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The Role of the CB1 Cannabinoid Receptor at the Frog Neuromuscular Junction

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ABSTRACT

Endocannabionoids are endogenous compounds that have been linked to the activation of endocannabinoid receptors, like CB1, in the brain. Endocannibinoids, via CB1 activation, are responsible for modulating inhibitory and excitatory responses through retrograde signaling. Here we show that CB1 activation through application of an endocannabinoid agonist, arachidonylcyclopropylamide (ACPA), causes a decrease in stimulus-induced end-plate potentials (EPPs) in the frog neuromuscular junction (NMJ). We found that EPP amplitude decreased significantly only after eight minutes post-application, and remained depressed for an additional 36 minutes. This study demonstrates that retrograde signaling through endocannabinoid activation of CB1 receptors may influence synaptic transmission at the frog NMJ.

INTRODUCTION

The frog neuromuscular junction (NMJ) provides an excellent model for studying the role of receptors in synaptic transmission. The preparation has a large postsynaptic element, making it relatively easy to monitor changes in synaptic transmission in the form of end plate potentials (EPPs). Unlike action potentials, EPPs are not all-or-none responses; instead, they reflect small changes in synaptic transmission. To observe EPPs, antagonists must be applied to the NMJ to compete with neurotransmitter binding to postsynaptic receptors. This competition prevents the depolarization of the postsynaptic membrane from reaching threshold and thus, eliminates action potentials.

In the NMJ, the primary signaling pathway involves the neurotransmitter acetylcholine (ACh); release of this neurotransmitter causes muscle contraction. However, there are many additional signaling pathways that can regulate ACh release. One regulatory pathway that has been recently studied throughout the central nervous system involves endocannabinoids. Endocannabinoids are lipid-derived molecules that activate brain cannabinoid receptors and are structurally similar to Δ^9-tetrahydrocannabinol (THC), the psychoactive ingredient in marijuana (Freund et al., 2003). Endocannabinoids are part of a retrograde signaling system; they are released postsynaptically and bind to receptors on the presynaptic membrane to alter neurotransmitter release (Brenowitz and Regehr, 2003). More specifically, they have been shown to modulate ion channels, change the firing of presynaptic cells, and decrease synaptic strength (Brenowitz and Regehr, 2003).

Endocannabinoids bind to the cannabinoid receptor, CB1, in the rat brain to inhibit voltage-gated calcium channels and activate potassium channels, decreasing signal propagation (Melis et al., 2004). In mice, CB1-Rs have been found throughout the central nervous system, but expression is highest in cortical areas such as the hippocampus, anterior olfactory nucleus, neocortex and amygdala (Freund et al., 2003). Additionally, they have been shown to be involved in smooth muscle contraction regulation in the mouse gastrointestinal tract (Storr et al., 2004).

Preliminary studies indicate that these cannabinoid receptors may be present in the frog NMJ (Van der Kloot, 1994). To confirm these results, we applied a CB1 agonist, arachidonylcyclopropylamide (ACPA), and recorded the resulting changes in EPP amplitude. We hypothesized that if cannabinoid receptors were present on the presynaptic membrane of the frog NMJ, we would expect to see a decrease in EPP amplitude due to the retrograde inhibition of ACh release. We found that application of ACPA did cause a decrease in EPP amplitude. Such results indicate that cannabinoid signaling occurs outside the central nervous system, and may explain the physiological effects of CB1 activation.

MATERIALS AND METHODS

NMJ dissection and preparation

To examine the role of cannabinoid receptors at the frog NMJ, the sartorius muscle and attached nerve were dissected from *Rana pipiens pipiens*. The muscle preparation was then bathed in standard perfusion fluid (frog Ringer's) containing 10 mM glucose. Using a glass suction electrode, the nerve was stimulated and threshold was determined. All subsequent stimulations were

supramaximal (at least four times threshold). The bathing solution was then removed and replaced with 8 μM curare in Ringer's. The muscle preparation was incubated in curare solution until supramaximal stimulation no longer yielded a physical twitch.

Recording EPPs

A glass microelectrode inserted into the curarized muscle near end plates was used to record the resulting change in membrane potential upon stimulation of the motoneuron. The electrode was repeatedly inserted to locate areas of the highest stimulation-induced depolarization, which were assumed to indicate the location of an NMJ. The resulting EPPs were recorded and compared to EPPs from approximately the same location after addition of the cannabinoid agonist, ACPA (Tocris-Cookson) to the solution. EPPs were recorded beginning immediately following ACPA application until 44 minutes post-application.

Statistical Analysis

EPPs were measured during four time periods: Time 0 (control: Ringer's solution with glucose and curare) and then Times 1-4 minutes, 8-14 minutes and 25-44 minutes following the application of ACPA. For each EPP, we measured both amplitude and the time (milliseconds) that elapsed between the stimulus artifact and the apogee of the EPP. T-tests were run on amplitude and time to determine significance both compared to Time 0 and between different time intervals.

RESULTS

We hypothesized that the mean amplitude of EPPs would decrease with the application of ACPA. The mean amplitude of the EPPs was measured at Time 0, 1-4, 8-14, and 25-44 (Figure 1).

