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Simultaneous application of H_2S and inhibition of its endogenous production induces disparate synaptic response at the crayfish neuromuscular junction

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ABSTRACT

Hydrogen sulfide (H_2S) has recently been identified as a gaseous neuromodulator of synaptic transmission. Within the cell, H_2S is synthesized via two distinct enzymatic pathways, one beginning with cystathionine β-synthase (CBS) and the other with cystathionine γ-lyase (CSE). The objective of our research was to examine the role of H_2S, CBS, and CSE in synaptic transmission of the crayfish, a model organism in which, to our knowledge, no previous studies of this sort have been performed. To do so, we electrically induced an excitatory post-synaptic potential (EPSP), the amplitude of which is indicative of the extent of synaptic transmission. Measurements were taken under various chemical treatments: exogenous H_2S, CBS inhibition, CSE inhibition, or any combination thereof. Our results suggest that CBS plays a minor role in H_2S production whereas the role of CSE is more substantial. The presence of exogenous H_2S alone appears to have little impact, but in conjunction with a CBS or CSE inhibitor, H_2S has two divergent responses. Most significant are the enlarged EPSPs that result from the application of a CSE inhibitor along with exogenous H_2S.

INTRODUCTION

H_2S, a gas well-known for its unpleasant stench, has long been studied because of its toxic properties and its affect on the cardiovascular system. Recent findings suggest that it is produced in considerable amounts in mammalian tissues and has important physiological effects (Lowicka and Beltowski 2007). As a result, it has been classified as a gaseous neuromodulator, joining the ranks of carbon monoxide (CO) and nitric oxide (NO). Like CO and NO, H_2S is not stored in synaptic vesicles, easily permeates cell membranes, and relaxes smooth muscles. Similar to NO, it also acts by means of calcium-calmodulin enzyme activation (Boehning and Snyder 2003).

As determined by Abe and Kimura (1996), naturally occurring H_2S is synthesized from L-cysteine by cystathionine β-synthase (CBS) or alternatively by cystathionine γ-lyase (CSE). The former has been found to play an important role in the production of H_2S in the rat's central nervous system, whereas the latter has been found to play an important role in mammalian cardiovascular systems. CBS and CSE, enzymes crucial to the production of endogenous H_2S, can be completely inhibited by hydroxylamine hydrochloride and DL-propargylglycine, respectively (Abe and Kimura 1996).

Extensive research has been carried out in mammals to determine the connection between hydrogen sulfide and synaptic transmission. In particular, Chen et al. (2007) investigated its role in the enhancement of NMDA receptor-induced currents and the resulting changes in long-term potentiation. Other studies suggest that H_2S may regulate neuron-astrocyte signaling, as it has been shown that the presence of H_2S coincides with an increase in intracellular Ca^{2+} levels in astrocytes (Lowicka and Beltowski 2007).

These aforementioned findings have all been acquired through experimentation on mammals. Our intention is to use this background to conduct similar experiments in crayfish and to determine if indeed H_2S has a similar effect at the neuromuscular junction (NMJ). Experiments carried out in crayfish are of interest because similarities between crayfish NMJs and human glutamatergic synapses allow us to extend our conclusions to humans.

We hypothesized that increasing concentrations of exogenous H_2S will lead to an increase in EPSP amplitude, indicating enhanced synaptic transmission. Conversely, inhibition of CBS and CSE will lead to a reversible decrease in EPSP amplitude. Our results supported this hypothesis with respect to the inhibition of CSE; however, they did not support our intuition about exogenous application of H_2S and inhibition of CBS.

MATERIALS AND METHODS

Organisms and Dissection

Crayfish (*Oroconectes rusticus* or *Pacifisticus leniusculus*) were stored on ice for approximately 10 minutes prior to dissection. The tail of a crayfish was removed and its exoskeleton was cut along the lateral edges. The ventral exoskeleton, entrails, and large muscles were removed by peeling them away by hand, leaving only the dorsal exoskeleton and the attached extensor muscles. The crayfish tail was pinned to a dissection dish to prevent excessive motion during the

experiment. In order to mimic the natural intercellular environment, this dish was filled with standard saline solution (see Table 1). A Leica Zoom 2000 microscope was used to observe the muscle tissue in finer detail.

Preparation of Chemicals

- Standard Saline

The components of the physiological saline are summarized in Table 1. The volume of solution used was 100mL (enough to fully cover the specimen).

Component	Concentration (mM)
KCl	5.4
NaCl	196
MgCl	2.6
CaCl	13.5
Hepes Buffer	10

Table 1. Composition of standard saline meant to mimic the ordinary ionic environment of a crayfish muscle cell.

- H_2S

A 1mM stock solution was prepared by dissolving appropriate quantities of sodium hydrosulfide hydrate (NaHS) (Sigma-Aldrich St. Louis, MO) into standard saline solution. The stock solution was then diluted to 200, 400, and 600μM concentrations immediately before use.

