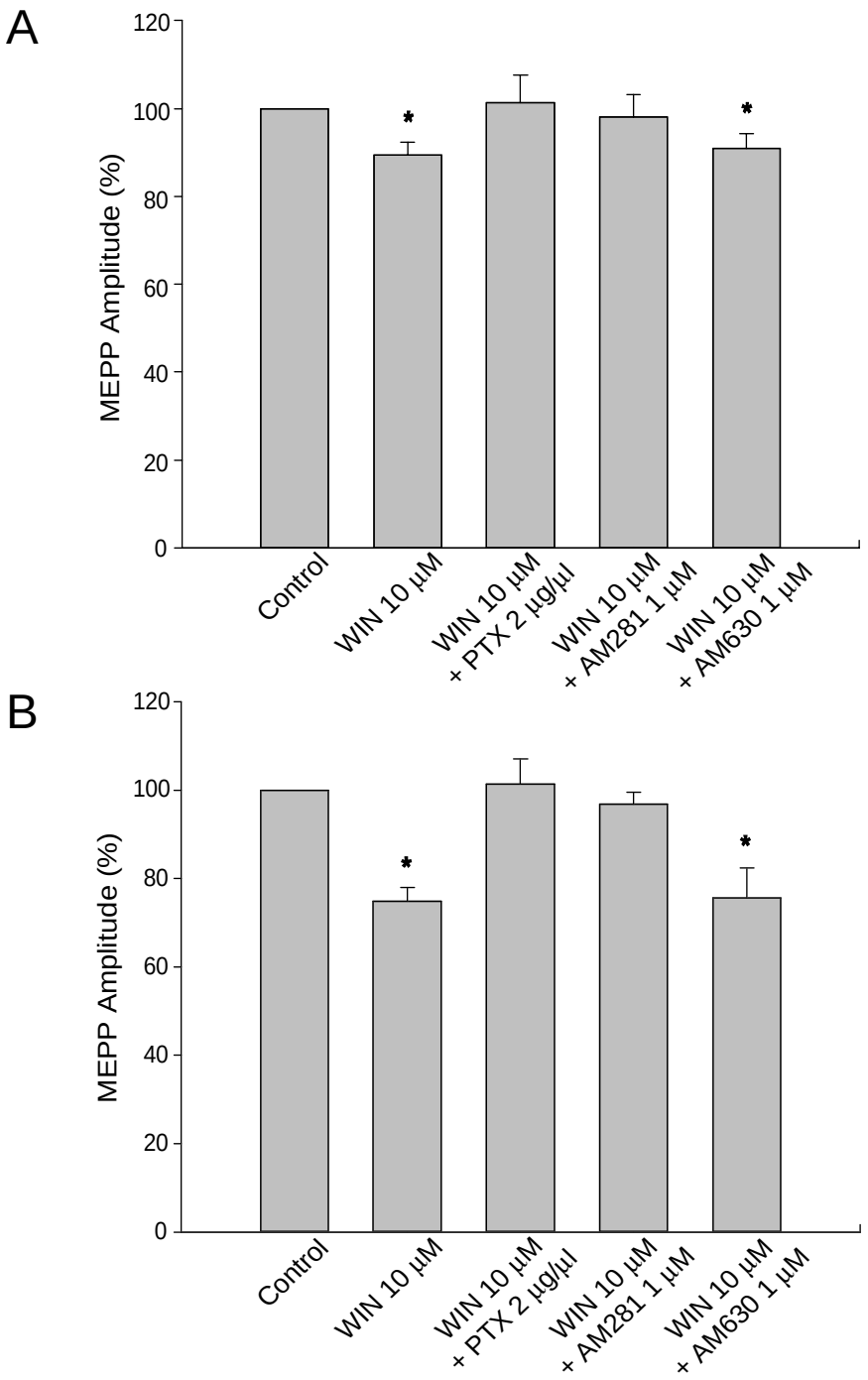

Figure 2

