

Carbon Monoxide Modulation of Nitric Oxide Induced Retrograde Signaling in Crayfish Neuromuscular Junction

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ABSTRACT

We tested the effects of nitric oxide (NO), and nitric oxide with carbon monoxide (CO) on retrograde signaling in crayfish extensor muscle cells. Nitric oxide is known to increase retrograde signaling, and therefore increase the amplitude of excitatory post-synaptic potentials (EPSPs). We predicted that EPSPs would increase in amplitude when exposed to NO, and when NO was paired with CO we predicted that the EPSPs would further increase. We tested this hypothesis through intracellular recording of excitatory postsynaptic potentials (EPSPs). We found that in comparison to baseline measurements (without NO and NO+CO), the experimental groups did show significant increases in the amplitudes of the measured EPSPs. Furthermore, the NO+CO experimental group had the greatest average increase in EPSP amplitude, and therefore the greatest increase in retrograde signaling.

INTRODUCTION

Until recently, synaptic transmission was believed to occur only by a pre-synaptic cell sending messages to a postsynaptic cell. It was thought that transmission could only occur in one direction. Then three scientists—Robert Furchgott, Louis Ignarro, and Ferid Murad—found that nitric oxide (NO) acts as a signaling device between cardiovascular cells (“The Nobel prize”), and it was observed that NO was released by the post-synaptic cell onto the pre-synaptic cell. This marked the beginning of the retrograde signaling theory, and these three scientists shared the 1998 Nobel Prize in medicine for their discovery (“The Nobel prize”). Also, it was believed that neurotransmitters had to be stored in synaptic vesicles before being released into the synaptic cleft via exocytosis; NO is not stored in such vesicles, but is simply formed on demand and released by diffusing through the cell membrane (Jaffrey and Snyder, 1995). This latter property is due to the fact that while most neurotransmitters are large molecules, NO molecules are relatively miniscule, which allows them to easily pass through the lipid bilayer (Bear, Connors, & Paradiso, 2001). In addition, NO differs from other neurotransmitters because it is inactivated by reacting with other molecules, rather than an enzymatic mechanism or reuptake (Jaffrey and Snyder, 1995). NO is inactivated automatically when it reacts with oxygen in the body. This generally happens within seconds after its release.

NO is synthesized from the amino acid L-arginine (Bear, Connors, & Paradiso, 2001). It activates the enzyme guanylyl cyclase, which catalyzes the dephosphorylation of GTP to cGMP

(Klabunde, 2008). cGMP is a second messenger for many important cellular functions, including smooth muscle relaxation and vasodilation (Klabunde, 2008). Since NO leads to the production of cGMP, an increase in NO causes an increase in blood flow (Schuman and Madison, 1994).

There are certain drugs that enhance vasodilation. Perhaps the most well known of these drugs is Viagra®, which works by inhibiting phosphodiesterase (PDE), an enzyme that breaks down cGMP (Brain). This helps treat erectile dysfunction in males.

NO is not the only neurotransmitter with these distinctive characteristics. There is evidence that carbon monoxide (CO) may play a role in retrograde signaling as well (Ewing & Maines 1992, Venna et al 1993). It most likely operates like NO as it is very similar in size and chemical structure to NO.

However, the specific effects of CO on synaptic transmission, as well as the effects of the interaction of CO and NO, have been relatively unknown. By comparing an NO group with an NO+CO group, we hope to uncover some mystery on the effects of CO and how NO and CO work together in retrograde signaling. In a 1996 study, NO was found to function as a retrograde messenger for CO signaling in ischemic rat hearts (Maulik, Engelman DT, Watanabe, Engelman RM, & Das, 1996).

The effect of CO on retrograde signaling is a curious topic with a fair amount of significance. Most people know carbon monoxide as a colorless, odorless, and deadly gas that is a component of cigarette smoke and exhaust fumes from automobiles. It prevents oxygen from reaching cells throughout the body by taking up binding sites on hemoglobin, the oxygen-transporter molecule (Starr & Taggart, 1998).

Even in tiny amounts, CO can be extremely dangerous, as its binding capacity is at least 200 times greater than that of oxygen (Starr & Taggart, 1998). However, if kept outside of the bloodstream and away from hemoglobin, CO perhaps is a helpful or even an essential molecule involved in synaptic transmission.

By measuring Excitatory Post-Synaptic Potentials (EPSPs) in the superficial extensor muscles in the tails of crayfish (*Procambarus clarkii*), we will observe the effects of NO and NO+CO on retrograde signaling. Comparing a baseline control group with experimental NO addition and NO+CO groups, we will demonstrate what and how much of an effect NO and NO+CO have as retrograde signalers on synaptic transmission.

We found that the addition of NO significantly increased EPSP amplitudes. We also found that when NO and CO were combined, it even further enhanced the EPSP amplitudes than just the addition of NO.

MATERIALS AND METHODS

Experiment Preparation.

We used crayfish (*Procambarus clarkii*) purchased from Carolina Biologicals, a company based in North Carolina. We removed the tails from the crayfish and exposed the superficial extensor tail muscles and nerves. We did this by making cuts on either side of the crayfish towards the abdomen on the ventral side. We then separated the ventral side of the tail from the dorsal side of the organism.

