Effects of Cannabinoids on Synaptic Transmission in the Frog Neuromuscular Junction

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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 WIN55212-2 (WIN; R-[-2,3-dihydro-5-methyl-3-[(morpholino)]-pyrrolo[1,2,3-de]-1,4-benzoazinyl]-1-naphthalenyl)methanone) and arachidonylcyclopropylamide (ACPA; N-(2-cyclopropyl)-5Z,8Z,11Z,14Z-eicosatetraenamide) and the cannabinoid antagonists 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1H-pyrazole-3-carboxamide (AM281) and 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl[4-methoxyphenyl)methanone (AM630). Adding WIN to the external medium decreased the frequency and amplitude of the miniature end-plate potentials (MEPPs); the WIN EC50 value was 5.8 ± 1.0 μM. Application of ACPA, a selective agonist of cannabinoid receptor CB1, also decreased the frequency of the MEPPs; the ACPA EC50 value was 115.5 ± 6.5 nM. The CB2 antagonist AM630 did not inhibit the effects of WIN, indicating that its action is not mediated through the CB2 receptor. However, the CB1 antagonist AM281 inhibited the effects of WIN and ACPA, suggesting that their actions are mediated through the CB1 receptor. Pretreatment with the pertussis toxin inhibited the effects of WIN and ACPA, suggesting that their effects are mediated through Gi/o protein activation. The N-type Ca2+ channel blocker ω-conotoxin GVIA (ω-CgTX) diminished the frequency of the MEPPs, with an ω-CgTX EC50 value of 2.5 ± 0.40 μM. Blocking the N-type Ca2+ channels with 5 μM ω-CgTX before addition of ACPA to the bath had no additional inhibitory effect on the MEPPs, whereas in the presence of 1 μM ω-CgTX, ACPA had an additional inhibition effect. These results suggest that cannabinoids modulate transmitter release in the end-plate of the frog neuromuscular junction by activating CB1 cannabinoid receptors in the nerve ending.

Cannabinoids, the active components of marijuana (Cannabis sativa) cause psychoactive and motor effects when the plant is consumed. These effects are produced by the interaction of these compounds with the cannabinoid membrane receptors named CB1 and CB2 (Howlett et al., 2002). Both subtypes of cannabinoid receptors alter cellular activity through activation of G proteins of the Gs subtype, which are sensitive to pertussis toxin (Soderstrom et al., 2000), although some effects are thought to occur through Gαs (Bayewitch et al., 1995; Glass and Felder, 1997). Activation of Gs proteins by CB1 and CB2 receptors decreases adenylyl cyclase activity, leading to decreased levels of intracytoplasmic 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 CB1, but not CB2, is negatively coupled through Gsα 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, emerged early in evolution, and conservation of the CB1 receptor seems to be restricted to vertebrate species. Therefore, in amphibians, only the CB1 receptor ortholog has been reported to date (Soderstrom et al., 2000; McPartland et al., 2006). The endocannabinoids (endogenous cannabis-like substances) are fatty acids derived from arachidonic acid, and they notably include anandamide and 2-arachidonylglycerol. Once anandamide and

ABBREVIATIONS: CB, cannabinoid; MEPP, miniature end-plate potential; ω-CgTX, ω-conotoxin GVIA; WIN, WIN55,212-2, R-[-2,3-dihydro-5-methyl-3-[(morpholino)]-pyrrolo[1,2,3-de]-1,4-benzoazinyl]-1-naphthalenyl)methanone; ACPA, arachidonylcyclopropylamide [N-(2-cyclopropyl)-5Z,8Z,11Z,14Z-eicosatetraenamide]; AM281, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1H-pyrazole-3-carboxamide; AM630, 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl[4-methoxyphenyl)methanone; PTX, pertussis toxin; ACh, acetylcholine.
2-arachidonoylglycerol are formed, in some cases they target the CB₁ 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 past 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 (Iₖ) or by blocking voltage-dependent Ca²⁺ 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 Δ⁹-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, whereas Turkanis and Karler (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.

In contrast, 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.

