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Voltage Dependency of Neurotransmitter Release at the Crayfish Neuromuscular Junction

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ABSTRACT

To test the new hypothesis that a calcium independent mechanism is required for neurotransmitter release, we measured excitatory post-synaptic potentials (EPSPs) at the neuromuscular junction of the crayfish tail extensor muscle. Stimulation of the nerve was accomplished with a suction electrode and measurements were made with a micropipette placed in a post-synaptic muscle cell. The tail was bathed in Co^{2+} to effectively eliminate EPSPs. Next, the calcium ionophore A23187 was added to raise the intracellular concentration of Ca^{2+}. Upon addition of A23187 a small recovery of EPSP was observed in dilute cobalt concentrations but this was reversed using a higher concentration of Co^{2+}. Our results neither confirm nor deny either theory.

INTRODUCTION

At the neuromuscular junction, depolarization is known to initiate a multi-step process culminating in the release of neurotransmitter in quantal packets. This release eventually leads to contraction of the muscle fiber (Katz, 1962).

In the late sixties, scientists recognized the importance of calcium inside the pre-synaptic nerve terminal to elicit a post-synaptic response (Katz, 1969). Since then, the majority of the scientific community has held that calcium influx through voltage-gated channels is both necessary and sufficient for neurotransmitter release (Katz an Miledi, 1967). It is thought that upon depolarization of the pre-synaptic membrane, voltage gated calcium channels open, allowing influx of calcium ions that then bind to the SNARE complex, initiating vesicle fusion with the synaptic membrane (Byrne, 2009).

This conception of neurotransmission has recently met serious resistance. Evidence now points to a calcium-independent and voltage-dependent component of release (Dudel 2009). Voltage-sensitive G-protein coupled receptors (GPCRs) implicated in removal of the steady-state tonic block on release (Kupchick, 2008) have also been identified. In light of these findings, a new mechanism has been proposed in which the voltage-sensitive GPCR remains bound to synaptic vesicles until membrane depolarization initiates dissociation of the GPCR and vesicle, allowing release. Following these events, Ca^{2+} binds to the SNARE complex, effecting release as has traditionally been understood (Kupchick, 2008, Parnas, 2007., Ben-Chaim, 2006, 2003., Parnas, 2005., Slutsky, 2003., Slutsky, 2001). It is important to note that the new theory does not necessarily deny any part of the old theory, but rather, adds a new level of complexity to it.

Here, using the neuromuscular junction of the crayfish tail extensor muscle, a typical model for the study of synaptic transmission, we employed the well-known calcium channel blocker, cobalt, and the calcium ionophore, A23187, to test the new voltage hypothesis. We first determined the concentration of cobalt needed to effectively eliminate calcium influx through V-gated channels and then applied the ionophore to introduce calcium into the presynaptic cell independent of depolarization. The ionophore elicited weak recovery of the post-synaptic response in dilute Co^{2+} but not in concentrated Co^{2+} and we observed no paired pulse facilitation. Our results neither confirm nor deny either theory.

MATERIALS AND METHODS

Dissection/Bathing

The tail of a crayfish was cut and dissected to expose the extensor muscles and the nerves leading to them. The prep was placed in standard crayfish Ringer's solution during all experiments. Experiments were carried out in two different ringer's solutions. Week one's experiements were conducted in a bath containing 5.4 mM KCl, 196 mM NaCl, 2.6 mM MgCl, 3.375 mM $CaCl_2$, and 10mM HEPES. Week two's experiments were conducted in a similar ringer's with 4x as much calcium.

Electrophysiology

All post-synaptic recordings of EPSPs were taken with a 1.2 mm borosilicate glass electrode and an A-M Systems Model 3100 Intracellular Electrometer (resistance < 200 mega ohms). Pre-synaptic nerves were

stimulated through a suction electrode connected to a Grass SD4 Stimulator. The pre-synaptic nerve was stimulated using pulses of current applied through a wide-tipped suction electrode in pulses spaced approximately 2 seconds apart. The amplitude of depolarization was increased until a response was registered or we had surpasses a reasonable estimate of physiological depolarizations.

Measurements

Following initial characterization, 50uL of 13.5mM $CoCl_2$ was added to the dish resulting in a final concentration of cobalt of 13.5uM. Next, 50uL of 2mM A23187 (in 50:50 DMSO:Ethanol) was added to the same dish, resulting in a final concentration of A23187 of 2uM. Finally, another dose of $CoCl_2$ was added to bring the final concentration of cobalt to 27uM.

