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M_2 receptor mediates a decrease in ACh release at the frog neuromuscular junction.

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ABSTRACT

Muscarinic receptors have been implicated in modulating ACh release at vertebrate neuromuscular junctions (NMJ). We characterized the roles of M_2 and M_3 receptors at the frog NMJ by pharmacologically isolating each receptor and measuring ACh release in terms of EPP amplitude. We observed an M_2 mediated inhibition of ACh release and no apparent involvement of M_3 receptors. We conclude that muscarinic responses in the frog are unlike those observed in the lizard or the rat.

INTRODUCTION

Muscarinic acetylcholine receptors act via several second-messenger pathways to produce either inhibitory, excitatory or retrograde effects at the nerve terminal, depending on the type of cell and the combination of sub-types that are expressed (Hulme, et al., 1990; Caulfield, 1993; Felder, 1995). Generally, M_1, M_3 and M_5 receptors activate the phospholipase C pathway, while M_2 and M_4 receptors inhibit adenylyl cyclase activity (Caulfield, 1993). The M_1 and M_2 receptors have been implicated in differentially modulating acetylcholine (ACh) release at the frog NMJ (Slutsky et al., 1999). Specifically, activation of the M_1 receptor enhances ACh release while activation of the M_2 receptor inhibits release. Furthermore Slutsky et al. found that coapplication of M_1 and M_2 antagonists abolishes the immediately observable muscarinic response and simulates application of atropine, thereby suggesting no involvement of other muscarinic receptor sub-types (1999). However, Graves et al. report inhibition of ACh release in the lizard NMJ, mediated by the M_3 receptors (2004). These results build on earlier studies conducted in the rat corticostriatal neurons by Hsu et al., which showed that specific inhibition of the M_3 receptors by 4-DAMP results in a reduction of synaptic transmission (1995). Therefore, the regulation of ACh release by muscarinic receptors at the frog NMJ may also involve the M_3 sub-type, as has been established in the rat and the lizard.

In the present study, we used electro-physiological recording techniques to isolate an M_2 and M_3-induced modulation of ACh release at the frog NMJ. We measured amplitudes of curarized, muscarine-evoked end-plate potentials (EPPs) in the presence of the specific M_1 antagonist pirenzepine and either the M_2 antagonist AF DX-116 or the M_3 antagonist 4-DAMP. We report a significant decrease in EPP amplitude in the presence of the M_3 antagonist but no difference in synaptic transmission and release of ACh using the M_2 antagonist, which indicates that the muscarine-induced synaptic depression is solely mediated by the M_2 and not the M_3 receptor at the frog NMJ.

MATERIALS AND METHODS

Frog preparation and solutions

Prior to experimentation all frogs were double pithed. The sartorius muscle was isolated from *Rana pipiens* (Carolina Biological Supply, Burlington, NC) and pinned in a Slygard-coated chamber containing Ringer solution composed of 112 mM NaCl, 3.2mM KCl, 2.7mM $CaCl_2$-$2H_20$, 0.5mM $NaHPO_4$, 2.0mM Tris buffer and 2.0mM dextrose (pH adjusted to 7.2). Fresh Ringer solution was made for each lab using stock solutions. The bath solution was changed every 15-20 minutes to increase the life of the preparation. All drugs applied to the muscle were dissolved at a given concentration in the fresh Ringer solution bath. Evoked end-plate potentials (EPPs) were reduced below the action potential threshold by applying 5-8μM turbocurarine chloride (Sigma-Aldrich). M_1 receptors were blocked with 5μM pirenzepine (Sigma-Aldrich). Either 10μM AF-DX 116 (Tocris Chemical) or 5μM 4-DAMP (Sigma-Aldrich) were added in the presence of 10μM muscarine (Sigma-Aldrich) to block M_2 or M_3 receptors, respectively.

Electrophysiology and data analysis

EPPs were elicited by stimulating the motor nerve axon with continuous suprathreshold depolarizing square pulses at 0.2 Hz (Grass SD9 Stimulator) and measured using standard intracellular recording techniques and glass microelectrodes filled with 3M KCl (resistance >2MΩ). Membrane potentials were recorded with a MacLab data acquisition system (AD Instruments). Each

EPP amplitude measurement represents the average of 8 EPPs. For control and experimental conditions EPP amplitudes were measured at 3-10 different neuromuscular junctions. Resting potentials were between -50 and -110 mV. We evaluated statistical significance using a t-test assuming unequal variances ($P< 0.05$ considered significant).

RESULTS

Activation of M_2 receptors mediates decrease in EPP amplitude.

To determine the effect of M_2 and M_3 receptors on end-plate potential (EPP) amplitude at the frog neuromuscular junction (NMJ) we pharmacologically isolated each receptor prior to muscarine activation. We initially isolated the M_2 response by blocking M_1 and M_3 receptors using pirenzepine and 4-DAMP, respectively. As shown in Figure 1, the first twelve minutes of muscarine activation showed a decrease of greater than 50 percent in EPP amplitude compared to the control, pirenzepine alone. This significant decrease in EPP amplitude is caused by a decrease in acetylcholine (ACh) release. After twelve minutes, the EPP amplitude increases and is not statistically different from the control which is consistent with the presumed decrease in muscarine effect over time. This decrease in EPP amplitude in the first twelve minutes could indicate the activation of inhibitory M_2 receptors or the inhibition of tonically activated excitatory M_3 receptors.