Solutions and Chemicals.

After we removed the tail from the crayfish, we placed the sample in a Ringer's solution, a general stock saline solution, intended to help preserve the cells and keep them viable. The Ringer's solution we used in this particular experiment was prepared prior to use, and immediately before the solution was used during each trial, the pH was neutralized to 7.4. The molarity of the solution used was as follows:

KCl 5.4 mM
NaCl 196 mM
MgCl₂·6H₂O 2.6 mM
Sodium Hepes Buffer 10 mM
CaCl₂·2H₂O 13.5 mM

This Ringer's solution was used for both the control and experimental groups. The chemicals that were used in this particular experiment were sodium nitroprusside and carbon monoxide gas. The sodium nitroprusside dihydrate (5) was purchased from the Sigma-Aldrich Company. For the first experimental group, the Ringer's solution was concentrated with the nitroprusside; 100 mL of Ringer's stock solution was concentrated with 100 microliters of the sodium nitroprusside. The second experimental group used

the same nitroprusside-Ringer's solution, in the same quantities, however the carbon monoxide gas (which was procured from the Grinnell College Chemistry Department) was infused (via bubble infusion) into the solution under a hood.

Electrodes.

The electrodes we used to measure the voltage difference across the cell membranes were Borosilicate glass capillary tubes, 1.2 millimeters in diameter, which had been pulled to a sharp point. These particular high resistance microelectrodes were used to penetrate the cell membrane and record the voltage in millivolts (mV). After the electrodes had been pulled to a point, we filled them with 3 molar KCl (potassium chloride). Because of the fragility of the electrode tips, the electrodes often needed to be replaced and the points extracted from the muscle tissue. The suction electrodes that were used were made from the same capillary tubes that had a flexible hollow tip super glued to the glass tube. The resistance of the microelectrodes had some variation and was not controlled. Throughout the experiment, we tested the resistance, and we kept the resistance above 4 MΩ (Megaohms).

Recording Procedure.

For the baseline measurements, the nerve was isolated and sucked up into the suction electrode. We then placed the glass electrode into a muscle cell in the same segment of the crayfish muscle as the nerve. Once in a muscle cell, the voltage difference was measured in mV. We stimulated the nerve with a shock, at a low frequency (.4 PPS-pulses per second). If a nerve was located and the electrode was in a cell, in the correct segment of the organism, an EPSP (excitatory postsynaptic potential) was observed using the Scope program. The EPSPs were measured in millivolts, and the amplitude of the EPSPs varied significantly between crayfish samples and individual cells. The same recording procedure was followed for the nitric oxide experimental group and the nitric oxide/carbon monoxide experimental group. We then returned the crayfish saline to general saline, and the EPSPs were measured to determine whether time was a confounding factor.

Methods of Data Analysis.

In analyzing the measured data, we averaged the amplitudes of the control and experimental group EPSPs, and compared those means to one another. The method we used to compare these differences was a two sample with variance t-test. To further test the hypothesis, p-values were used to test the probability that there is no difference between the

various groups. To interpret the p-value, an alpha value of .05 was used.

RESULTS

Preliminary evidence from past experiments suggested that the crayfish, *Procambarus clarkii*, tail neuromuscular junction was ideal for investigating retrograde signaling and retrograde messenger effects. Using microelectrodes of high resistance ($>10\text{ M}\Omega$), we could measure EPSPs in crayfish tail muscle cells and evaluate the effect that known retrograde messengers Nitric Oxide and Carbon Monoxide have on EPSPs through retrograde signaling. Based on the results of an experiment analyzing NO and CO as retrograde messengers in the hippocampus, which showed that NO and CO both enhanced EPSP after tetanic stimulation in the long run, we anticipated that NO and CO with low frequency stimulation would be excitatory retrograde messengers in the short term as well, producing enhanced EPSPs. This expectation would infer that NO would enhance EPSPs and the combination of NO and CO would enhance the EPSPs more than the NO induced retrograde signaling. This occurrence then would support the hypothesis that CO modulation of NO induced modulation is excitatory and not inhibitory, with NO retrograde modulation being excitatory and not inhibitory as well.

Carbon Monoxide modulation of Nitric Oxide induced retrograde signaling enhances the excitatory effect of Nitric Oxide modulation alone on the EPSP. Prior to examining these two modulation condition, we confirmed, with a t-test, that NO had an excitatory effect on baseline EPSP (Table 1). After using a two sample mean of differing variances t-test for statistical analysis of the differences between EPSP means of the modulation conditions, we discovered that CO modulation of NO induced retrograde signaling produced EPSPs legitimately (statistically confirmed) greater than those of NO modulation alone. The t-test results gave extremely small two - tail P-values for tests for the hypothesized difference of zero between the two modulation conditions of Nitric Oxide alone and the combination of Nitric Oxide and Carbon Monoxide (Table 1). These low probabilities indicate that we can reject the null hypothesis of no difference between NO and NO+CO modulation conditions and accept the hypothesis that CO and NO together enhance EPSPs more than NO alone, further indicating CO to not impede but amplify the excitatory effect of NO retrograde signaling. In solely comparing the mean EPSP in each of the three conditions, a general trend is evident of NO's EPSP enhancement and then CO's

enhancement of the NO induced retrograde signaling EPSP (Figure 1).