RESULTS

To determine whether or not membrane depolarization is required for neurotransmitter release, we began recording EPSPs from the muscle in both 3.375 and 13.5 mM $CaCl_2$. We were able to successfully record post-synaptic potentials following stimulation of the corresponding nerve with a suction electrode in both concentrations, though greater stimulus was needed in 3.375 mM $CaCl_2$ (Fig 1). Figure two shows that addition of 13.5 uM cobalt chloride was sufficient to completely eliminate the EPSP in both concentrations of Ca^{2+}.

Before the application of the ionophore, the bathing medium was switched to 13.5 mM $CaCl_2$. Application of 2µm of the Ca^{2+} ionophore A23187 temporarily restored the post-synaptic response (Fig. 3). Though we did not test for paired pulse facilitation in our control, we also found that paired pulses did not reveal any facilitation, suggesting calcium influx through V-gated channels was not substantial enough to generate the residual calcium necessary for facilitation (Fig 4). Subsequent doubling of the concentration of Co^{2+} resulted in the elimination of the post-ionophore response (Fig 5). Neither the recording electrode nor the stimulating electrode was moved during this process to ensure that we maintained contact with the nerve and muscle cell.

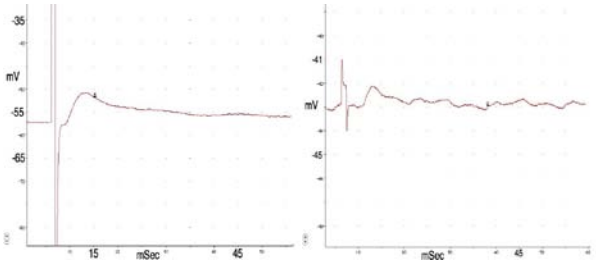

Figure 1: Sample traces of an initial EPSP from crayfish tail extensor muscle in standard crayfish Ringer's solution. The ordinate (y-axis) represents EPSP amplitude in mV; the abscissa (x-axis) represents time in mS. Week 1 shown on left (3.37 mM $CaCl_2$), week 2 on the right (13.5mM Ca).

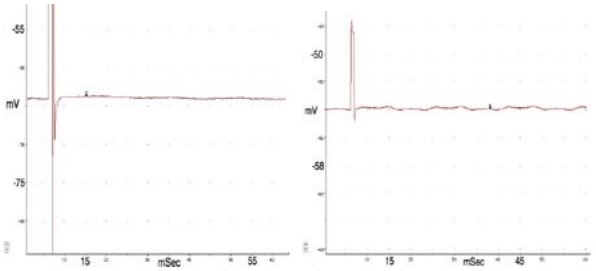

Figure 2: Sample trace of an EPSP from crayfish tail extensor muscle in standard crayfish Ringer's solution after the addition of 13.5mM $CoCl_2$. Week 1 (3.375 mM $CaCl_2$) shown on left, week 2 (13.5mM $CaCl_2$) on the right. The ordinate (y-axis) represents EPSP amplitude in mV; the abscissa (x-axis) represents time in mS.

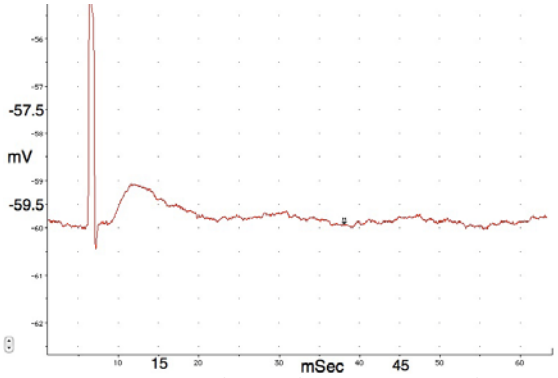

Figure 3: Sample trace of EPSP after the application of both 13.5uM cobalt and 2uM A23187. The ordinate (y-axis) represents EPSP amplitude in mV; the abscissa (x-axis) represents time in mS. The ionophore appears to partially restore excitability (neurotransmitter release).