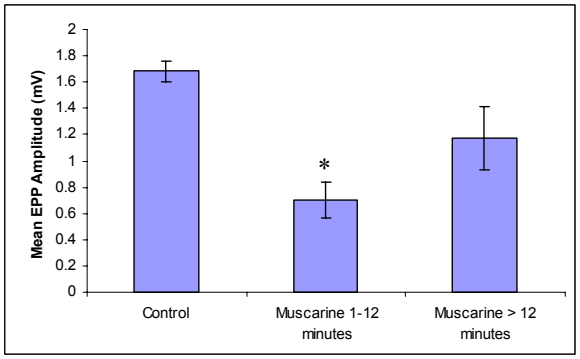

Figure 1. Decrease in EPP amplitude is mediated by M_2 receptors. The effect of muscarine in the presence of pirenzepine, an M_1 antagonist, and 4-DAMP, an M_3 antagonist, was examined. The first 12 minutes represents maximum muscarine activity, with presumed decrease in muscarine effect after 12 minutes. Each bar represents mean EPP of 4-8 independent EPPs. *Statistical significance relative to control. Error bars represent standard error of the mean.

In order to distinguish between M_2 activation and M_3 inhibition we repeated this experiment in the presence of pirenzepine and AF DX-116, an M_2 antagonist, therefore isolating the M_3 response. We would expect to see an increase in EPP amplitude during the first twelve minutes of muscarine activation if M_3 mediates an excitatory response. However, we saw no statistical difference between the control, the period of muscarine activation and the period after muscarine activation (Figure 2). This indicates that the decrease originally seen (Figure 1) is in fact mediated by inhibitory M_2 receptors and not excitatory M_3 receptors.

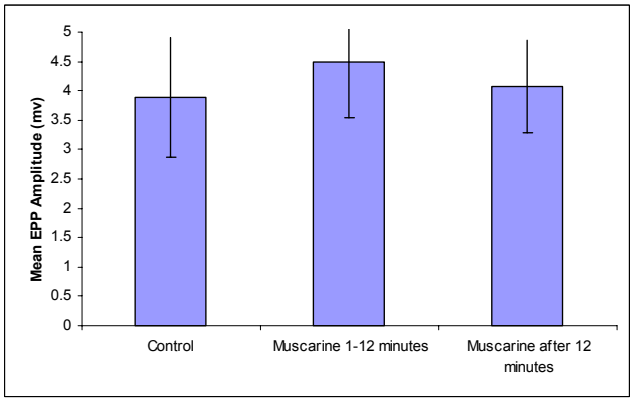

Figure 2. M_3 receptors do not alter EPP amplitude. The effect of muscarine in the presence of pirenzepine, an M_1 antagonist, and AF DX-116, an M_2 antagonist, was examined. Each bar represents mean EPP of 4-8 independent EPPs. The first 12 minutes represents maximum muscarine activity, with presumed decrease in muscarine effect after 12 minutes. Error bars represent standard error of the mean.

DISCUSSION

Previous research indicates that M_1 and M_2 receptors are involved in the enhancement and release of acetylcholine (ACh) at the frog neuromuscular junction (NMJ); Slutsky et al. (1999) show that specific inhibition of both receptors mimics application of atropine, completely blocking the effect of muscarine. However, studies have not directly tested for the involvement of M_3 receptors at the frog NMJ, which are known to cause inhibition of ACh release at the rat corticostriatal neurons and the lizard NMJ (Hsu et al. 1995; Graves et al. 2004). In order to clarify the roles of the M_2 and M_3 receptors we selectively inhibited each receptor and observed muscarine-induced effect on ACh release. In addition, we inhibited M_1 receptors at all phases of the experiment to eliminate their known enhancement of ACh release and possible nonspecific interaction with the M_3 antagonist (Slutsky et al. 1999). We measured the end-plate potential (EPP) amplitudes resulting from coapplication of antagonist and muscarine. The main result of our study is that M_2 receptors mediate inhibition of ACh release at the frog NMJ with no apparent involvement of M_3 receptors, indicating that the frog muscarinic response is unlike that of the lizard and the rat (Hsu et al. 1995; Graves et al. 2004).

Blocking M_3 receptors with 4-DAMP resulted in a greater than 50 percent decrease in EPP amplitude. This result could indicate that either the M_3 antagonist blocks tonic excitatory M_3 receptors or that muscarine activates

inhibitory M_2 receptors. It is unlikely that this decrease in EPP amplitude is mediated by excitatory M_3 receptors because Graves et al. (2004) and Hsu et al. (1995) have shown that M_3 receptors are in fact inhibitory in the lizard and the rat. To completely rule out this possibility, we blocked the M_2 receptor to unmask M_3 activity. Therefore, we expected to see an increase in EPP amplitude if M_3 were excitatory. However, we observed no change in EPP amplitude suggesting no M_3 involvement. Slutsky et al. (1993) indicate that M_2 is inhibitory and suggest no involvement of M_3 receptors in the frog. Our results agree with Slutsky's model, indicating a difference between frog muscarinic responses and those of the rat and lizard. This may indicate an evolutionary divergence in neuromodulation of amphibians versus reptiles and mammals. These interesting differences merit further study to establish a clear understanding of muscarinic effects on ACh release.

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