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Inhibiting Endogenous Hydrogen Sulfide Production Decreases Amplitudes of EPSPs at the Crayfish Neuromuscular Junction.

Cory Brooke-deBock, Justyn King, and Sam Sherwood
Department of Biology, Grinnell College, Grinnell, Iowa

ABSTRACT

H_2S has been shown to have an effect at the neuromuscular junction. The enzymes CBS and CSE both produce H_2S, but at the neuromuscular junction CBS is mainly responsible for its production. We determined that H_2S alters synaptic transmission at the crayfish neuromuscular junction by decreasing the amplitude of EPSPs. This was accomplished by inhibiting the enzyme CBS and comparing inhibited EPSPs to baseline EPSPs. In order to inhibit the enzyme we used hydroxylamine. In addition to inhibiting CBS, hydroxylamine is known to create nitric oxide (NO). This variable was eliminated by the application of Carboxy-PTIO, a NO chelator. These data show that H_2S acts as neuromodulator at the crayfish neuromuscular junction, however it might have different effects in crayfish than in mammals.

INTRODUCTION

The article "Hydrogen sulfide: Neurochemistry and neurobiology" (Qu et al, 2007) mentions that H_2S plays a very important role in the brain. It can act as a neuromodulator as well as an intracellular messenger. H_2S affects the levels of Calcium in the cell and has an effect on the NMDA receptors present in the post-synaptic cell. It also acts as a messenger between Glial cells by helping in the creation of calcium waves that can communicate with other cells (Qu et al, 2007).

Hydrogen sulfide is produced throughout the body by two enzymes. One enzyme is cystathionine β-synthase (CBS) and the other is cystathionine y-lyase (CSE). Previous studies have shown that the enzyme CBS is mainly responsible for producing H_2S in neurons (Abe & Kimura, 1996). Inhibition of CBS and consequently the inhibition of H_2S can be accomplished by the application of the compound Hydroxylamine (Abe & Kimura, 1996).

According to Southam and Garthwaite (1991) an additional effect of applying hydroxylamine is the production of nitric oxide (NO). Nitric oxide, like H_2S, is a known neuromodulator (Giraldi-Guimarães et al, 2007). The production of NO is another variable that, in addition to H_2S, could affect the EPSPs. In order to eliminate this variable we applied Carboxy-PTIO, a NO chelator, to the saline solution.

Previous studies have shown that H_2S exists in the rat, human and bovine brain (Abe & Kimura, 1996). However, we are unaware of any studies confirming the existence of H_2S in crayfish. Therefore we sought to determine if H_2S alters synaptic transmission at the crayfish neuromuscular junction.

In order to achieve this goal we used the CBS inhibitor hydroxylamine. Our hypothesis was that when CBS was inhibited, the absence of H_2S would affect the synapse at the crayfish neuromuscular junction. To test this hypothesis we first recorded Excitory Post-Synaptic Potentials (EPSPs) at the uninhibited synapse. After this was accomplished we applied Hydroxylamine to inhibit CBS and then we recorded EPSPs at the inhibited synapse. The inhibited EPSPs were then compared to the uninhibited and analyzed for potential changes. We have found that when CBS is inhibited at the crayfish neuromuscular junction there is a significant reduction in the amplitude of inhibited EPSPs therefore verifying our hypothesis.

MATERIALS AND METHODS

Preparation

Two different types of crayfish were used: *Pacifisticus leniusculus, Oroconectes rusticus*. The crayfish were taken off ice and their tails were removed from their torso. The tail was then dissected to expose the extensor muscles on the dorsal surface. The dissected tail was then pinned in a dish and submerged in 100 mL of a saline solution. We used either a normal crayfish saline solution (2, 6) or a low calcium saline solution (3,4,7,8). Both of them were produced in our lab. In addition to the saline solution, a NO chelator, Carboxy-PTIO, was added in order to eliminate the potential extra variable, NO, however some of our preliminary data did not have Carboxy-PTIO added.

Chemicals

We used two general types of saline solutions. One was the normal crayfish saline solution and the other was a low calcium saline solution. The chelator Carboxy-PTIO was added to all the solutions. The CBS inhibitor, Hydroxylamine was added to half of the saline solutions. All this is summarized in the chart below (all values in mM unless otherwise noted).