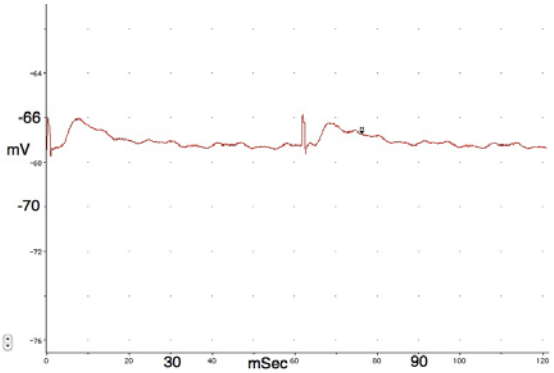

Figure 4: Sample trace of a paired-pulse experiment conducted after the addition of cobalt and A23187 ionophore in 13.5 mM Ca^{2+}. Paired pulse stimulation revealed no facilitation following the addition of cobalt and the ionophore. The ordinate (y-axis) represents EPSP amplitude in mV; the abscissa (x-axis) represents time in mS.

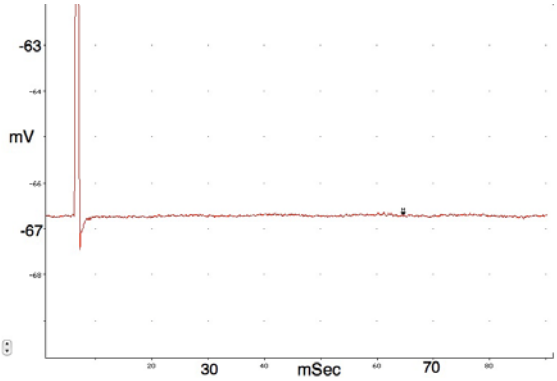

Figure 5: Sample trace of EPSP following the addition of a second dose of cobalt (final concentration of cobalt is 27uM). Application of additional cobalt resulted in a loss of the regained EPSP function. The ordinate (y-axis) represents EPSP amplitude in mV; the abscissa (x-axis) represents time in mS.

DISCUSSION

We successfully demonstrated that the application of extracellular cobalt to the crayfish neuromuscular junction results in the disappearance of a post-synaptic response following stimulation of the neuron (Fig 1 and 2). We assumed the cobalt bound and thus blocked pre-synaptic voltage-gated calcium channels and eliminated EPSPs by depriving the pre-synaptic terminal of the calcium needed for neurotransmitter release.

The subsequent addition of the ionophore A23187 resulted in the re-emergence of the post-synaptic response (Fig 3). A23187 is a small molecule that allows calcium ions to pass freely through membranes. By allowing calcium to enter the pre-synaptic nerve terminal, independent of depolarization, we hoped to show that membrane depolarization could generate a post-synaptic a response, even with the V-gated channels blocked. This result would suggest a voltage dependent, but calcium independent, aspect of neurotransmitter release. We did observe this expected result in Fig 3, though later addition of more cobalt eliminated the response.

As an additional test, we conducted a paired-pulse experiment in the presence of cobalt and A23187. Under normal conditions, the second response of a paired-pulse stimulus should be larger due to residual calcium in the pre-synaptic nerve terminal. Under our experimental conditions, with the cobalt and ionophore, the intracellular concentration of calcium should remain constant and we should see no paired-pulse facilitation. Though we did not demonstrate paired pulse facilitation in our control due to time restrictions, the fact that we did not see it in our experimental conditions is compatible with the voltage hypothesis (Fig 4).

To ensure that the post ionophore response did not result from incomplete Ca^{2+} blockage, we added another dose of cobalt, doubling the extracellular concentration. If our hypothesis were correct, we would expect this addition to have no effect, because we already assumed the V-gated calcium channels to be completely blocked. The additional cobalt abolished the post-synaptic response, despite the continued presence of the ionophore. Thus, any results we obtained that supported a calcium-independent component of neurotransmitter release should be attributed to an incomplete block of the voltage-gated calcium channels.

Our results support neither theory. Though it seems that the observed post-ionophore response was the result of incomplete Ca^{2+} channel blockage, it could just as well be that the second dose of cobalt interfered with the pre-synaptic neuron, preventing it from firing and thus eliminating a response that actually was independent of calcium influx. In the future, this experiment might be replicated using other, specific Ca^{2+} channel blockers less likely to have non-specific effects.

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