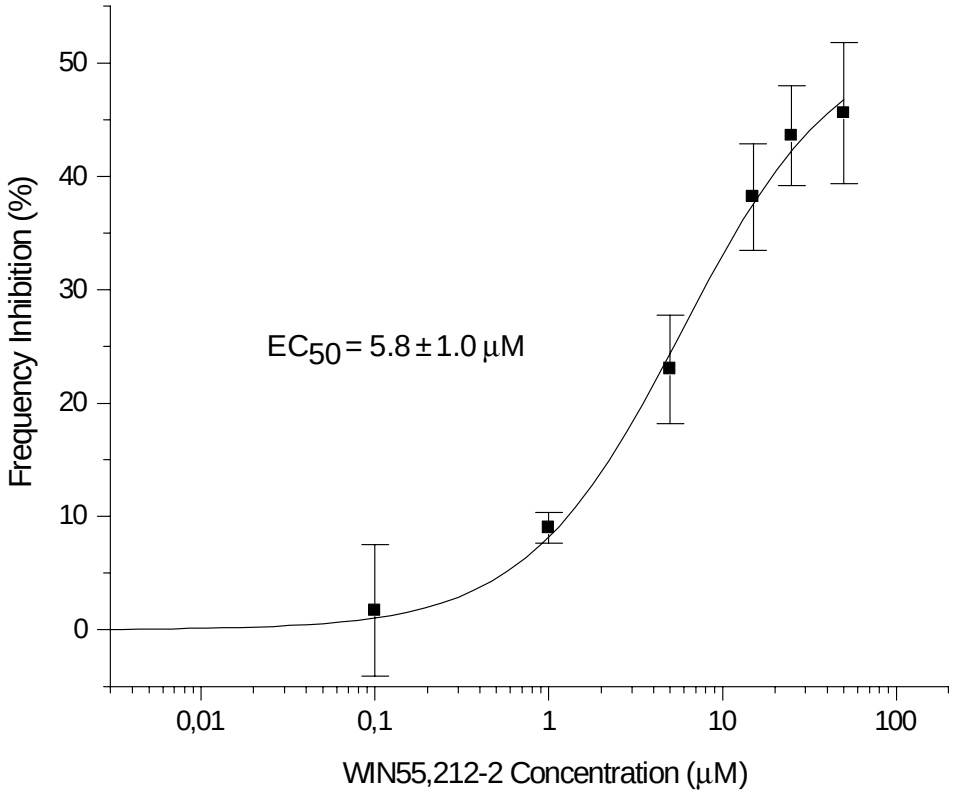

Figure 3

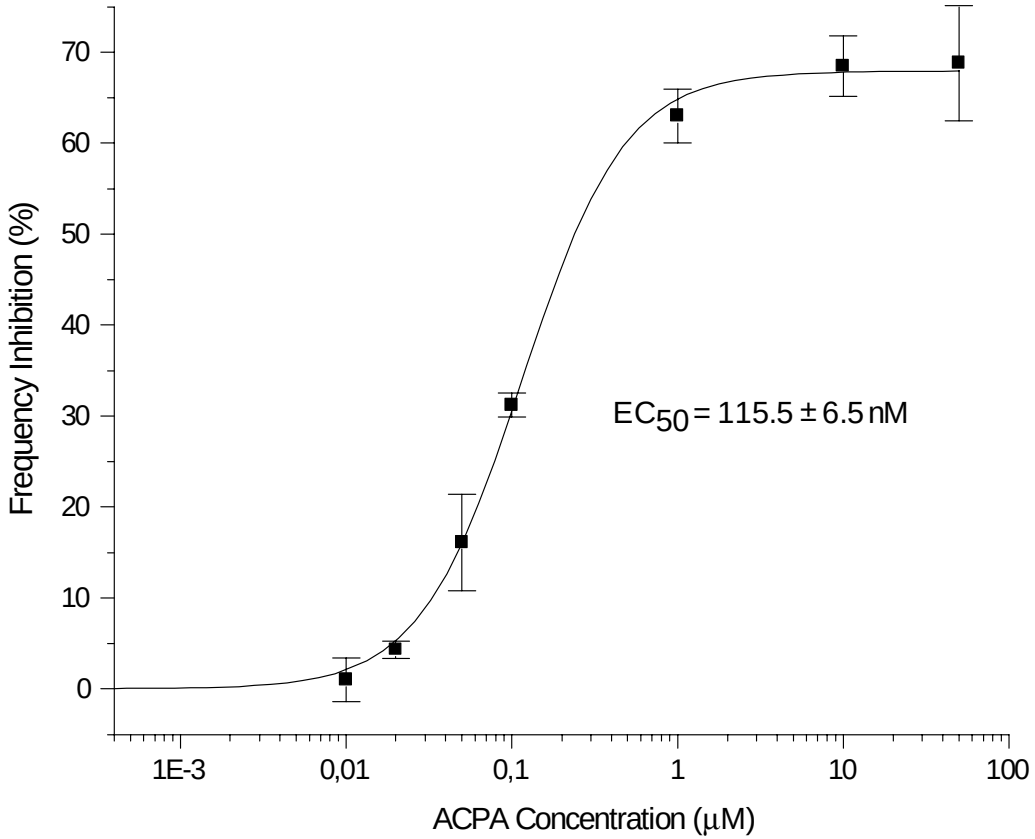