#	**Saline** solution	**KCl**	**NaCl**	**Na hepes buffer**	**$MgCl_2 6H_2O$**	**$CaCl_2 2H_2O$**	**Hydro-xyla-mine**	**Car-boxy-PTIO (nM)**
1	Ca	5.4	196	10	2.6	13.5	0	0
2	Ca	5.4	196	10	2.6	13.5	0	250
3	Low Ca	5.4	196	10	14.1	2	0	250
4	Low Ca	5.4	196	10	10.5	5.6	0	250
5	Ca	5.4	196	10	2.6	13.5	1	0
6	Ca	5.4	196	10	2.6	13.5	1	250
7	Low Ca	5.4	196	10	14.1	2	1	250
8	Low Ca	5.4	196	10	10.5	5.6	1	250

We used two different chemicals, Hydroxylamine and Carboxy-PTIO. Hydroxylamine was obtained from the company Sigma Aldrich online. The stock form of Hydroxylamine was 100 mM and was diluted at a ratio of 1:1000 with normal crayfish saline. It inhibits the enzyme CBS, this enzyme is responsible for the production of H_2S. However a side effect of the inhibition process may be the production of NO (Abe & Kimura, 1996). The other chemical used in this experiment was Carboxy-PTIO, a NO chelator. It was attained from the chemical supply company Invitrogen. Its purpose was to prevent any effect produced by hydroxylamine. It did this by chelating NO in the crayfish saline.

Measurements

To measure the EPSPs we used microelectrodes with 1.2-millimeter diameters made from Borosilicate Glass capillaries produced by WPI. To create these electrodes we used a pipette puller manufactured by WPI. These electrodes were then filled with 3 M KCl and then rinsed with a crayfish ringer solution to remove excess KCl on the exterior of the tip. The resistances of the microelectrodes were optimally between 1 and 25 mega ohms.

To stimulate the nerves of the crayfish tail, and generate EPSPs, we used a suction electrode to capture a nerve bundle. An electrical pulse was applied to the nerve bundle through the suction electrode. A Grass SD9 Stimulator manufactured by Grass Instruments generated the electrical pulse. We stimulated the nerve at a frequency of 1 Hz for around .8 ms at a voltage ranging between 2.5 and 3.5 volts.

The EPSPs were recorded by the microelectrodes that were placed in the same tail segment as the nerve. A PowerLab 4/25, manufactured by ADInstruments, interpreted these EPSPs, and displayed them on the computer with the program Scope version 4.03 (ADInstruments). For each cell the nerve was stimulated four times and the resultant EPSPs were averaged by Scope.

Analysis

We measured the averages of EPSPs in at least three different cells within each dissected crayfish in the uninhibited saline. After the crayfish tail was saturated with the inhibited saline for at least five minutes we measured the inhibited EPSPs in a minimum of three different cells. In some cases we reapplied the uninhibited saline and measured at least three more EPSPs in muscle cells.

The amplitudes and the duration of the uninhibited EPSPs were measured and used as baseline data in which to compare with inhibited EPSPs. This was to examine the change in the EPSPs between uninhibited and CBS inhibited cells in each individual crayfish. We then used a t-test to determine the statistical significance of our data.

RESULTS

In examining the effects of H_2S at the crayfish neuromuscular junction the enzyme CBS was inhibited. This halted the production of H_2S in the crayfish, therefore allowing us to determine if H_2S played a role at the crayfish neuromuscular junction. This was accomplished by comparing H_2S inhibited EPSPs to baseline EPSPs collected from five different crayfish.

The first method used to analyze the data was to compare the mean of the inhibited EPSPs to the baseline EPSPs and then to the wash EPSPs (fig. 1). This analysis demonstrates that time does not have an effect on the change in the EPSPs because figure 1 shows that when the wash is applied, the H_2S inhibited EPSP amplitudes return to amplitudes similar to the baseline.

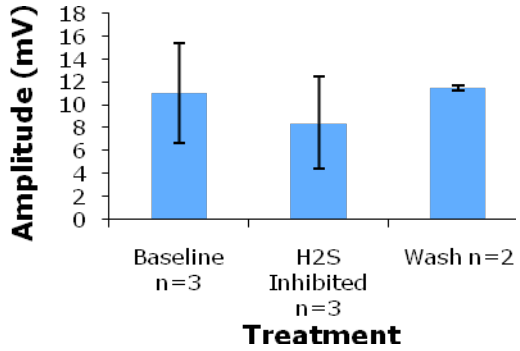

Fig. 1: The mean amplitude of EPSPs, before and after H_2S inhibitor was applied. Error bars represent the Standard Error of the means of the EPSPs.

Another way the collected data were analyzed was by comparing the percent change of the H_2S inhibited EPSPs to the baseline EPSPs (fig. 2). The graph shows that there was a noticeable drop in amplitude of the EPSPs when the CBS inhibitor was applied. The figure also shows that the addition of Carboxy-PTIO had little effect on the percent change of the EPSPs. However, these data were unable to be statistically supported through a t-test because the factor of time could not be accounted for. The figure still demonstrates that the inhibition of H_2S could have an effect on EPSPs at the crayfish neuromuscular junction.

