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Inhibiting BK channels at the crayfish neuromuscular junction decreases amplitudes of single pulse EPSPs and increases synaptic facilitation.

DAVID KRIST, STACY MCCARTHY, and JOCELYN NEWHOUSE
Department of Biology, Grinnell College, Grinnell, Iowa

ABSTRACT

Since BK channels serve to regulate neurotransmitter release at synapses in the rat hippocampus (Raffaelli et al., 2004), inhibition of the channels could have significant effects on synaptic activity at the crayfish neuromuscular junction. We studied the effects of charybdotoxin, a BK channel inhibitor, on the resting membrane potential, action potential threshold, excitatory post-synaptic potentials (EPSPs), and synaptic facilitation in the crayfish neuromuscular junction (NMJ). We measured the effects of the neurotoxin by observing the crayfish tail dissection in standard crayfish Ringer's solution (control), Ringer's solution with charybdotoxin (toxin), and again in standard Ringer's solution (wash) using suction and recording electrodes in conjunction with an electrical stimulator and a data collection program. With the addition of charybdotoxin, we observed an increase in the resting membrane potential, a slight decrease of the action potential threshold, a significant decrease in the amplitude of the EPSPs, and an observed increase in the facilitation of EPSPs with a 20ms delay between pulses. Our results reveal that BK channels regulate neurotransmitter release in a manner distinct from other presynaptic voltage- and calcium-activated potassium channels in that they may function in the dampening of facilitation. In addition, the decrease in the amplitude of single pulse EPSPs indicates that BK channels may affect postsynaptic potentiation.

INTRODUCTION

Potassium channels, found within the somatodendritic, axonal, and terminal membranes of the neuron, serve a variety of purposes. In early studies, these channels were found to repolarize the cell membrane after action potential depolarization by allowing an efflux of potassium ions (Meir et al., 1999). Relatively recently, presynaptic potassium channels were found to regulate the release of neurotransmitters (Raffaelli et al., 2004). Presynaptic neurons release neurotransmitters via exocytosis upon the influx of calcium ions, which occurs primarily during membrane depolarization (Freeman, 2005). Thus, transmitter release halts once potassium channels allow the ion efflux, repolarizing the membrane.

Though a variety of presynaptic potassium channels exist, research on neurotransmitter release regulation continually implicates the involvement of voltage- and calcium-gated potassium channels. These potassium channels are grouped according to the degree of potassium conductance: small conductance (SK), and big conductance (BK). BK channels facilitate spike repolarization and fast afterhyperpolarization of the membrane. Research reveals that these channels regulate presynaptic excitability by limiting the duration of the presynaptic spike at the terminals of the frog neuromuscular junction, thereby reducing the calcium influx and transmitter release (Robitaille et al., 1992). Our study focuses on the consequences of inhibited BK channels on the resting membrane potential, the action potential threshold, excitatory post-synaptic potentials (EPSPs), and synaptic facilitation at the crayfish NMJ. However, most research to date has been limited to the general blocking of voltage- and calcium-activated potassium channels rather than targeting specific potassium channels, such as the BK channels.

Research shows that a general blocking of presynaptic potassium channels with the tetraethylammonium ion increases the amplitude of the second reaction of two test pulses (Sivaramakrishnan et al., 1991). This result supports the idea that voltage- and calcium-activated potassium channels limit the release of neurotransmitter. Additionally, an investigation revealed that general blocking of voltage- and calcium-activated potassium channels with 3, 4 diaminopyridine increases both the amplitude and the duration of depolarization (Blundon et al., 1995). Nevertheless, experimentation that specifically targets the BK channels shows that only the frequency and not the amplitude of spontaneous excitatory postsynaptic currents (EPSCs) increases when the channels are inhibited (Raffaelli et al., 2004). The disparity between these experiments shows that BK channels regulate neurotransmitter release in a manner distinct from other presynaptic voltage- and calcium-activated potassium channels. Our study employed the

use of charybdotoxin, a scorpion peptidyl toxin that is known to specifically block BK channels (Skinner et al., 2003).