Figure 4

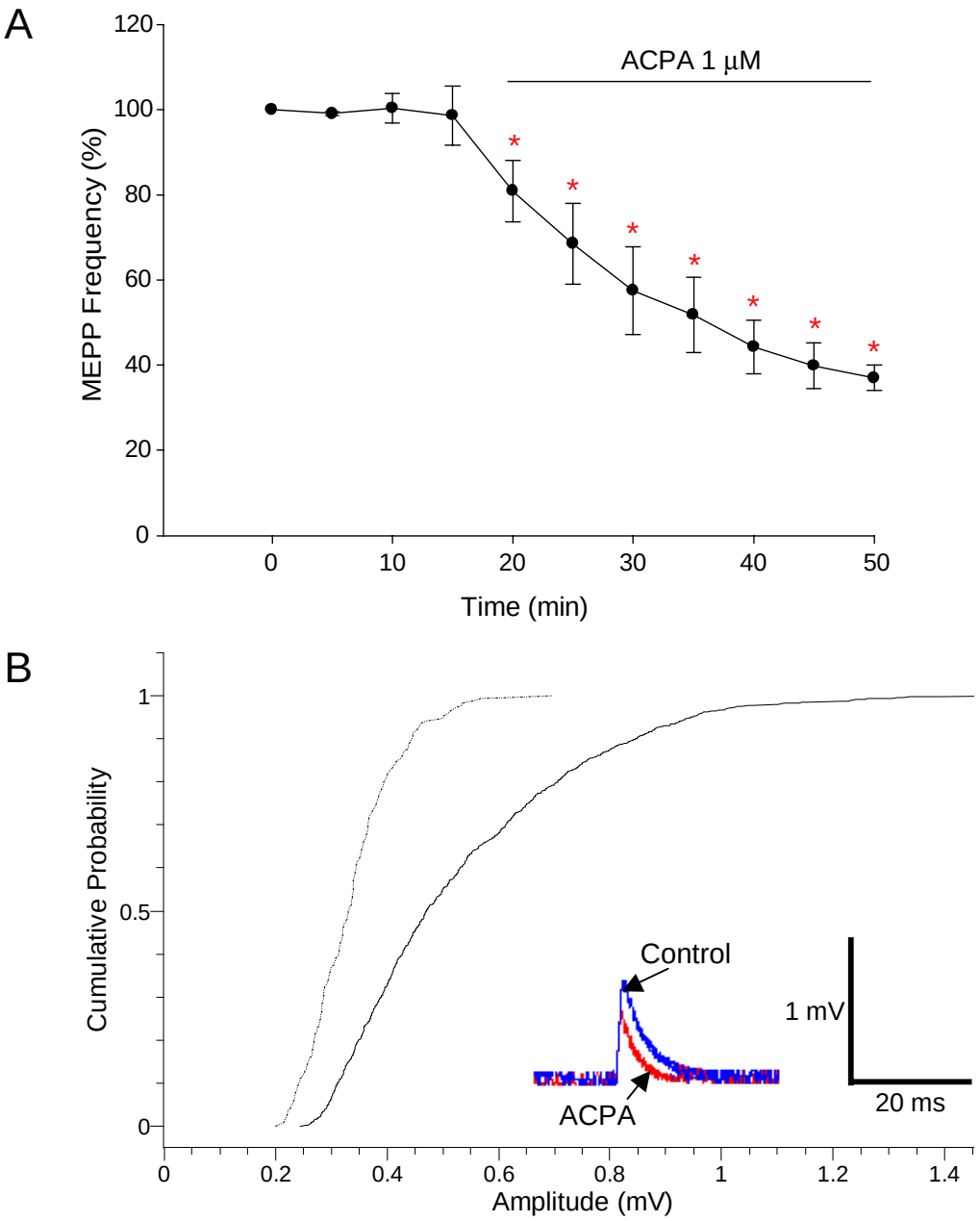