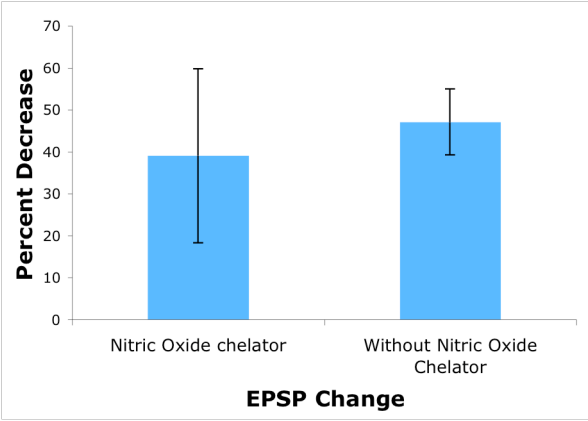

Fig. 2 Percent decrease of the H_2S inhibited EPSPs when compared to the Baseline EPSPs. The first bar represents data from solution with Carboxy-PTIO (n=3) and the second bar represents data from solutions without Carboxy-PTIO (n=2). Error bars represent Standard Error of the mean of the change in EPSPs

To determine if H_2S has a significant effect at the neuromuscular junction we compared the means of our baseline EPSPs to the H_2S inhibited EPSPs (fig. 3). This graph included the data collected without Carboxy-PTIO (solutions 1,5) because the NO chelator had little effect on the EPSPs at the neuromuscular junction (fig. 2). The t-test that was performed resulted in a p-value of .027, which allows us to reject the null hypothesis, meaning the differences are statistically significant. When all three figures are examined it becomes apparent that the inhibition of CBS and therefore the removal of H_2S from the neuromuscular junction lowers the amplitudes of EPSPs.

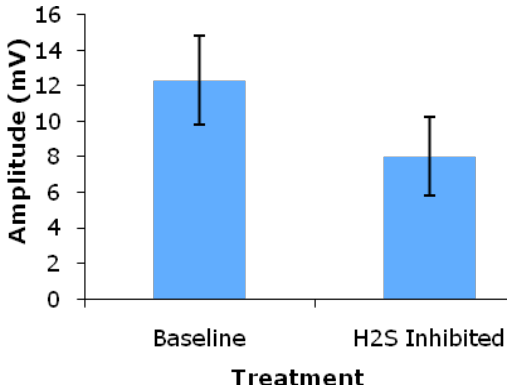

Fig 3: The effects of inhibiting the enzyme CBS and halting the production of H_2S. Graph includes treatment solutions that do not have an NO cheleator dissolved in it. n=5 and a paired two tailed t-test resulted in the number .026. The error bars represent the standard error of the mean of the EPSPs.

DISCUSSION

Hydrogen sulfide has been proven to be a neuromodulator in many different organisms. Our results demonstrate that it is also present at the crayfish neuromuscular junction. The enzymes CBS and CSE create H_2S. Studies have shown that CBS is mainly responsible for the production of H_2S at the neuromuscular junction (Qu et al, 2007). Hydroxylamine has been shown to inhibit the creation of H_2S by blocking the enzyme CBS (Abe & Kimura, 1996). We used hydroxylamine to inhibit CBS and to observe the effects of H_2S at the crayfish neuromuscular.

We hypothesized that when CBS was inhibited there would be a noticeable change in the EPSP when compared to an uninhibited EPSP. The results that were obtained demonstrate that there is indeed a noticeable change in the EPSP. The results also show (fig. 1,2,3) that the amplitude of the EPSPs following H_2S inhibition decreases in comparison to baseline EPSPs. These results are interesting and perhaps counter to the conclusions made by Abe and Kimura (1996).

Abe and Kimura (1996) performed experiments on the effect of H_2S on adult rat brains. One of the experiments Abe and Kimura (1996) performed was to measure EPSPs in rat brains at baselines levels and also with external H_2S applied. Their results showed that when the external H_2S was applied the EPSPs were suppressed. Their data suggest that as levels of H_2S increase the EPSPs shrink. Our data suggest the opposite. When we inhibited CBS, and therefore halted the production of

H_2S, we found that the inhibited EPSPs also decreased in amplitude. This is counter because in our experiment our neuromuscular junction was lacking H_2S and if our results had been consistent with Abe and Kimura (1996) the EPSPs would have increased.

Our data still holds valid even though it counters Abe and Kimura (1996). A possible reason for this discrepancy could be that H_2S affects crayfish and rats in different ways. More experiments at both the crayfish and rat neuromuscular junctions would have to be performed to verify if H_2S affects the two organisms differently. One possible way this could be done would be by testing other similar crustaceans beyond crayfish and also other rodents. This would be to see if H_2S acts in a similar matter throughout the two types of organisms.

Our results have suggested that H2S is present at the crayfish neuromuscular junction. It appears that when H_2S production is halted the EPSPs display a decrease in amplitude. This supports our hypothesis that the inhibition of CBS would affect the resulting EPSPs. With this knowledge there are several future experiments that could be performed. One experiment would be the addition of external H_2S to examine the trend that appears in our previous results that reducing H_2S decreases the amplitude of the EPSPs. Therefore if we increase H_2S we would to see an increase in the amplitude. Another experiment that could be performed would be to determine which receptor H_2S targets on the post-synaptic cell. In other organisms it is known to affect NMDA receptors (Qu et al, 2007)

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