Previous experimentation establishes relatively little on the role of presynaptic voltage- and calcium-activated potassium channels compared to the wealth of knowledge on somatic and axonal ion channels. Moreover, investigations that inhibited BK channels were, on the aggregate, inconclusive (Meir et al., 1999). Thus, our study's purpose was to elucidate the role of the BK channel by inhibiting it specifically.

Research shows that the inhibition of BK channels with paxilline increases success rates for the amplitude of both the first and second spikes (Raffaelli et al., 2004). Nevertheless, it remains unclear whether the inhibition of BK channels fundamentally increases facilitation. A greater amount of neurotransmitter release with the toxin present does not necessarily indicate an increase in facilitation—the amplitude ratios between the first and second pulse spikes truly reveal the degree of facilitation. We hoped that varied delay intervals for paired-pulse tests would provide further insight into these ratios and the degree with which facilitation changes when BK channels are blocked.

From this study we desired to establish a more concrete understanding of the contribution of BK channels to the regulation of neurotransmitter release at the crayfish neuromuscular junction (NMJ). Since investigations on the effects of BK channels on synaptic facilitation, resting membrane potential, and membrane potential threshold have been heretofore inconclusive, we anticipated that the results of our experiment would elucidate the basis for past findings. We hypothesized that the presence of charybdotoxin at the crayfish NMJ would cause an increase in synaptic facilitation, amplitude and duration of EPSPs and the resting membrane potential, and a decrease in action potential threshold. Notably, we observed an instance where facilitation increased when BK channels were blocked, suggesting that these channels regulate neurotransmitter release by dampening facilitation. Distinct from past research, our experiments indicated that EPSP amplitudes decreased in the presence of the toxin during single pulse stimulation. These results witness to the complexity of the interactions between synaptic ion channels and synaptic plasticity since they suggest that BK channels have postsynaptic influence.

MATERIALS AND METHODS

Dissection

Each day we performed a standard preparation of a crayfish, *Procambarus sp*, obtained from the Carolina Biological Supply Company. After icing the crayfish for 20 minutes, the tail was cut off and the ventral exoskeleton removed, making sure to execute the incisions high enough to leave functional nerves that innervate the dorsal extensor muscles. Excess tissue and muscle were removed to expose the nerve bundles and fast extensor muscles of the crayfish. The dissected tail was completely immersed in crayfish Ringer's solution (Table 1), which was replaced approximately every 15 min to extend the normal function of the nerve and muscle.

Chemical	**Concentration (mM)**
NaCl	205.0
KCl	5.40
$CaCl_2$	135.0
$MgCl_2$	2.6
Tris(pH 7.4)	10.0

Table 1. Composition of standard crayfish Ringer's solution

Measuring Membrane Potential

Recording electrodes were made with 1.2 mm diameter glass capillary tubes (Kwik-Fil™) pulled to a fine tip by an electrode puller (World Precision Instruments, Inc.). We then filled the electrode with 3M KCl solution and attached it to MacLab through a micro-manipulator (World Precision Instruments, Inc.). After inserting it into the solution with the help of a microscope, we tested the resistance to ensure that the electrode had an intact fine tip, there were no air bubbles, and the resistance was greater than 10MΩ. The equipment was then zeroed using the data collection program Scope v3.6.3. We obtained resting membrane potential readings by inserting the electrode in a fast extensor muscle.

Stimulation of Muscle

In order to stimulate the muscle, we used a suction electrode (a recording electrode with the fine tip broken off and sanded to an appropriate size). We sucked up a nerve bundle (making sure the nerve fit fairly tightly) along with some crayfish Ringer's solution. With the reference electrode in the crayfish Ringer's solution and the suction electrode attached to the SD9 Stimulator (Grass Medical Instruments), we stimulated the muscle until we observed a twitch that indicated a functional NMJ.

Measuring Action Potential Threshold and EPSPs

We recorded changes in the membrane potential corresponding to stimulation by inserting the recording electrode in the vicinity of the observed twitch. The action potential threshold was measured by recording, from the stimulator, the minimum voltage necessary to elicit an EPSP with a single pulse. The resulting EPSPs were recorded and analyzed using Scope. In order to measure facilitation, we used the Twin-pulse function of the stimulator. We varied the delay between pulses (20, 50, 80, 110ms) and collected readings of EPSPs for each.