To further support our hypothesis, we made sure that the observed increases in EPSP amplitude were in fact due to the changing conditions, and not to time. After we observed the increase in EPSP amplitudes when using the +NO and CO+NO groups, we returned to baseline. At first, the "return to baseline" group readings remained high, similar to the experimental +NO and CO+NO groups, but after a period of several minutes, the EPSP amplitudes began to decline.

A)

	Baseline	NO Induced Modulation
Mean EPSP	24.87	34.73
Hypothesized Mean Difference	0	
Two Tail P-Value	0.008	

B)

	NO Induced Modulation	CO + NO Modulation
Mean EPSP	34.73	44.73
Hypothesized Mean Difference	0	
Two Tail P-Value	0.005	

Table 1. A) Two Sample with differing variances t-test comparing baseline EPSPs with NO induced modulation EPSPs. The two-tail P value was less than .01, thus confirming that NO has an excitatory effect on EPSP. B) Two sample with differing variances t-test comparing NO induced modulation EPSP's with CO modulation of NO induced modulation EPSPs. The two-tail P value was also less than .01, well below the established alpha value of .05, therefore enabling us to reject the hypothesis that CO has no effect on NO induced retrograde signaling and accepting the hypothesis that CO modulation enhances the excitatory effect of NO induced retrograde signaling on EPSP in crayfish tail neuromuscular junction.

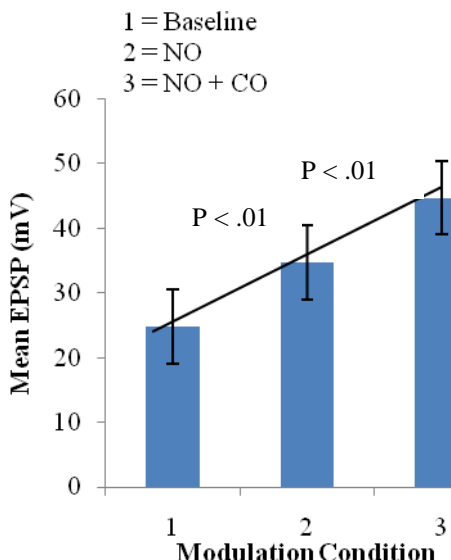


Figure 1. Mean EPSP for baseline, NO induced modulation, and CO modulation of NO induced retrograde signaling conditions. NO induced retrograde signaling has an excitatory effect on EPSP while CO modulation of NO induced retrograde signaling enhances the already enhanced EPSP from NO modulation alone.

DISCUSSION

Our results supported the widely accepted idea that NO acts as a retrograde signaler, as the average EPSP amplitude with added NO (+NO) was approximately 10 mV greater than the baseline group with no added NO. This is a significant increase in amplitude. Furthermore, our hypothesis that CO acts as a retrograde signaler much like NO was also strongly supported, as the average EPSP amplitude with both NO and CO was about 10 mV greater than the group with only +NO. It is interesting to note that the difference between the baseline and +NO groups (≈ 10 mV) is almost exactly the same as the difference between the +NO and CO+NO groups. Our results suggest that CO and NO work together to increase EPSP amplitudes, and perhaps that there is no significant difference between NO and CO as neurotransmitters.

A significant gap in our data is that there is no group with only added CO (and no NO). With a +CO group, we could further argue that CO acted similarly to NO if the average EPSP amplitudes for +CO were about the same as that for +NO. The problem with our experiment in creating a +CO group is that the methods for adding NO (decomposition of Sodium

Nitroprusside) were very different than for adding CO (directly bubbling CO gas into the solution). Simply put, we do not know exactly how much NO we added when we added the sodium nitroprusside, although we could measure exactly how much CO we added by looking at the gauge on the tank. Since the methods of delivery were different, we could not keep the amount of NO equal to the amount of CO; therefore, we couldn't directly compare the two. Because of this, we found it unnecessary to indicate in our experiment the precise amounts of NO and CO used.

Our findings are in agreement with the 1996 experiment that found NO to function as a retrograde messenger for CO in ischemic rat hearts (Maulik, Engelman DT, Watanabe, Engelman RM, & Das, 1996). They found cGMP levels to increase significantly after treatment by L-arginine. This makes sense, because L-arginine produces NO, which stimulates cGMP production. They also found that protoporphyrin, a chemical that inhibits the enzyme hemeoxygenase (an enzyme that breaks down heme into CO and other molecules), reduced the NO-mediated augmentation of cGMP. Their results strongly suggest that CO plays a similar role to NO.

In conclusion, we provide pharmacological evidence that CO has an excitatory effect on NO-induced retrograde signaling, and perhaps, there is no significant difference between NO and CO as neurotransmitters. A future experiment that compares +NO and +CO and carefully controls the amounts of NO and CO used will be useful to further support this hypothesis.

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