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Excitatory Effects of Benzamil on the Sodium-Calcium Exchanger at the Crayfish Neuromuscular Junction.

JOSH LAVIN, ALEX ODOM, and SAM MCCONNELL
Department of Biology, Grinnell College, Grinnell, Iowa

ABSTRACT

In crayfish neurons, the sodium-calcium exchanger (NCX) pumps calcium ions out of the cell in exchange for sodium ions. The chemical benzamil has been found to be inhibitory to the NCX in crayfish antennal gland basolateral membrane vesicles (BLMVs) at concentrations under 30μM and excitatory at higher concentrations (Wheatly et al. 2002). We predicted that concentrations of 60μM and 100μM would excite the neuromuscular junction (NMJ) of the crayfish tail, leading to lower EPSPs. We also attempted to determine whether the effects of benzamil are pre- or post-synaptic by analyzing ratios of EPSPs generated by paired-pulse stimulation. We found that benzamil was excitatory at 60μM and 100μM and the results of the paired-pulse stimulations suggest pre-synaptic effects.

INTRODUCTION

The NCX plays a critical role in the pre-synaptic neuron of the NMJ by removing intracellular calcium ions after an action potential. Therefore, excitation of this exchanger should facilitate the removal of some intracellular calcium, allowing the cell to return its calcium levels to their resting state more quickly. The effects of the drug Benzamil on the NCX, have been studied in crayfish hepatopancreas and antennal gland basolateral membrane vesicles. The NCX in the antennal gland was inhibited by concentrations under 30μM and excited by those over 30μM (Wheatly et al. 2002). However, no effect was observed on the NCX of the hepatopancreas (Wheatly et al. 2002). It has also been studied in protein kinases where it had the effect of slowing down PKC activity (Giambalvo 2004).We aim to add to these results by recording the effects of 60μM and 100μM of Benzamil on the NMJ of the crayfish tail and determining if the effects are pre- or post-synaptic. The crayfish NMJ is commonly used among scientists because they are inexpensive, have a fast and simple dissection, and exhibit many of the same neural properties and cellular functions as humans (Liu, Killilea and Ames, 2002).

Our research is important because it provides further insight into the NCX, adding to our understanding of the crayfish NMJ. It also generates new data on the excitation of the NCX which, allows us to see the effect that Benzamil can have on the NCX in crayfish. Previous studies have shown Benzamil to affect blood vessels in rats (Wang et al. 2008), and kidney cells (Fischer et al. 2002), so observing its effects in crayfish when compared to other studies can inform the effects it may have on other animals, and different types of cells in humans. To test the effect of excitatory levels of Benzamil on the NCX, our lab group inhibited the exchanger using 60μM and 100μM of Benzamil and recorded the EPSP amplitude after the first and second stimulation in paired-pulse facilitation. We used the amplitude of the first EPSP to determine whether Benzamil had an effect on the NMJ. We expected lower amplitudes in the Benzamil trials than in the control because the NCX would help to return internal calcium levels to normal more quickly than usual. Paired-pulse facilitation was used to determine whether the effects of Benzamil were pre- or post-synaptic. We hypothesized that if the effects were pre-synaptic, the ratio of the amplitude of the second EPSP to the first EPSP would be lower after application of Benzamil than in the control. Because paired pulse stimulation consists of two pulses in quick succession, the pre-synaptic cell doesn't have long to pump calcium out between pulses. This leaves extra calcium in the pre-synaptic cell when the second pulse occurs and causes the EPSP to be higher than normal. Therefore, excitation of the NCX would allow the pre-synaptic cell to pump out more calcium in that time period than normal, leading to a lower second EPSP. However, if the ratio of the amplitude of the second EPSP to the first EPSP were to stay the same or increase, then Benzamil might also be affecting the post-synaptic cell. We found that benzamil did indeed reduce the amplitude of the first EPSP and that its effects appeared to be pre-synaptic.