Toxin Application

The neurotoxin was applied to the crayfish tail using a Ringer's solution with a 5nM concentration of charybdotoxin. We then waited 15 minutes for the toxin to take effect before measuring membrane potential, action potential threshold and EPSPs.

Wash

We measured the reversibility of the toxin by removing the Ringer's solution containing the toxin and replacing it with standard crayfish Ringer's solution. We recorded the membrane potential, action potential threshold and EPSPs after waiting 15 minutes.

Statistical Analysis

We averaged our results from four successful data collection sessions for data analysis. To determine the statistical significance of each aspect of synaptic transmission, we performed two-tail t-tests (assuming equal variance) among the three conditions. We rejected the null hypothesis at the 95% confidence interval.

RESULTS

To elucidate the contribution of BK channels to the regulation of neurotransmitter release at the presynaptic terminal, this experiment studied the effects of charybdotoxin on resting membrane potential, action potential threshold, amplitude and duration of EPSPs, and synaptic facilitation at the crayfish NMJ. We recorded each of these measurements in control, toxin, and wash preparations on a constant NMJ. For the control preparation, the crayfish tail was bathed in a standard crayfish Ringer's solution (Table 1). In the toxin preparation, we used the same solution with an added concentration of 5nM charybdotoxin, and then for the wash preparation we reverted back to the standard Ringer's solution. We used a suction electrode and an electrical stimulator to stimulate a given nerve attached to the fast extensor tail muscles, and obtained readings via a recording electrode attached to a computerized data collection program.

There was an observable, but not significant, increase in the resting membrane potential after the addition of charybdotoxin (Figure 1). The mean resting membrane potential of the control was -52.2mV, whereas -43.8mV was the mean resting membrane potential for the experimental condition. In addition, the mean resting membrane potential for the wash, -43.2 mV, although again not significantly different than the control, showed the same increase as the toxin.

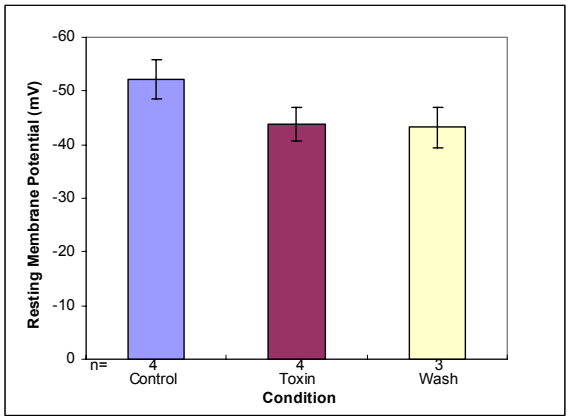

Figure 1. The effects of charybdotoxin on resting membrane potential (+/- 1 s.e.) in the crayfish neuromuscular junction. For all conditions, $p>0.05$ for the control (n=4) vs. toxin (n=4) and control vs. wash (n=3) and toxin vs. wash.

Concerning action potential threshold, we found no significant change from the control threshold mean (0.52V) to the toxin threshold mean (0.48V), or to the wash threshold mean (0.40V) (Figure 2). These values do not suggest that charybdotoxin appreciably affects action potential threshold at the crayfish NMJ because of the large variance of the toxin (Figure 2).

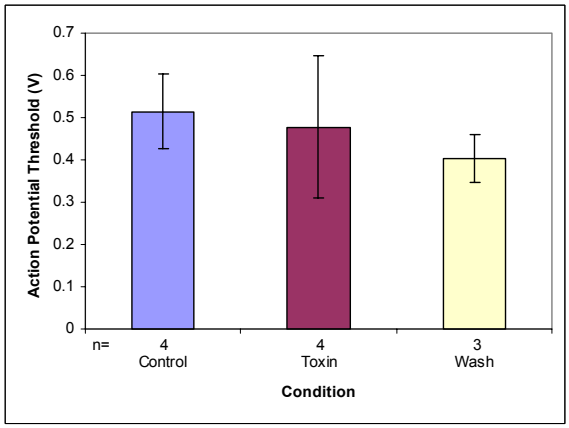

Figure 2. The effects of charybdotoxin on mean action potential threshold (+/- 1 s.e.) in the crayfish neuromuscular junction. For action potential thresholds, $p>0.05$ for control (n=4) vs. toxin (n=4) and control vs. wash (n=3).