Figure 5

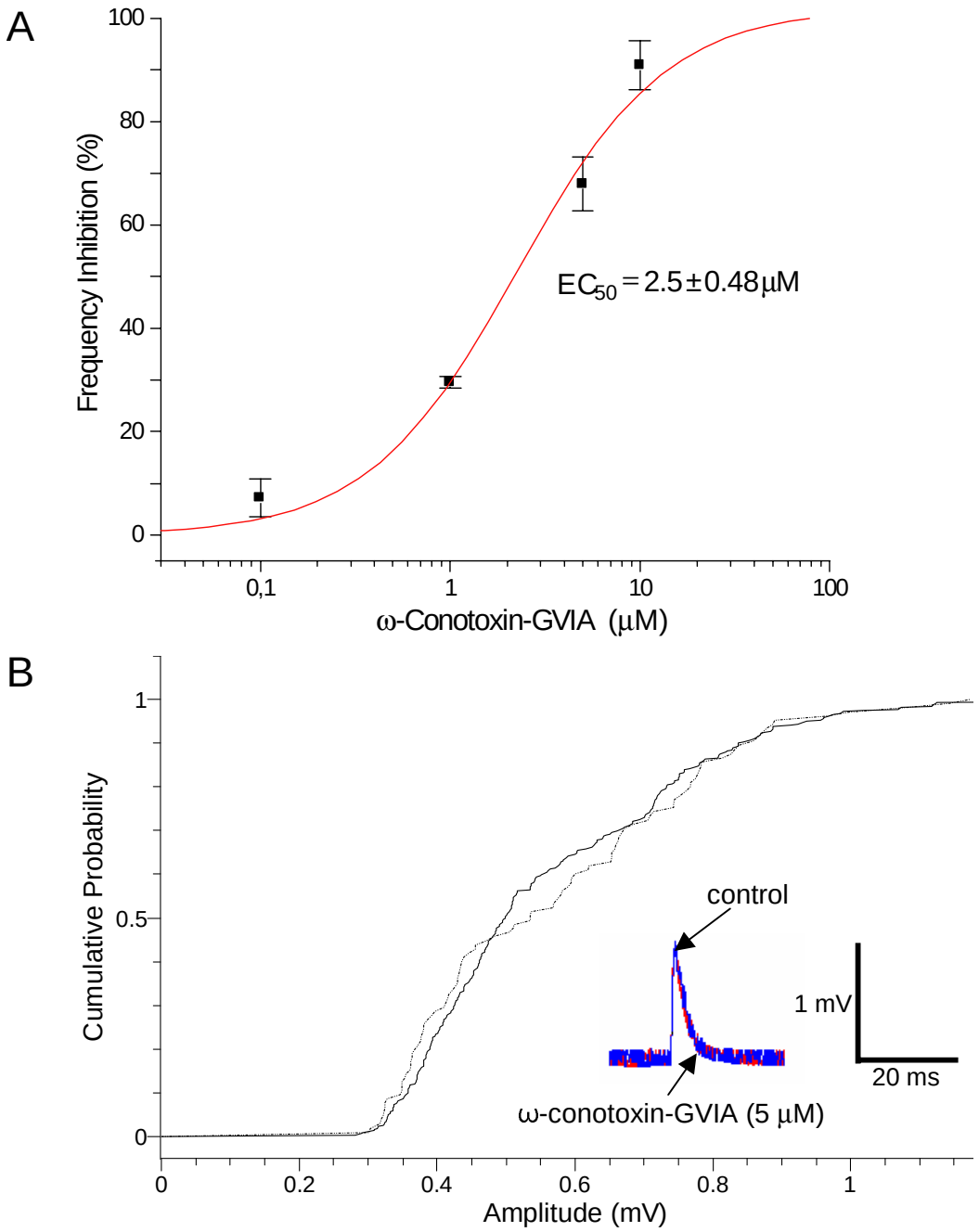

Figure 6