Pioneering Neuroscience, 2006, **7**, 17-21

The role of calcium-activated potassium channels in excitatory postsynaptic potential duration.

CARL KRAUSE, EVAN PETRIE, SMITA SHARMA
Department of Biology, Grinnell College, Grinnell, Iowa

ABSTRACT

Our research looks at the role of calcium-activated potassium channels (K_{Ca} channels) in synaptic transmission. K_{Ca} channels play a potentially significant role in repolarization duration of the presynaptic cell following an action potential. Additionally, focusing on the function of these channels may offer insights into the significance of extracellular calcium concentrations on the process of transmission. We expected that the time taken for repolarization of the presynaptic cell would correspond to the duration of excitatory postsynaptic potentials which we could then record. We used 3,4-diaminopyridine (3,4-DAP) to block delayed-rectifier potassium channels in the crayfish NMJ, so that we could study calcium-activated ones exclusively, and then modulated extracellular calcium concentrations to see its effect on EPSP duration. We hypothesized that by blocking delayed-rectifier potassium channels, we would inhibit repolarization of the presynaptic cell, resulting in a broadened EPSP in the postsynaptic cell. Our second hypothesis was that lowering extracellular calcium concentrations for 3,4-DAP-exposed crayfish cells would further inhibit repolarization as Ca^{2+} ions are required to activate K_{Ca} channels. Our results demonstrated a statistically-significant difference in repolarization time between 3,4-DAP-exposed crayfish and unexposed crayfish, but this difference contradicted our hypothesis. Instead of lengthening repolarization duration, 3,4-DAP shortened it. We also found extracellular calcium concentrations to have an effect on EPSP duration: the lower the extracellular calcium concentration, the broader the EPSP measured. This result was in keeping with our second hypothesis. One explanation we offer for these results is that K_{Ca} channels might become more efficient in the presence of 3,4-DAP and/or the blockage of other potassium channels, and that extracellular calcium concentrations might in turn regulate this efficiency.

INTRODUCTION

Chemical synaptic transmission is the process by which an axon, via the presynaptic terminal, transmits a message (either excitatory or inhibitory) to a neighboring cell via the receiving cell's postsynaptic terminal. When the depolarizing spread of the action potential reaches the nerve terminal of the presynaptic cell, voltage-gated calcium channels open and calcium ions flow into the cell, initiating a process by which neurotransmitters are expelled into the synaptic cleft. The influx of Ca^{2+} into the presynaptic terminal is somewhat delayed by the positive intracellular charge which repels the calcium cation (Dobbs, *et al*. 2002) but after this initial synaptic delay, influx is rapid, due to the steep concentration gradient that exists across the presynaptic membrane. This concentration gradient reflects the typical external and internal Ca^{2+} concentrations of 10^{-3} M and 10^{-7} M respectively (Augustine, *et al.* 1987). Elevation of the internal Ca^{2+} concentration facilitates the fusing of synaptic vesicles to the plasma membrane of the presynaptic neuron, thereby allowing neurotransmitter release. Repolarization occurs in the presynaptic terminal as potassium channels open and potassium ions are expelled, cutting the inflow of both sodium and calcium ions.

Our research looks at calcium-activated potassium channels and their role in the process of cell repolarization in the presynaptic terminal. These channels play an important part in the modulation of firing rates and the regulation of transmitter release (Meir *et al*, 1999). Large-conductance K_{Ca} channels (BK channels) are employed in the negative-feedback function and help repolarize the nerve ending following an action potential. BK channels link internal calcium concentration and membrane excitability and thus are believed to regulate the frequency and duration of action potentials (Lara *et al*, 1999). BK channels are blocked by the toxins tetraethylammonium (TEA) and charybdotoxin (CTX), but not 3,4-diaminopyridine (3,4-DAP), which blocks delayed-rectifier potassium channels (Meir *et al*, 1999; Sivaramakrishnan *et al*, 1991). 3,4-DAP, a potassium-channel blocking agent, is notable in that it allows the isolation and study of BK channels and creates a better understanding of the influence of calcium concentrations on repolarization processes. Sivaramakrishnan *et al* (1991) found that the application of 4-AP, a similar substance to 3,4-DAP, prolonged the length of action potentials by

blocking all potassium channels except those of the calcium-activated variety.