The addition of charybdotoxin to the crayfish solution revealed a lower mean EPSP amplitude (6.79 mV) than during the control condition (11.43 mV) (Figure 3). The mean EPSP amplitude was 6.38 mV for the wash condition. These results were significant ($p<0.05$) for the control vs. toxin and for the control vs. wash t-test. This suggests that the charybdotoxin caused a decrease in EPSP amplitude. The results were not significant ($p>0.05$) for the toxin vs. wash t-test, suggesting that the effects of charybdotoxin on the EPSP amplitude were irreversible.

Duration was measured as the width of the spike at half amplitude. Although there was some variation between the mean duration of the EPSPs of the control (15.08 ms), toxin (16.03 ms) and wash (12.86 ms), these results are not significant (Figure 3). In addition, the means show no observable trend if we assume the charybdotoxin to be irreversible, suggesting that the toxin does not effect the EPSP duration at the crayfish NMJ.

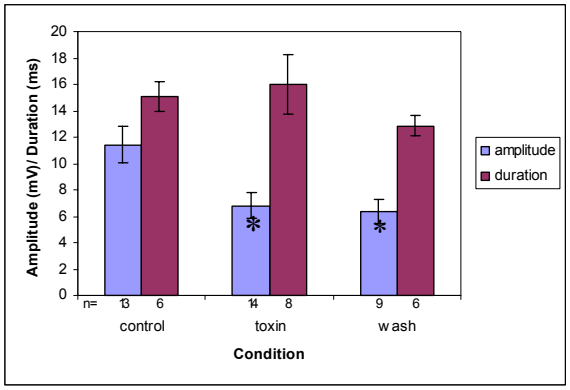

Figure 3. The effects of charybdotoxin on mean EPSP amplitudes and durations (+/- 1 s.e.) in the crayfish neuromuscular junction. For amplitude, $p<0.05$ (denoted by *) for the control (n=13) vs. toxin (n=14) and control vs. wash (n=9) and $p>0.05$ for the toxin vs. wash. For duration, $p>0.05$ for all conditions.

Facilitation was determined through the Facilitation Index (FI) where the amplitude of the second EPSP is divided by the sum of both amplitudes. Facilitation is acknowledged to occur if this index is greater than 0.5. The only observable increase in facilitation occurred for the 20 ms twin pulse delay where the mean FI for the toxin preparation was 0.59 compared to 0.53 for the control and 0.54 for the wash (Figure 4). The differences between these means are not significant, but do suggest that charybdotoxin may increase facilitation at the 20 ms delay. Additionally, a t-test value of 0.91 between the control and the wash means could signify that the effects of charybdotoxin are lost with the wash concerning facilitation at the 20 ms delay. The result at the 50 ms, 80 ms, and 110 ms delay intervals reveal no observable trends pertaining to facilitation.

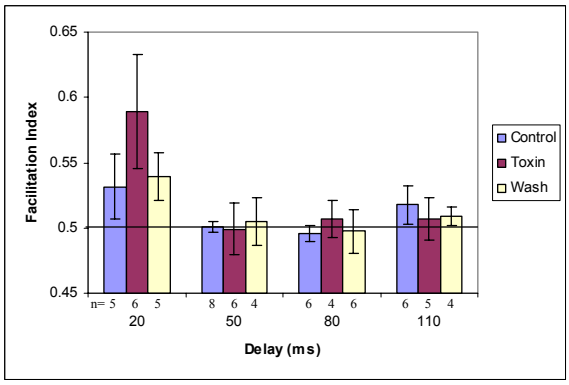

Figure 4. The effects of charybdotoxin on synaptic facilitation (+/- 1 s.e.) in the crayfish neuromuscular junction. The horizontal line at 0.5 indicates the smallest index value for which facilitation occurs. For all delays (20, 50, 80, and 110 ms), $p>0.05$ for the control (n=5, 8, 6, 6) vs. toxin (n=6, 6, 4, 5) and control vs. wash (n=5, 4, 6, 4).