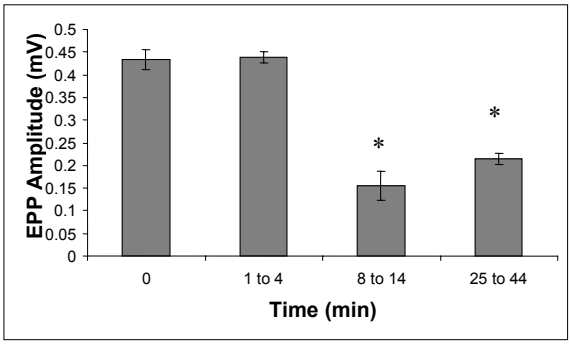

Figure 1. The effect of ACPA on mean EPP amplitude. At Time 0, only standard solution was applied. Times 1-44 are the minutes after the application of ACPA. Error bars indicate s.e.m. Asterisks indicate significance when compared to Time 0. ($p<<.001$ for all significant results, n=1)

There was no significant difference between Time 0 and Time 1-4 (Figure 1). However, there was a significant decrease when Time 8-14 and Time 25-44 was compared to Time 0. Furthermore, both of these time intervals were significantly different from Time 1-4, and there was a significant increase between Time 8-14 and Time 25-44 (all $p<<.001$)

Although not statistically significant, an interesting trend was observed in the values obtained for the time required to reach peak EPP amplitude (Figure 2). An initial decrease in the time required to reach maximum amplitude was followed by a step-wise increase.

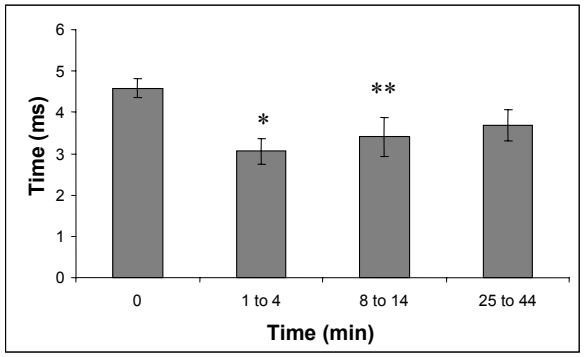

Figure 2. The effect of ACPA on time (ms) to reach the apogee of EPP. A significant difference ($p=.007$) was found between Time 0 and Time 1-4 (indicated by an asterisk). Significance was also found between Time 1-4 and 8-14 (indicated by double asterisks). Error bars indicate s.e.m. ($p=.01$, n=1)

DISCUSSION

To confirm the presence of the cannabinoid receptor, CB1, in the frog NMJ, we applied ACPA to the muscle preparation and examined the change in EPP amplitude. We observed a significant decrease in EPP amplitude starting at eight minutes post-application continuing through 44 minutes post-application. This indicates that CB1 receptors are indeed present in the frog neuromuscular junction and may inhibit the release of acetylcholine through retrograde signaling by endocannabinoids.

This retrograde signaling mechanism involving CB1 has been observed in other model systems. In the ventral tegmental area of the rat brain, endocannabinoids inhibit the release of glutamate through the activation of CB1 receptors (Melis et al., 2004). In rat hippocampal slices, a cannabinoid receptor agonist decreased ACh release when the slices were electrically stimulated (Gifford and Ashby, 1996). Additionally, in the mouse gastrointestinal tract, CB1 activation reduces the amount of ACh released from cholinergic nerve endings, subsequently influencing neurotransmission (Storr et al., 2003). Therefore, the reduction in postsynaptic EPP

amplitude that we observed may be due to the decrease in ACh release as a result of CB1 activation. Determining whether or not the change in synaptic transmission is due to quantal or frequency changes will provide more clues to the mechanism of retrograde inhibition by endocannabinoids in the frog NMJ.

Interestingly, this decrease in EPP amplitude was not observed immediately following application of the CB1 agonist, ACPA. The amplitudes of EPPs recorded 1-4 minutes post-application were not significantly different from the control responses (Time 0). Similarly, when WIN 55,212-2 (WIN), a CB-R agonist, was applied to the rat ventral tegmental area, the decrease in EPP amplitude also increased over time (Melis et al., 2004).

It is possible that during the initial few minutes post-application, ACPA could not bind to a sufficient number of CB1 receptors to inhibit neurotransmitter release. Alternatively, the mechanism by which CB1 activation affects the release of ACh may require more than four minutes. More conclusive data on the details of the mechanism are necessary to make this distinction. Furthermore, the increase in EPP amplitude from the 8-14 to the 25-44 time interval indicates that the ACPA and endocannabinoids may have a transient effect.

While no change in EPP amplitude was observed between 1-4 minutes and Time 0, there was a significant decrease in the amount of time it took for the EPP to reach its peak amplitude (Figure 2). All readings were taken at the same distance from the end plate within 1 mm. Assuming a speed of conduction of 10 m/sec, the maximum change in time to reach peak amplitude could be estimated to be 0.1 milliseconds. We observed time changes on the order of 0.3-1.5 seconds; therefore, we can assume changes in the time it took for the EPPs to reach their peak amplitude were due to a mechanistic difference. Because this effect was observed before the change in neurotransmitter release at the nerve terminal, it is possible that CB1 receptors were also affecting the rate of axonal propagation. Additionally, following the significant decrease between control responses and Time 1-4, there was a step-wise increase in time required to reach maximum amplitude. Assuming that ACPA-mediated activation of CB1 receptors does affect the rate of axonal propagation, it appears as if this effect lessens over time.

The primary findings of this study indicate that CB1 receptors are present at the frog NMJ and that a retrograde signaling pathway may regulate the release of acetylcholine. Future research on the mechanism of CB1 inhibition through the examination of changes in ionic conductance following endocannabinoid agonism is essential to further characterization of the role of CB1 receptors in neurotransmitter release. Furthermore, investigation of the mechanism responsible for the changes in the observed pattern of time required to reach maximum amplitude could indicate an additional role for CB1 receptors at the frog NMJ.

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