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Effects of CO as a Signaling Molecule at the Frog Neuromuscular Junction.

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ABSTRACT

Carbon monoxide (CO) has been characterized as a neurotransmitter in the gut, blood vessels and brain. However, little is known about the effects of CO as a neurotransmitter on the neuromuscular junction. In order to test these effects, the motor neuron of the frog *Rana pipiens* sartorious muscle was stimulated in Ringer's solution containing CO. The resulting decrease in end plate potentials (EPPs) taken from the sartorious muscle tentatively indicate that the presence of CO causes decreased EPP intensity. This suggests that CO acts to increase the amount of cGMP formed and thus causes hyperpolarization and relaxation of the muscle.

INTRODUCTION

The importance of carbon monoxide (CO) as a neurotransmitter has been demonstrated to varying degrees in many different organisms. In all creatures, CO is created as a byproduct of the heme oxygenase (HO) catalyzed break down of heme to bilirubin. CO is characterized by its ability to permeate cell membranes and its relatively short life-span.

CO first came to the attention of researchers as a possible neurotransmitter when it was observed that HO2 (a type of heme oxygenase) was associated with soluble guanylyl cyclase (sGC) and that HO2 inhibitors lead to lower levels of cGMP in olfactory neurons (Verma et al. 1993). These observations indicated the possibility of CO as a neurotransmitter because sGC changes GTP to cyclic GDP (cGDP). In turn, cGDP activates cGMP-dependent protein kinase (PKG) which leads to an increase in protein phosphorylation, finally resulting in a decrease in intracellular calcium concentrations (Nicholls, et al. 2001).

Therefore, CO was first positively identified as a signaling molecule when it was found to activate sGC, which in turn released cGMP (Boehning and Snyder 2003). This relationship has been characterized mainly in the enteric nervous system with HO2-knockout mice demonstrating the effect of CO on nonadrenergic/noncholinergic (NANC) neurotransmission (Boehning and Snyder 2003). These studies indicate that in mouse intestines, cGMP levels are reduced in mice lacking HO2, but that normal levels can be recovered when CO is added exogenously (Snyder and Ferris 2001).

While there is a clear relationship between CO and cGMP release in enteric nervous system, the effects of CO on other parts of the nervous system are not as well understood. For example, While HO2 knockout mice display normal cGMP levels in the brain as a whole, the olfactory bulb and the cortex are both affected by the lack of CO (Boehning and Snyder 2003). In the central nervous system the strongest evidence for CO as a neurotransmitter is in olfactory neurons (Verma et al. 1993).

To further complicate matters, it has been found that CO in rat tail arteries can react directly on K^+ channels by binding to a histidine mole on the surface of the channel. This binding causes the K^+ channels to open, causing hyperpolarization and relaxation of the cells (Wang and Wu 1997).

While the effects of CO have been studied to varying degrees in the brain, gut and tail of certain animals, their effects on the neuromuscular junction (NMJ) is relatively unknown. However, the presence of HO2 has been demonstrated at rat neuromuscular junctions (Kusner, LL et al. 1999), suggesting that CO may act as a neurotransmitter at the NMJ.In order to determine if CO acts as a neurotransmitter at the frog NMJ, CO was applied exogenously to a frog NMJ and the EPP intensity measured. This study, which aimed to determine if CO acts as a neurotransmitter at a frog NMJ, tentatively reports that frog EPP intensity drops after exposure to CO, supporting the hypothesis that CO acts as a neurotransmitter in a similar fashion at the NMJ as it does in the enteric nervous system.

MATERIALS AND METHODS

The experiments for this paper were performed on the neuromuscular junction of the sartorious muscle of the frog, *Rana pipiens*. The frogs were doubled pithed and the sartorious muscle and its accompanying nerve were dissected out and pinned in a recording chamber. Unless otherwise noted, all experiments were conducted in standard frog Ringer's solution (120 mM NaCl, 3.2 mM KCl, 2.7 mM $CaCl_2$-$2H_2O$, 0.5 mM $NaHPO_4$, 2.0 mM Tris buffer, and 10.0 mM D-Glucose). The pH was adjusted to 7.2 with NaOH. All experiments were performed at room temperature.

Electrophysiology

The motor nerve of the satorious muscle was stimulated just above threshold using a Grass SD9 Stimulator connected to a suction electrode. Synaptic responses in the form of EPPs were recorded intracellularly using glass microelectrodes filled with KCL (3 M). The electrodes were connected to an ADInstruments Bridge Amplifier connected to a MacLab/4 recording device interfaced with Scope v3.6.3 software (both ADInstruments). The software was also used for data analysis. All experiments were performed on muscle fibers which maintained a membrane potential of less than –60 mV.