Materials and Methods

The experiments were performed on the neuromuscular junction of the cutaneous pectoris muscle of R. pipiens. Frogs were used in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals. Frogs were sacrificed by decapitation and demedulated. The nerve and muscle were dissected, pinned on a Sylgard-coated dish (WPI, Sarasota, FL), and bathed in normal frog Ringer’s solution consisting of 117 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, and 2 mM 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) filled with 3 M KCl gave 10- to 20-MΩ resistance. Stock solutions for each drug (see “Drug Preparations”) were added to the bath solution by a three-way changer, we first used the cannabinoid agonist WIN, which binds to both types of cannabinoid receptors. Adding this agonist (0.1–50 μM) to the external media decreased both the frequency and amplitude of the MEPPs compared with control conditions. Figure 1 shows that adding 10 μM WIN to the bath reduced the amplitude of the MEPPs by 10.6 ± 2.9% and reduced their frequency by 25.3 ± 3.2% in all five cells tested (Fig. 1, A and B). Maximal inhibition by WIN was reached 25 to 30 min after its application (Fig. 1), and this effect was sustained for at least 15 min 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 (Gₐ and Gᵢ) (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 2 μg/ml PTX pretreatment for 22 to 24 h 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 CB₁ or CB₂ could reverse the effect of WIN. Adding the CB₁ receptor antagonist AM630, and AM281 (Tocris Cookson Inc., Ellisville, MO)—was prepared as a 0.5 mg/ml stock solution in water. Stock solutions were stored at 20°C. The L-type Ca²⁺ channel blocker nifedipine (Sigma-Aldrich) was dissolved in ethanol to make a 10 mM stock solution in dimethyl sulfoxide or ethanol (diluent concentration was <0.01%; Sigma-Aldrich, St. Louis, MO). Stock solutions were stored at −20°C. The L-type Ca²⁺ channel blocker nifedipine (Sigma-Aldrich) was dissolved in ethanol to make a 100 mM stock solution. The N-type Ca²⁺ channel blocker ω-CgTX (Sigma-Aldrich) was prepared as a 0.5 mg/ml stock solution in water. Experiments using ACPA and nifedipine were performed in the dark.

Pretreatment with PTX. PTX (Sigma-Aldrich) was reconstituted as a 50 μg/ml stock solution with 5 mg/ml bovine serum albumin (Sigma-Aldrich) in water. The muscle was incubated with 2 μg/ml PTX in normal frog Ringer’s for 22 to 24 h 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 (OriginLab Corp., Northampton, MA), and the data were adjusted to a Hill equation of the type I = Iₘₐₓ/(1 + (EC₅₀/x)ⁿ).

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 (0.1–50 μM) to the external media decreased both the frequency and amplitude of the MEPPs compared with control conditions. Figure 1 shows that adding 10 μM WIN to the bath reduced the amplitude of the MEPPs by 10.6 ± 2.9% and reduced their frequency by 25.3 ± 3.2% in all five cells tested (Fig. 1, A and B). Maximal inhibition by WIN was reached 25 to 30 min after its application (Fig. 1), and this effect was sustained for at least 15 min after washout (data not shown).

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antagonist AM281 at 1 μM reversed the inhibitory effect of WIN on the frequency of the MEPPs was 1 μM, and the maximal effect was reached with 50 μM WIN. Each point in the curve (Fig. 2) corresponds to the frequency averaged from five experiments. Data were adjusted to a Hill equation, and the EC50 value for the inhibition of the MEPPs frequency by WIN was 5.8 ± 1.0 μM. The I_max value calculated by the Hill equation was 52.68 ± 3.26%, and the Hill coefficient was 0.96 ± 0.11.