- CBS inhibitor

A 100mM stock solution of hydroxylamine hydrochloride (Sigma-Aldrich St. Louis, MO) in standard saline was prepared and refrigerated until use. 100μL of stock was added to 100mL saline solutions each containing a different NaHS concentration (0, 200, 400, or 600μM) to make solutions of 100μM hydroxylamine hydrochloride in addition to the specified NaHS concentration. This concentration is known to fully inhibit CBS activity (Abe and Kimura, 1996).

- CSE inhibitor

A 100mM stock solution of DL-propargylglycine (Sigma-Aldrich St. Louis, MO) in standard saline was prepared and kept at a subfreezing temperature until use. 100μL of stock was added to a 100mL saline solution containing the desired NaHS concentration (0, 200, 400, or 600μM) to create a 100μM DL-propargylglycine concentration. This concentration is known to fully inhibit CSE activity (Abe and Kimura, 1996).

Recording EPSPs

To measure voltages across the membrane of an extensor muscle, it is necessary to create a conducting circuit with the cell membrane as one of its elements. Simply letting an exposed wire sit in the saline creates part of the desired circuit. The remaining part must make electrical contact with the inside of the cell. This is accomplished using a glass capillary that is heated and pulled to a sharp point by a pipette puller. Filling this tip with 3M KCl allows it to conduct and become part of the circuit. Microelectrode tips were rinsed with normal saline prior to use in order to prevent any changes of extracellular ionic concentrations that could occur when the electrode is placed in the saline solution that covers the specimen. Additionally, to ensure quality and consistency of measurements, air bubbles were avoided and only tips with an electrical resistance between 5 and 20 MΩ were used. For precise placement of the tip, we used a World Precision Instruments MM301 micromanipulator attached to a magnetic stand.

To actually take measurements from this circuit, an additional wire was connected from the electrode holder to a MacLab Bridge Amplifier, which in turn was connected to a PowerLab 4/25. This fed the data into the program *Scope (v4.0.3),* which created a trace exemplified by Figure 1. ADInstruments manufactured all three of these products.

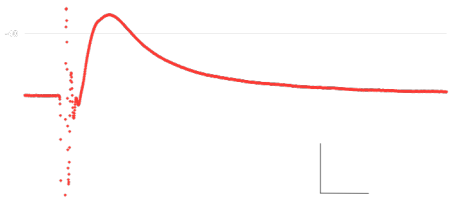

Figure 1. An example trace of an EPSP measured by Scope 4.0.3 and ADInstruments PowerLab. The axes shown represent a scale of 5mV by 5ms.

The amplitude of an EPSP was calculated using the "Max - Min" function in *Scope*'s DataPad over an interval that excluded the stimulus artifact. Data was imported into Microsoft *Excel* for further analysis.

Stimulus

A Grass Instruments SD9 Stimulator acted as the source of an electrical signal that would create an action potential in the motor axon, which then released neurotransmitters onto the muscle and induced the measured EPSPs. This device allows for the regulation of the frequency, delay, duration, and amplitude of the stimulus as well as sends out a prepulse to trigger the recording device. The standard settings used were, respectively, 0.5 pulses per second, 8.0 milliseconds, and 0.08 milliseconds, with the voltage amplitude varying between 1 and 100 Volts (the value used was the minimum required to elicit an EPSP). This stimulus was

then applied at a lateral point across the ridge that exists between tail segments using a two pronged stimulating device attached to a second MM301 on a magnetic stand. The stimulus was applied here because the nerve that controls the extensor muscle resides in this area.

Statistical Tests

To consolidate the raw data, we calculated the arithmetic mean of the EPSP amplitude measurements taken from each individual cell. A two-tailed, paired Student's T-Test was used to determine whether or not the chemical treatment made a significant impact on the EPSP amplitude (when the data allowed).

Additionally, a further mean was taken over all the cells under the same conditions, that is, the same crayfish specimen and applied chemicals. In order to compare these values to one another we had to take into account the natural difference in EPSP amplitude between any two crayfish. We did this by calculating the percent difference between the experimental amplitude and the control as given by the equation:

$$P.D. = \frac{A_{Experimental} - A_{Control}}{A_{Control}}$$

RESULTS

Our experiment tested the effects of H_2S on synaptic transmission at the crayfish neuromuscular junction. Specifically, the quality of synaptic transmission was quantified by measuring EPSP amplitudes under various predetermined conditions. These conditions included inhibition of endogenous H_2S production through CBS and CSE both alone and in conjunction with exogenous application of H_2S. Average measurements of EPSP amplitudes under these conditions are presented in Figure 2 as percent differences from control values.

As the concentration of applied H_2S was increased, the corresponding EPSP amplitudes decreased. However, due to extremely limited experimental data, no conclusion can be drawn about the addition of 200µM H_2S.

Adding the CBS inhibitor to a normal saline solution considerably increased EPSP amplitude. When H_2S was added to this inhibitor solution, the amplitudes decreased moderately relative to the control. As the H_2S concentration was increased, the percent difference from the control became steadily less negative.