In our experiment, we expected modulation of external Ca^{2+} concentrations to impact chemical synaptic transmission. As shown by Fatt and Katz (1952), when the neuromuscular junction is bathed in a solution that has a low Ca^{2+} concentration, stimulating the motor neuron evokes end-plate potentials with reduced amplitudes. Modulation of extracellular Ca^{2+} concentrations becomes even more interesting in the presence of 3,4-DAP. We would expect to find an impact on repolarization time as a result of chemical blocking of the voltage-gated potassium channels, which effectively forces the cell to depend upon calcium-activated potassium channels to achieve repolarization. We postulated that a decrease in extracellular concentration would cause a decreased calcium intake which would, in turn, reduce the activity of the calcium-activated potassium channel. We expected results to show that a decrease in the activation of K_{Ca} channels would delay presynaptic cell repolarization and this delay would be reflected in a prolonged repolarization of the postsynaptic cell. We found that, contrary to our hypothesis, crayfish presynaptic cells exposed to 3,4-DAP exhibited a faster repolarization rate than crayfish cells in normal Ringer's solution. Additionally, repolarization duration was slower when extracellular calcium concentrations were halved or eliminated. These results point to a possible alternative hypothesis that K_{Ca} channels, either under the pressure of being the only active potassium channels or while exposed to 3,4-DAP, might perform their task more efficiently and thus manage to repolarize the cell at a faster rate than normal. This efficiency is lowered as extracellular calcium concentrations decrease.

We chose to use crayfish for this investigation as the crayfish neuromuscular junction (NMJ) proves an excellent preparation for research on presynaptic facilitation. Crayfish are cheap and easily attainable. They are also, and importantly, poikilothermic animals. This characteristic allows the cooling down of crayfish to a level of inactivity at which point they can undergo dissection painlessly. Further, crayfish motor axons and muscle bundles are easily isolated for stimulation and recording. Crayfish NMJ nerve terminals are fairly uncomplicated, with only two known types of potassium channels: delayed-rectifier and calcium-activated ones. Finally, crayfish NMJ have large magnitudes for short-term synaptic enhancement (Vyshedskiy *et al*, 2000; Cattaert, Le Ray, 2001).

MATERIALS AND METHODS

Crayfish Preparation

The experiment was carried out using muscle cells of an isolated crayfish tail. We first dissected the crayfish tail along the edges of the fast extensor muscles according to standard procedure. Using small scissors, we separated the ventral part of the abdomen from the rest of the abdomen and removed the large mass of muscle at the dorsal part of the abdomen. The crayfish tail was placed in a Sylgaard dish and pinned down at each end in a slightly stretched position to ensure that the muscle bundles and motor neurons were both clearly visible. The crayfish tail was covered with standard Ringer's solution (see Table 1). When possible, the solution was replaced with fresh solution of the same composition approximately every ten minutes over the course of the experiment in order to preserve the crayfish preparation. Nonetheless, variation in crayfish and crayfish dissection, as well as inability to replace the saline solution regularly once 3,4-DAP had been added, complicated our preparation. The Sylgaard dish containing the preparation was placed under the microscope.

Chemical Component	Amount (mM)
NaCl	205
KCl	5.4
$CaCl_2$	13.5
$MgCl_2$	2.6
Tris Buffer (pH 7.4)	10.0

Table 1. Chemical components of normal crayfish Ringer's. The column on the left gives the chemical components composing normal crayfish saline while the column on the right column shows the amount (in mM) of each component.

Chemical Component	Amount (mM)
NaCl	205
KCl	5.4
$CaCl_2$	0.0
$MgCl_2$	16.1
Tris Buffer (pH 7.4)	10.0

Table 2. Chemical components of calcium-free Ringer's. The column on the left gives the chemical components composing normal crayfish saline while the column on the right column shows the amount (in mM) of each component.

Chemical Preparation

We used standard Ringer's solution (see Table 1) and/or calcium-free Ringer's solution (see Table 2) to create our solutions. In all cases, we bathed the crayfish NMJ in 125mL of solution. We used either standard Ringer's, calcium-free Ringer's,

or a half-and-half solution (61.875mL of standard Ringer's and 61.875mL of calcium-free Ringer's). We added 1.25mL of 100mM 3,4-DAP to 123.75mL of solution in each of our 3,4-DAP-exposed experiments in order to create 125mL of solution with 1mM 3,4-DAP, a molar amount used by Sivaramakrishnan et al (1991). We used 3,4-DAP because it blocked delayed-rectifier K^+ channels, but not K_{Ca} channels.

Electrode Preparation

Using an electrode puller, we made some measuring glass microelectrodes which we filled with 3.0M KCl, making sure to prevent the formation of bubbles that would increase the level of resistance in the voltage readings. We filled the electrode holder with 3.0M KCl and attached one microelectrode to it and fixed this to a micromanipulator. We set up a second micromanipulator, this time with a fire-ground stimulating electrode. Both the stimulating electrode and the recording electrode were grounded to a metal grounding plate. The stimulating electrode was then connected to a Square-pulse generator and the recording electrode was connected to a microamplifier and interfaced with a computer running the MacLab software suite. Both grounding electrodes were fixed to the Slygaard dish. Using MacLab's "bridge amplifier" tool, we inserted the recording electrode into the Ringer's solution and checked to ensure that resistance was at an acceptable level (we aimed for a resistance between 5 and 20 MΩ). We used Maclab software to measure resting potential with a microelectrode to pierce the cell membrane and a base electrode placed in the extracellular solution to measure potential difference. Using the micromanipulator, we then positioned the tip of the stimulating electrode near the motor axon of a crayfish tail muscle bundle. The recording electrode was placed in the Ringer's solution.