Inhibitory Effects of ACPA on Spontaneous Transmitter Release. We next examined whether the synthetic CB1-selective agonist ACPA, which is analogous to the endocannabinoid 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 equa-

Fig. 1. A, effect of 10 μM WIN on the amplitude of the MEPPs. WIN attenuated MEPP amplitude by 10.6 ± 2.9% compared with control conditions (compare first versus second columns). Pretreatment with 2 μg/ml PTX for 22 to 24 h and 1 μM CB1 antagonist AM281 reversed the effect of WIN (third column). The CB2 antagonist AM630 at 1 μM had no additional effect on the inhibition by WIN on MEPPs (see fifth column). B, effect of 10 μM WIN on the frequency of MEPPs. WIN decreased MEPP frequency compared with control conditions (first versus second columns). Pretreatment with PTX and the CB2 antagonist AM281 at 1 μM reversed the effect of WIN (third and fourth columns). The CB2 antagonist AM630 at 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.

Fig. 2. Dose-response curve for the synthetic cannabinoid WIN. The EC50 for the inhibition of MEPP frequency by WIN was 5.8 ± 1.0 μM.

Fig. 3. Dose-response curve for the selective agonist of CB1 receptor ACPA. The EC50 for inhibition of MEPP frequency by ACPA was 115.5 ± 6.5 μM.
tion, and the EC_{50} value for the inhibition by ACPA of the frequency of the MEPPs was 115.5 ± 6.5 nM. The I_{max} value calculated by the Hill equation was 67.94 ± 0.78%, and the Hill coefficient was 1.40 ± 0.10.

Adding 1 μM ACPA to the bath diminished both the mean frequency and amplitude of the MEPPs compared with control conditions (Fig. 4, A and B). Maximal inhibition was reached 30 min after its application, when the mean inhibition of MEPP frequency was 63.0 ± 2.9%. ACPA decreased mean MEPP amplitude 35.2% ± 10.1%. Figure 4 shows that ACPA significantly decreased the cumulative amplitude distribution compared with the control condition (Fig. 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 2 μg/ml PTX for 22 to 24 h. ACPA at 1 μM had no effect on the frequency or amplitude of the MEPPs. The mean frequency of MEPPs after 30-min exposure to the presence of ACPA was 100.8 ± 3.6% 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 CB1 receptor antagonist AM281 at 1 μM 5 min before adding ACPA did not reduce MEPP frequency or amplitude. Mean MEPP frequency was 95.9 ± 4.1%, and mean MEPP amplitude was 95.2 ± 3.3%; these results were not statistically significant. Likewise, the cumulative amplitude did not differ significantly from control after 30 min in the presence of ACPA. These results suggest that ACPA activates pre- and postsynaptic CB1 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 End-Plate 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 min in the presence of 10 μM nifedipine, mean MEPP frequency was 103.7 ± 5.8%, and mean MEPP amplitude was 98.6 ± 5.1%. These data indicate that L-type Ca^{2+} channels do not participate in spontaneous transmitter release and that they do not affect acetylcholine receptors in the postsynaptic membrane. In contrast, Fig. 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

![Fig. 4. A, effects of the CB1 agonist ACPA at 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 1 μM ACPA is shown. ACPA attenuated MEPP amplitude by 35.2 ± 10.1% 30 min after application (n = 5; P < 0.001, Kolmogorov-Smirnov test). The inset shows original MEPPs recorded at time 0 and 30 min after the addition of ACPA.](https://example.com/fig4)

![Fig. 5. A, dose-response curve for the N-type Ca^{2+} channel blocker ω-CgTX. The EC_{50} value 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 5 μM ω-CgTX. 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 min after the addition of ω-CgTX.](https://example.com/fig5)
we used 1 postsynaptic site of action for this cannabinoid agonist. When however, the presence of the CB1-selective antagonist are not involved in the effects observed after addition of WIN.