MATERIALS AND METHODS

To determine whether excitatory concentrations of the drug benzamil would have an effect on the NMJ of crayfish tails (and whether this effect is pre- or post-synaptic), EPSPs of deep abdominal extensor muscles of

crayfish were observed at three different time points while exposed to two different concentrations of benzamil.

Preparation/Dissection

Before any incisions were made we anesthetized the crayfish by covering it with ice for 15 minutes. Then we cut off the tail and removed its ventral surface. To isolate the dorsal superficial extensor muscle, we cut the exoskeleton just shallow of the ventral surface and pulled off the ventral exoskeleton and muscles, scraping our finger along the exoskeleton to remove all muscle mass but the superficial extensor.

Solutions

The initial measurement was done in 100mL of standard crayfish saline (205 mM NaCl, 5.4 mM KCl, 13.5 mM CaCl2 2.6 mM, and MgCl2 10.0 mM with a pH of 7.4). Since the Benzamil was first diluted with DMSO and then diluted into standard crayfish saline, we used the standard ringer solution mixed with the same dilution of DMSO that would be applied if we added 60µM of Benzamil as our control (833.:1 ml). Our Benzamil solutions were 60µM and 100µM both diluted in 100ml of Standard Ringer Solution.

Electrophysiology

We used glass microelectrodes created by a PUL-1 pipette puller in two ways. The first was to record the EPSPs in the muscle cells; for this we used a sharp microelectrode and an electrode holder backfilled with 3M KCl to record intracellularly. We recorded the resistance to be 146mv- 158mv for all of our results. The tip of the other microelectrode was filed down to make a suction electrode with a diameter of approximately .6mm. We used this electrode to suck up the nerve in order to expose it to electrical stimulus. Before suction, this electrode was filled with the current solution in the dish in order to provide a constant medium for stimulation. The electrical stimulus was provided by a Grass SD9 electronic timulator.

RESULTS

Our goal was to determine whether exciting the NCX with 60µM and 100µM of Benzamil would decrease the amplitudes of EPSPs over time, and if so, whether this effect was pre or post-synaptic. We did this by using paired-pulse facilitation (stimulating the nerve twice in rapid succession). We expected that the amplitudes of the EPSPs caused by the first pulse would decrease after application of benzamil, and that the ratio of the second EPSP to the first EPSP would also decrease if the effects were pre-synaptic. We expected that DMSO would have no effect on the EPSPs.

The results in Fig. 1 show that DMSO had negligible effects on EPSPs at 5 and 10 minutes, as we expected. However, we unexpectedly observed a change in EPSPs at 15 minutes, meaning that we have to take into account its effects at that time point.

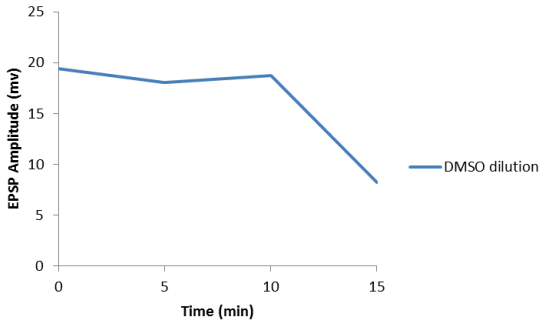

Figure 1. EPSP amplitude at 0, 5, 10, and 15 minutes of exposure to DMSO diluted 833.3:1 ml (dilution equal to 60µM Benzamil). There are .12ml of DMSO in 100ml of Standard Ringer Solution to give the same dilution as the 60µM Benzamil. EPSP amplitude for each time interval is the average of 5 recordings.

Since we determined the effects of DMSO did not change the amplitude from 0-10 minutes we can use the Standard Ringer Solution as the baseline at those time points for the experiments containing Benzamil. Fig. 2 shows the decrease in amplitude after the application of both concentrations of Benzamil.