Introduction of CO at the NMJ

In order to obtain a standard EPP without the presence of CO, the muscle and nerve were first placed in Rigner's solution to which curare (7µM) was added to prevent muscle contraction. The muscle was allowed to bathe in the curarized Ringer's for approximately 20 minutes to ensure the sufficient blocking of the acetocholine receptors.

In order to measure the affects of CO on the amplitude of the EPP, the curarized Ringer's was removed and replaces with a curarized Ringer's contating CO. To prepare the CO solution, CO gas (Linweld Inc., Omaha, NE) was bubbled through a glass tube into the curarized Ringer's for 20 min. at room temperature, resulting in curarized Ringer's with a CO concentration of approximately 1µg/mL. Readings were taken every minute for 15 min. beginning immediately after the muscle was exposed to the CO solution. After 15 min., the CO Ringer's was removed from the recording chamber and the muscle was rinsed twice with curarized Ringer's. This was done to determine the presence of any EPP recovery. During the rinsing, 2 EPPs were recorded; one immediately after the first rinse, the second 4 minutes after the second rinse.

RESULTS

CO Might Cause a Decrease in EPPs

To determine the affect of CO on the NMJ, the EPP of the sartorious muscle was first found to be 3.475 mV in standard Ringer's solution. The muscle was then bathed in a Ringer's solution containing 1µg/mL CO, and an average of 4 EPP's was taken every minute for 15 minutes (Figure 1). As the figure indicates, there was a general decline in EPP amplitude after exposure to CO.

The overall decrease in the amplitude of the EPPs indicates that the CO has an inhibitory effect on the firing of end plate potentials. Using the findings in mouse myenteric plexus as a model (Boehning and Snyder 2003), it can be assumed that the CO causes an increase in cGMP which in turn causes hyperpolarization of the postsynaptic cell and a decrase in the amplitudes of the EPPs.

Lack of a Purely Linear Relationship Between CO Exposure and Size of EPPs

The data indicates that the relationship between length of CO exposure and EPP size is not entirely linear. This could be attributed to the diffusion of CO across its concentration gradient.

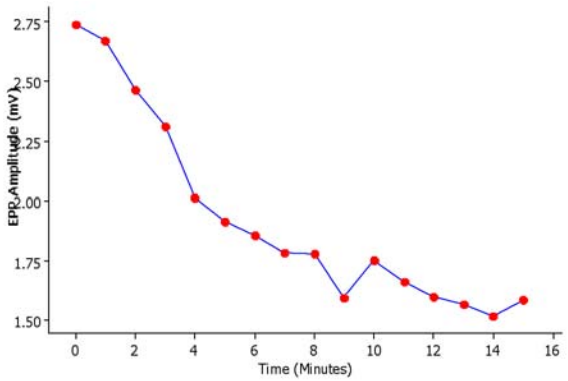

Figure 1. Effect of Time on EPP amplitude of a *Rana pipens'* sartorious muscle bathed in 1µg/mL CO curarized Ringer's solution. Each data point represents the average value of four EPPs.

CO Might Not Cause a Decrease in EPPs

After the final reading, the CO-containing Ringer's was replaced by curarized Ringer's and allowed to bathe for 15 minutes, after which time, the EPP was recorded to be 1.2375 mV. This curarized Ringer's was then replaced with fresh curarized Ringer's and the EPPs were found twice more; once immediately after replacement (1.1812 mV) and once 4 minutes after replacement (1.2638 mV).

In order to be sure that the observed decrease was due entirely to the CO, the EPP after washing would have had to have returned to the original control level (3.475 mV). Because the EPPs that were collected after

the wash were not nearly to this level, there is a possiblilty that an outside factor, and not the CO, caused the decrease in the amplitude of the EPPs.

DISCUSSION

In this paper, tentative evidence is presented suggesting that CO may act as a neurotransmitter at the frog NMJ. However, these results can only be interpreted in light of the significant deficiencies in experimental procedure.

The Inadequacy of Procedure and Results

Due to unforeseen circumstances, (namely the loss of a group member, the lack of CO, and the lack of frogs), the data obtained, while interesting, was inconclusive.

No control

In order to have a more thorough study, EPPs should have been measured every minute for 15 minutes in the curarized Ringer's solution absent of CO. This would have allowed for a control and would have demonstrated if EPPs naturally decrease over time due to external factors such as nerve fatigue or an inability to synthesize sufficient neurotransmitters.