To determine whether ACPA decreases transmitter release by a mechanism involving inhibition of the presynaptic N-type Ca\textsuperscript{2+} channels, we explored the effects of adding 1 \mu M ACPA, which reduced MEPPs frequency by 63.1 \pm 2.9%, after blocking the N-type Ca\textsuperscript{2+} channels with \omega-CgTX with maximal subthreshold concentrations of 1 and 5 \mu M. Thirty minutes after the addition of \omega-CgTX, we recorded MEPPs for 15 min, and then we added the ACPA to the bath and recorded MEPPs for an additional 30 min. With the N-type Ca\textsuperscript{2+} channels blocked (5 \mu M \omega-CgTX), which reduced MEPPs frequency by 67.9 \pm 5.2%, ACPA did not additionally decrease MEPP frequency (99.6 \pm 2.05%). However, MEPP amplitude was still diminished (25.4 \pm 3.5%) by ACPA (Fig. 6, B and C), suggesting a postsynaptic site of action for this cannabinoid agonist. When we used 1 \mu M \omega-CgTX, which reduced MEPPs frequency by 29.6 \pm 1.0%, an additional decrease in the MEPPs frequency by 60% in the presence of 1 \mu M ACPA was observed (Fig. 6A). These results suggest that ACPA decreases spontaneous transmitter release by blocking the presynaptic N-type Ca\textsuperscript{2+} channels. However, we do not discard that ACPA has a postsynaptic inhibition of the amount of acetylcholine (ACh) released.

**Discussion**

The present results show that the cannabinoid WIN over a concentration range of 1 to 50 \mu M (the WIN EC\textsubscript{50} value was 5.8 \pm 1.0 \mu M), 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\textsubscript{i,0} proteins, which can be blocked by PTX (Bokoch et al., 1983). Pretreating the muscles with 2 \mu g/ml PTX before adding WIN caused WIN to have no significant effect on the MEPPs, indicating that WIN acts through G\textsubscript{i,0} protein-coupled receptors. In contrast, because WIN has similar affinity for CB\textsubscript{1} and CB\textsubscript{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\textsubscript{2} antagonist 1 \mu M AM630 (Ross et al., 1999), WIN had a similar effect to that observed in the absence of AM630, indicating that the CB\textsubscript{2} receptors are not involved in the effects observed after addition of WIN.

However, the presence of the CB\textsubscript{1}-selective antagonist AM281 at 1 \mu M (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\textsubscript{1} receptors. These results are accord with only the CB\textsubscript{1} receptor ortholog

\omega-CgTX on the frequencies of the MEPPs was 0.1 \mu M, and the mean of the maximum effect was reached with 10 \mu M \omega-CgTX. Data were adjusted to the Hill equation, and the EC\textsubscript{50} value for the inhibition of the MEPPs frequency with \omega-CgTX was 2.5 \pm 0.48 \mu M. The \textit{I} \textsubscript{max} value calculated by the Hill equation was 102 \pm 4.72%, and the Hill coefficient was 1.02 \pm 0.16. These results indicate that the Ca\textsuperscript{2+} current through the N-type Ca\textsuperscript{2+} channels is involved in spontaneous transmitter release in our preparation. Alternatively, \omega-CgTX had no significant effect on the cumulative amplitude, Fig. 5B shows an example when we used 5 \mu M \omega-CgTX.

**N-Type Ca\textsuperscript{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\textsuperscript{2+} channels, we explored the effects of adding 1 \mu M ACPA, which reduced MEPPs frequency by 63.1 \pm 2.9%, after blocking the N-type Ca\textsuperscript{2+} channels with \omega-CgTX with maximal subthreshold concentrations of 1 and 5 \mu M. Thirty minutes after the addition of \omega-CgTX, we recorded MEPPs for 15 min, and then we added the ACPA to the bath and recorded MEPPs for an additional 30 min. With the N-type Ca\textsuperscript{2+} channels blocked (5 \mu M \omega-CgTX), which reduced MEPPs frequency by 67.9 \pm 5.2%, ACPA did not additionally decrease MEPP frequency (99.6 \pm 2.05%). However, MEPP amplitude was still diminished (25.4 \pm 3.5%) by ACPA (Fig. 6, B and C), suggesting a postsynaptic site of action for this cannabinoid agonist. When we used 1 \mu M \omega-CgTX, which reduced MEPPs frequency by 29.6 \pm 1.0%, an additional decrease in the MEPPs frequency by 60% in the presence of 1 \mu M ACPA was observed (Fig. 6A). These results suggest that ACPA decreases spontaneous transmitter release by blocking the presynaptic N-type Ca\textsuperscript{2+} channels. However, we do not discard that ACPA has a postsynaptic inhibition of the amount of acetylcholine (ACh) released.