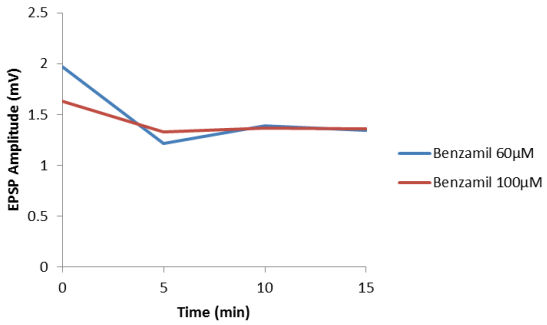

Figure 2. EPSP amplitude at 0, 5, 10, and 15 minutes of exposure to 60µM and 100µM of Benzamil. EPSP amplitude is the average of 5 recordings. The baselines of the controls are different because a different muscle cell was used.

The decrease appears to be fairly constant even at higher concentrations. Both concentrations decreased to around the same values.

Another question that arose during our research was whether the effects of benzamil were pre- or post-synaptic. We deduced that if the ratio of the second EPSP to the first EPSP were to decrease, then Benzamil most

likely affects the exchangers in the presynaptic cell. However, if the amplitude were to be different but the ratio remained constant, then the effects could also be located in the postsynaptic cell. The results depicted in Fig. 3 suggest that the results are pre-synaptic.

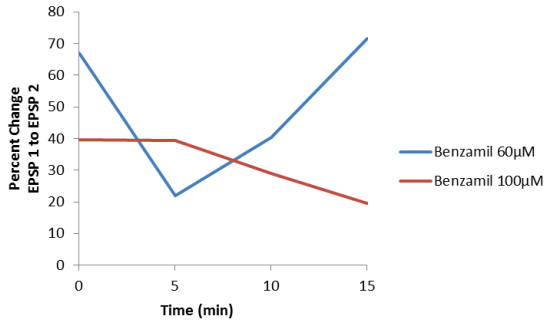

Figure 3. Change in EPSP amplitude in paired-pulse facilitation at 0, 5, 10, and 15 minutes of exposure to 60 and 100µM of Benzamil. The ratios at each time interval are an average of 5 recordings. The baselines of the controls are different because a different muscle cell was used.

Since we are disregarding the results at 15 min because we cannot rule out the effects of DMSO there, the ratio is lower in both concentrations. The second EPSP increases from the first to the second stimulus in all cases, but the ratio of the second EPSP to the first is lower at both 5min and 10min after application of benzamil.

DISCUSSION

Our data indicated that our hypothesis, that Benzamil would excite the NCX in the pre-synaptic cell, leading to lower amplitudes of initial EPSPs and lower ratios of the second EPSPs to the initial EPSPs, was correct. The amplitudes of the EPSPs decreased when exposed to both 60µM and 100µM of Benzamil for 5min and 10min, but did not change at those same time points when exposed to DMSO. This indicates that Benzamil caused the effect, and the fact that it decreased is consistent with our hypothesis that the benzamil excited the NCX. However, this does not tell us if the effect is pre- or post- synaptic. We determined that the effect was pre-synaptic because the ratio of the second EPSP to the first EPSP decreased at 5min and 10 min in Benzamil trials. This is consistent with our prediction that the benzamil would excite the NCX in the pre-synaptic cell which would cause a reduction in its intracellular calcium levels.

Our results are consistent with the findings of Wheatly et al. that when benzamil has an effect, it is excitatory at concentrations above 30µM. Future work should be done in the crayfish NMJ at concentrations below 30µM to determine if those concentrations are inhibitory to the NCX, as the Wheatly et al. study would suggest.

One question that arises is why is Benzamil inhibitory at under 30µM yet excitatory? We predicted that when the exchangers are inhibitory to a certain degree it causes other exchangers and channels to become excitatory. So it is possible that the Na+-Ca+ exchanger is completely inhibited and the results we recorded were from other channels and exchangers excitation.

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