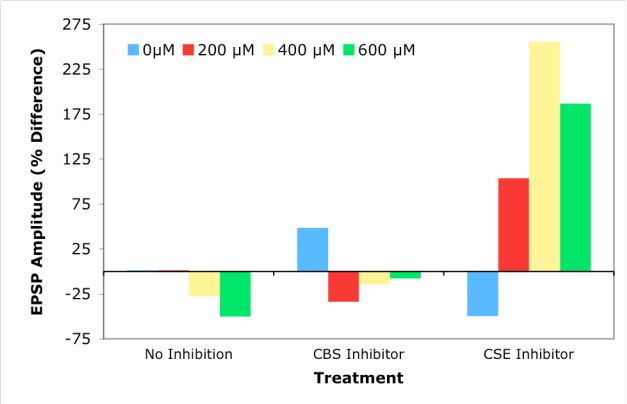

Figure 2. Values indicate the percent difference between the average EPSP amplitude post-treatment and that of the average control amplitudes measured in the same specimen.

Addition of a CSE inhibitor decreased EPSP amplitude by nearly 50%. As we increased the concentration of applied H_2S while maintaining CSE inhibition, EPSP amplitudes increased dramatically, reaching levels more than 250% higher than the control.

In addition to the percent differences in the average amplitudes, a Student's T-Test was used to assess the significance of amplitude differences. The resultant p-values are presented in Table 2. This test predominantly failed to reject the null hypothesis, except in the two cases of CSE and higher concentrations of H_2S.

	0µM	**200µM**	**400µM**	**600µM**
None	N/A	N/A	0.254*	0.124*
CBS	0.109	0.228	0.258	0.382
CSE	0.066	0.101	0.002	0.002

Table 2. A Student's T-Test was used to compare the Average EPSP amplitudes post-treatment (protein inhibition is designated by the row of the entry and concentration of exogenous H_2S by the column) to those of the control values measured in the specimen used that specific day. (* indicates n=3 for the experimental array and n=2 for the control array and is obtained through an unpaired T-Test, all other points are from a paired T-Test with n=4)

DISCUSSION

In biological systems other than the crayfish, H_2S has been shown to facilitate synaptic transmission. The presence of endogenous H_2S in the rat central nervous system was due to an enzyme reaction with CBS. However, in smooth muscle (such as the heart), CSE was the primary source leading to endogenous production of H_2S. Through this study, we hoped to find the primary source of endogenous H_2S in the crayfish NMJ as well as the effects of exogenous H_2S.

As shown in Table 2, the addition of exogenous H_2S did not cause any statistically significant change in EPSP amplitudes. This is potentially due to the fact that

crayfish tail extensor cells are already saturated with endogenous H_2S. We suspect that the cell produces at least as much H_2S as it is able to utilize for synaptic facilitation. Therefore, any extraneous H_2S is moot. Another explanation could be that because the H_2S was not always fresh it may not have been acting as we had expected. We suspect this because our results were more conclusive when the stock was prepared on the same day as it was used.

Contrary to our hypothesis, addition of hydroxylamine hydrochloride, a CBS inhibitor, led to an increase in EPSP amplitudes. Furthermore, Figure 2 shows that as H_2S was added, the EPSP amplitudes decreased, but then became less negative as the H_2S concentration was increased. In both cases, however, the data gathered lacked statistical significance. Thus, while the data does not support our hypothesis, it is also unable to adequately disprove the null hypothesis. This leads us to the conclusion that CBS may not play as significant role in the production of endogenous H_2S as previously suspected. However, our results may be affected by unaccounted for effects of hydroxylamine hydrochloride, such as the production of NO (Southam and Garthwaite 1991). Accordingly, to verify this conclusion additional research must be conducted with these effects in mind or with an alternative CBS inhibitor.

Inhibition of CSE with DL- propargylglycine decreased EPSP amplitude by 49.2 percent (see Figure 2). The results of experimentation with only this chemical were nearly significant (Table 2, P=0.066) and obtained from a single specimen. A larger sample size would allow us to make a more definitive conclusion.

Addition of H_2S at the same time that CSE was inhibited, universally led to an increase in EPSP amplitude. The increase was significant for 400 and 600µM H_2S (P=0.002 for both, n=4) but not for the 200µM (P=0.101, n=4). However, we believe that this is representative of the struggle between CSE (trying to lower EPSP amplitude) and H_2S (trying to increase EPSP amplitude). To take this into consideration, we also calculated a p-value between the various H_2S concentrations and the inhibitor (rather than the control). The values were all significant (P=0.036, 0.003, and 0.006 for 200, 400, and 600µM H_2S respectively, n=4 for all). This leads us to believe that an unknown response, due at least in part to the application of the CSE inhibitor, causes a drastic increase in the effect of H_2S on synaptic transmission. We believe, nonetheless, that H_2S plays an important role in the crayfish NMJ. Further experimentation would likely lead to greater insight into the effects of H_2S on humans, with potentially important physiological or medical implications.

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