DISCUSSION

We hypothesized that the presence of charybdotoxin at the crayfish NMJ would cause an increase in synaptic facilitation, amplitude and duration of EPSPs and the resting membrane potential, and a decrease in action potential threshold. The experiment supported the hypothesis that the blocked BK channels would cause increased synaptic facilitation, but noteworthy results were found only during the pulses with a 20ms delay. We also observed the predicted increase in resting membrane potential in the presence of charybdotoxin, yet the results were not significant. We did see significant results for the toxin's effect on EPSP amplitude—in the presence of charybdotoxin, the EPSP amplitude decreased, contrary to our hypothesis. In addition, our results did not support our predictions about charybdotoxin's effects on EPSP duration or action potential threshold.

The observed increase in resting membrane potential mirrors our prediction. Additionally, the similarity between the toxin and wash conditions suggests that either the effect of charybdotoxin on the resting membrane potential is irreversible, or the increase is a function of time. The latter conclusion, however, suggests that there would also be an increase in resting membrane potential from the toxin to the wash because the elapsed time between toxin and wash readings was the same as the time between control and toxin readings. Subsequently, this study partially implicates BK channels in the regulation of the resting membrane potential.

Although the slight decrease in action potential thresholds agrees with our hypothesis, the continuing decrease in the wash condition suggests other factors may be involved in action potential threshold maintenance.

Time or the tiring of a repeatedly excited nerve may have caused the observed effect. Thus, we cannot conclude whether charybdotoxin, and therefore BK channels, have an effect on the action potential threshold.

The only statistically significant result of our study, the decrease of single pulse EPSP amplitude upon the addition of charybdotoxin, is supported by previous research where iberiotoxin was shown to increase the frequency, but not the amplitude of EPSCs (Raffaelli, 2004). Interestingly, however, we have not encountered any past instances where the amplitude actually decreased. Also, in our experiment, the mean duration observably increased in the toxin condition despite the fact that this increase was not significant. Therefore, our results diverge from the research that employed 3,4 diaminopyridine to produce a general blocking of voltage- and calcium-activated potassium channels. Here, both the amplitude and the duration of depolarization increase (Blundon et al., 1995). The disparity between these experiments supports previous notions that BK channels regulate neurotransmitter release in a manner distinct from other presynaptic voltage- and calcium-activated potassium channels.

Although not statistically verified, facilitation increased in the presence of charybdotoxin for the 20ms delay. This finding not only verifies previous research where a general blocking of presynaptic potassium channels increases the amplitude of the second reaction of two test pulses, but also offers an insight that BK channels may function in the dampening of facilitation (Sivaramakrishnan et al., 1991). Unlike past studies, our findings reveal that facilitation—not just the amplitudes following the twin pulse—increases.

Overall, some of our findings suggest that BK channels regulate neurotransmitter release at the crayfish NMJ. The observed increase in facilitation at 20ms supports this regulating role the most despite the significant amplitude decrease that occurred when the channels were blocked. This amplitude decrease during single pulse stimulation suggests that a greater neurotransmitter release (allowed by blocked BK channels) does not directly relate to greater postsynaptic potentiation. This suggests that BK channels, although observed to be localized presynaptically, exhibit postsynaptic influence. Future experiments should explore this connection by variably inhibiting BK channels and postsynaptic channels simultaneously.

Some limitations to our study include that isolated crayfish preparations deteriorate over time, giving comparatively different results at the beginning and the end of the experiment. In addition, charybdotoxin may have unforeseen effects, such as the alteration of other ion channels. To account for these, we could conduct further research using charybdotoxin and conduct toxin measurements immediately after dissection.

Further research could also be done to elucidate the effect of BK channels on action potential threshold by observing the action potential directly as opposed to through the firing of EPSPs. Additionally, different neurotoxins could be used to determine the function of the BK channels being studied. The effects of BK channels could also be studied by enhancing instead of inhibiting them. Similar to other studies on BK channels, our results do not conclusively point to the channels' regulatory role in the synapse. Thus, this area of study lends itself well to different angles of experimentation.

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