**Fig. 6.** A, blocking the N-type Ca\textsuperscript{2+} channels with 1 \mu M \omega-CgTX for 45 min before adding 1 \mu M ACPA did not inhibit the additional effect of ACPA on MEPPs frequency. B, blocking the N-type Ca\textsuperscript{2+} channels with 5 \mu M \omega-CgTX for 45 min before adding 1 \mu M ACPA did not inhibit the effect of ACPA on MEPP amplitude. In the presence of 1 \mu M ACPA, MEPP amplitude was reduced by 25.4 \pm 3.5% after 30 min (\textit{P} = 0.002; \textit{n} = 4). C, cumulative probability plots of the MEPPs before and during application of 5 \mu M \omega-CgTX. \omega-CgTX caused not significant change in MEPP amplitude (dashed line) (\textit{P} > 0.05, Kolmogorov-Smirnov test). The inset shows original MEPPs recorded at time 0 and 30 min after the addition of ACPA, with the N-type Ca\textsuperscript{2+} channels blocked by \omega-CgTX.

To confirm that cannabinoids inhibit neurotransmitter release in the frog neuromuscular junction by their interaction with CB\textsubscript{2} receptors, we also used the synthetic CB\textsubscript{2}-selective agonist ACPA (the ACPA EC\textsubscript{50} value was 115.5 \pm 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 clarify this mechanism. These effects were more pronounced than

having reported in amphibians (Soderstrom et al., 2000; McPartland et al., 2006).

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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 the amphibian CB$_1$ receptor lacks some amino acid residues necessary for the binding of WIN and for its efficacious functional coupling of the receptor to the G protein. Furthermore, it has been suggested that 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 CB$_1$ (Song and Bonner, 1996). With respect to the WIN concentration used in this study, similar concentrations (10 $\mu$M) have been used in studies of synaptic transmission in the cerebellar cortex of rats (Szabo et al., 2004), and an IC$_{50}$ value of 4.6 $\mu$M 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 Kanthamm, 2001). This diminution in transmitter release could generate, as a consequence, a subliminal plate potential as was reported by Kumbaraci and Nastuk (1980) using 0.3 to 30 $\mu$M $\Delta^8$-tetrahydrocannabinol. In this way, action potential cannot be generated.

Furthermore, 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 at 10 $\mu$M 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 $\omega$-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 in agreement with previous data reported by Grinnell and Pawson (1989). The amplitude of MEPPs was not affected by $\omega$-CgTX at all concentrations, and although the frequency decreased by blocking the N-type of Ca$^{2+}$ channels with $\omega$-CgTX at 5 $\mu$M before addition of ACPA, it had no additional inhibition effect on the frequency of the MEPPs. However, MEPPs amplitude was diminished, whereas, in the presence of 1 $\mu$M $\omega$-CgTX, ACPA had an additional inhibition effect.

These data suggest that 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$_1$ receptors. We have reported the presence of mRNA for CB$_1$ in frog skeletal muscle fibers (Sánchez-Pastor et al., 2004). Cannabinoid receptors are frequently located presynaptically, but they also occur postsynaptically, e.g., in the hippocampus (Schweitzer, 2000). Another possibility is that ACPA acts presynaptically, inhibiting the ACh release by diminishing the quantal content of vesicles; therefore, the amplitude of MEPPs should be diminished. However, reduction in the amplitude occurs within a few minutes, and 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 et al., 1969).

To explain these postsynaptic effects, one possibility is that cannabinoids might act in form similarly to how 5-hydroxytryptamine acts on the nicotinic 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 nicotinic ACh receptors was reported in Xenopus oocytes expressing $\alpha_7$-ACh receptors (Oz, 2006). However, Nojima et al. (2000), using arachidonic acid and prostaglandin D$_2$, cooperatively accelerated desensitization of the nicotinic ACh receptor 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.

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