Had the EPP remained largely unchanged during the course of the control, it would confirmed what this study only suggests, that CO decreases the amplitude of EPPs. Had the EPP decreased over time, the amount of decrease could have been compared to the amount of decrease due to CO. This would provide a better gauge of the decrease caused by CO versus the decrease caused by factors unaccounted for in this study.

No repetitions

A further limitation of this study is that the data was obtained from a single trial, allowing any number of outside factors (ranging from nerve death to a faulty electrode) to possibly cause the decrease in EPP. As the muscle examined in this study had been out of the frog for over 2 hours at the time of the reading, the study would have been greatly bolstered by additional trials.

In order to be more thorough, a freshly dissected muscle should have been used. Also, EPPs should have been collected from a number of different end plates on the same muscle. By collecting numerous readings, external forces or experimental error could have been ruled out as the cause of the decrease in EPP.

More readings would have also helped further articulate the linearity of the relationship between EPP size and the length of CO exposure.

CO Might Cause a Decrease in EPPs.

This experiment allowed us to measure the size of EPPs at the frog NMJ after it was exposed to a steady amount of CO over a long period of time. When taken as accurate, the results of this experiment are quite striking. These findings show a strong negative relationship between EPP amplitude and the amount of time a frog NMJ has been exposed to CO, indicating that CO acts as an inhibitory neurotransmitter.

This finding indicates that CO acts in a similar fashion on the frog NMJ as it does in mouse myenteric plexus cells. Upon take up of CO from the Ringer's by the cell, the CO activates sGC and triggers the release of cGMP. The cGMP then activates cGMP-dependent protein kinase (PKG) which decreases the concentration of intracellular calcium thus relaxing the cell and causing the EPPs to become lower (Nicholls et. al. 2001). While, this mechanism is speculative, these findings indicate that CO is worth further examination as a neurotransmitter at the NMJ.

CO Might Not Have a Linear Effect on the Size of EPPs

When taken as accurate, the data also indicates that there is not necessarily a linear relationship between the amount of CO exposure and the size of the EPP. This could be attributed to the diffusion of CO across its concentration gradient.

One possibility is that as time increases, the cells' ability to uptake CO decreases. As CO would diffuse across the cells' membranes, there would be a great initial response as CO would rapidly diffuse down its concentration gradient. This large influx of CO would cause a correspondingly large increase in cGMP levels and a drop off in EPP size. After the CO had innervated the cell, however, less and less would enter until finally equilibrium would be reached at which time the EPP size would level off.

While on the surface these readings look to be highly significant, the lack of depth to this experiment must also be considered when examining these results.

No Return to Normalcy

However, the tentative finding that exogenous CO caused lower EPPs conflicts with other data from the study. Specifically, even after the muscle was washed twice with curarized Ringer's absent of CO, the EPP amplitude did not return to the pre-CO levels. While, this lack of rebound might suggest that CO was not the only factor contributing to the decrease in the EPP amplitude, the muscle and nerve had been out of the frog for nearly 3 hours at the time of these readings. Therefore, it is

probable that the muscle and its corresponding nerve tissues were starting to die and were thus resulting in inaccurate EPP readings..

Future Areas of Research

Once the experimental issues with the control and the repeatability of the results have been addressed, these readings (if they agree with the findings suggested in this paper) will provide an excellent starting point for further experimentation. Much work needs to be done in characterizing the exact mechanisms by which CO acts as a neurotransmitter at the NMJ. Specifically, if CO does or does not the same cGMP creating mechanisms that have already been characterized at the neuronal synapses in other parts of the organism (Boehning and Snyder 2003).

There are also larger questions concerning the role of CO in the nervous system which have yet to be adequately addressed. The similarities of how NO and CO act in the nervous system has been noted (Nicholls, et al. 2001, and how they interact is not fully understood. HO2 and nNOS (the enzyme responsible for forming NO *in vivo*) knockout mice have been used to demonstrate that CO and NO have an additive effect on the level of inhibition of NANC neurotransmission in mouse intestinal cells (Snyder and Ferris 2000). In the penis, however, CO and NO have very different targets. Experiments have determined that NO is responsible for erection, while CO is responsible for ejaculation (Boehning and Snyder 2003). The brain provides a third example of possible interactions between NO and CO where studies have shown that cerebellar granule cells indicate that CO antagonizes NO signaling (Boehning and Snyder 2003).

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