Recording

A measurement was taken to determine the electrode resistance. We zeroed our recording device (MacLab) before sucking a small amount of Ringer's solution in the stimulating electrode. We then sucked up the motor axon to be stimulated. Using the micromanipulator, we inserted the tip of the recording electrode into a portion of medial muscle in the muscle bundle closest to the stimulating electrode. Beginning with a low voltage, we used the Square-pulse generator to stimulate the motor axon, slowly increasing the voltage until an EPSP was observed, at which point we recorded both the amplitude and duration of the polarization, measuring duration as the time between peak amplitude and half amplitude (see Figure 1). Recording half-amplitude instead of complete repolarization minimized the confounding effect of electrical noise and the possibility of skewed results due to the subjective nature of the decision regarding what might constitute full repolarization (because repolarization does not always mean returning to *exactly* the same voltage as before the EPSP). Each recording was taken from a different muscle cell and the recording device was zeroed between recordings.

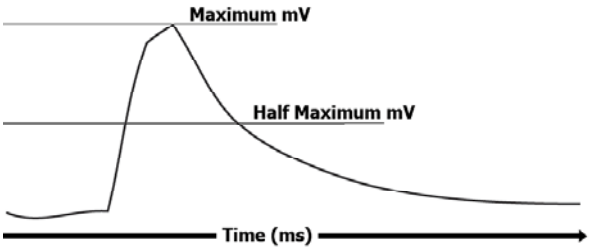

Figure 1. In order to minimize the confounding effect of electrical noise, we recorded at the half maximum mV (half amplitude) level.

RESULTS

This experiment was designed to answer two questions: Firstly, is there a difference in the duration of repolarization of the presynaptic cell between crayfish exposed to blocking agent 3,4-DAP and unexposed crayfish as reflected in EPSP repolarization duration? Secondly, does the modulation of extracellular calcium concentration produce a change in duration of EPSP repolarization for crayfish exposed to 3,4-DAP?

We were able to reject the null hypothesis when comparing our control to each of our experimental conditions with greater than 95% confidence in each case, meaning we found a statistically significant effect for each of our experimental conditions. The addition of 3,4-DAP, as well as modulation of extracellular calcium concentration in the presence of 3,4-DAP, had an impact on EPSP duration. Surprisingly, we found 3,4-DAP-exposed crayfish cells in standard Ringer's solution exhibited a shortened EPSP repolarization duration when compared to unexposed crayfish cells. This result contradicted our first hypothesis. Among 3,4-DAP-exposed crayfish with modulated extracellular calcium concentrations, we found that lower extracellular calcium concentrations resulted in longer EPSP repolarization duration. In sum, we found external Ca^{2+} concentration to be a key determinant of repolarization time in the presence of 3,4-DAP. Our results are summarized in Figure 2.

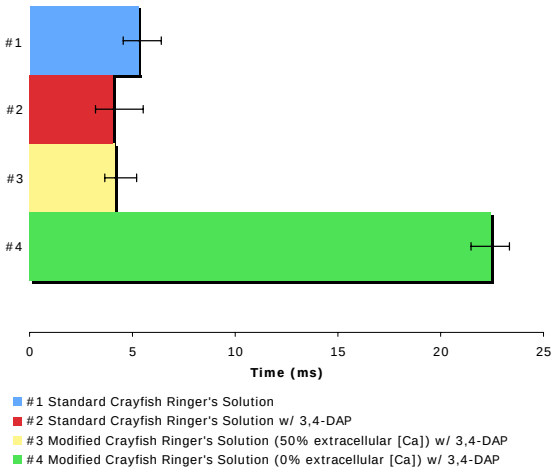

Figure 2. Mean time to half repolarization for control and each of our three experimental conditions. Bars represent Standard Error. For solution #1 n=6, for solution #2 n=21, for solution #3 n=4, and for solution #4 n=5. For solution #1 compared to solution #2, p=.001647768; for solution #1 compared to solution #3, p=.000373619; for solution #1 compared to solution #4 p=5.09063E-09.

DISCUSSION

Our experiment compared the EPSP repolarization duration in cells exposed to 3,4-DAP as well as crayfish cells exposed to different levels of extracellular calcium concentration in the presence of 3,4-DAP.

We postulated that introduction of 3,4-DAP to the crayfish neuromuscular junction would induce a change in EPSP duration in comparison to standard, unmodified crayfish Ringer's solution. Our hypothesis was that the 3,4-DAP-exposed crayfish NMJs would produce longer-duration EPSPs, reflecting lengthened duration of repolarization at the presynaptic terminal. The experiment did not support our hypothesis that EPSP duration would be longer in 3,4-DAP-exposed crayfish NMJs. EPSP duration was shorter, rather than greater, in the 3,4-DAP solution: EPSP duration fell from an average of 5.304ms in unmodified Ringer's solution to an average of 4.058ms in the 3,4-DAP solution. The presence of 3,4-DAP might have prompted calcium-activated potassium channels to become much more efficient in assisting in repolarization. It is also possible another unstudied ion channel is replacing the function of the potassium channels.

Our second experimental question asked whether a difference in extracellular calcium concentrations in 3,4-DAP-exposed crayfish would produce a significant difference in EPSP repolarization time. We hypothesized that modified Ringer's with lower calcium concentrations, specifically half-calcium and zero-calcium concentrations, would prolong repolarization time. Our data supported this hypothesis: the average EPSP duration for standard, 3,4-DAP-exposed Ringer's solution was 4.058ms; for half-and-half solution, was 4.144ms; and for calcium-free solution, was 22.45ms. Our results in the second experiment suggest that extracellular calcium concentrations might help regulate the level of activity of the 3,4-DAP cell.

While in our second experiment we learned the duration of the repolarization was lengthened when extracellular calcium concentrations were reduced to zero, we found it was not possible, at least within the scope of our study, to demonstrate how repolarization could be halted altogether. Our results lead us to believe that repolarization will almost always happen. Nevertheless, it might be interesting to find out whether there is a necessary timeframe within which repolarization must happen. Future work in this area might seek to determine the duration of repolarization deemed "good enough" for the cell to be considered as still functioning well. This means that if a cell takes a longer period to repolarize, the cell would be considered unviable. Having determined this length of time, research could be carried out to show the minimum level of extracellular calcium concentration necessary to maintain cell viability.

ACKNOWLEDGEMENTS

We would like to thank Professor Clark Lindgren, our lab assistant Sue Kolbe, and our student assistant Zach Newman for their time and assistance. We also thank our reviewers, Abby Griffith, Hanna Liebl, Matthew Clarke and Clark Lindgren, for their constructive feedback on an earlier draft of this paper.

REFERENCES

Augustine, G.J., M.P. Charlton, S.J. Smith. 1987. Calcium Action in Synaptic Transmitter Release. *Annual Review of Neuroscience* **10**: 633-693.

Cattaert, D., D. Le Ray. 2001. Adaptive Motor Control in Crayfish. *Progress in Neurobiology* **63**: 199-240.

Dobbs, L., J. Kawasaki, P. Malik. 2002. Broadened Action Potentials in the Presynaptic Cell Increase Synaptic Delay in Crayfish Neuromuscular Junction. *Pioneering Neuroscience* **4**: 31-36.

Fatt P., B. Katz. 1952. Spontaneous Subthreshold Activity at Motor Nerve Endings. *Journal of Physiology* **117**:109-128.

Lara J., J.J. Acevedo, C.G. Onetti. 1999. Large-Conductance Ca^{2+}-Activated Potassium Channels in Secretory Neurons. *Journal of Neurophysiology* **82**: 1317-1325.

Meir A., S. Ginsburg, A. Butkevich, S.G. Kachalsky, I. Kaiserman, R. Ahdut, S. Demirgoren, R. Rahamimoff. 1999. Ion Channels in Presynaptic Nerve Terminals and Control of Transmitter Release. *Physiology Review* **79**: 1019-1088.

Sivaramakrishnana, S., M.S. Brodwick, G.D. Bittner. 1991. Calcium-Activated Potassium Conductance in Presynaptic Terminals at the Crayfish Neuromuscular Junction. *Journal of General Physiology* **98**: 1161-1179.

Sivaramakrishnana, S., M.S. Brodwick, G.D. Bittner. 1991. Presynaptic Facilitation at the Crayfish Neuromuscular Junction: Role of Calcium-Activated Potassium Conductance. *Journal of General Physiology* **98**: 1181-1196.

Vyshedskiy, A., T. Allana, J.W. Lin. 2000. Analysis of Presynaptic Ca2+ Influx and Transmitter Release Kinetics during Facilitation at the Inhibitor of the Crayfish Neuromuscular Junction. *Journal of Neuroscience* **20**(17): 6326-32.