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Nitric oxide synthase is present in the crayfish tail.

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ABSTRACT

Until the recent discovery of the varied and critical roles played by nitric oxide (NO) in the human body, it was known only in negative context. Its role as a neurotransmitter in vertebrates has since been determined and thoroughly researched, but our study was one of few to investigate the presence of NO in the crayfish superficial extensor muscle. NO is difficult to locate in tissue so we used histochemistry to establish the presence of NO synthase (NOS), which suggests the presence of NO. We found that NOS is present in the superficial extensor muscle of the crayfish tail, which suggests that NO could play a neuromodulatory role in crayfish.

INTRODUCTION

For many years, nitric oxide (NO) was almost entirely known in a negative context. This noxious gas was a known environmental pollutant that destroyed ozone, preceded acid rain and was thought to cause cancer (Culotta & Koshland 1992). Yet, beginning in the late 1980s, a new face of nitric oxide came to light. It was found to play an array of crucial roles throughout the human body (Ignarro *et al.* 1987; Palmer *et al.* 1987). A list of the roles of NO includes, but is not limited to, its affects on blood pressure regulation; reproduction; assimilation of odors in the olfactory bulb; strokes; antimicrobial defense; and digestion (1992; Barañano *et al.* 2001). By 1992, nitric oxide was definitively accepted into the ranks of messenger molecules (Culotta & Koshland 1992).

Nitric oxide was the first gas to be recognized as a neurotransmitter. It is a simple molecule and the smallest and lightest known neurotransmitter. Consequently, NO has expanded the definition of neurotransmitter to include substances without specific post-synaptic receptors, for it diffuses directly into the nearby neurons, where it influences cellular events (e.g. stimulates guanylyl cyclase). Its mechanism of release is also unique, for NO does not have presynaptic storage vesicles, so it must be synthesized on demand (Barañano *et al.* 2001). A unique enzyme called nitric oxide synthase (NOS) fills this role and biochemically produces NO. The enzyme is present in three forms in cells, neuronal NOS, inducible NOS and constitutive NOS (Culotta & Koshland 1992). NOS is thought to be present in only 1 % of the cerebral cortex's cell bodies, yet the NOS nerve terminals branch enough that they interact with almost all the neurons in the cortex (Barañano *et al.* 2001).

The unpaired electron on NO renders it a free radical that diffuses away almost as rapidly as it interacts with other molecules. Due to its short lifespan and intense reactionary nature, NO is exceedingly difficult to locate in tissue, so most studies establish the presence of NOS as evidence of the presence of NO (Kusner & Kaminski 1996). Henceforth when we refer to the location of NOS we implicitly refer as well to the location of NO.

It has been shown that NO is active at the neuromuscular junction (NMJ) of a variety of vertebrates, including rabbits, lizards, rats and cows (Palmer *et al.* 1987; Graves *et al.* 2004; Kusner & Kaminski 1996; Ignarro *et al.* 1987). If we find NOS is present in the crayfish superficial extensor muscle, it will suggest a similar neuromodulatory role in crayfish as in vertebrates.

Given the absence of previous experimentation related to the distribution of NO in crayfish, we hoped to determine whether NOS is present by using histochemistry. We looked for evidence of NOS by trying to detect $NADP^+$, one of the products of the NOS reaction:

L-ARG + O_2+NADPH → L-citrulline + NO + $NADP^+$.

Previous experiments have used histochemistry procedures to locate NADPH-diaphorase (NADPH-d). They added nitroblue tetrazolium (NBT) to the products, which reduced the $NADP^+$ into NADPH. The process stained the NBT a vibrant blue color. Since locations where NBT staining is present also contain $NADP^+$, a product of the NADPH-d, the NBT staining is a localization method for NOS

(Kusner & Kaminski 1996). Since few enzymes in animals use NADPH as a co-factor, evidence of $NADP^+$ strongly suggests the presence of NOS (C. Lindgren, personal communication, 8 Nov 2007).

The histochemistry resulted in uniform staining in the superficial extensor muscles. This provides strong evidence that nitric oxide (NO) is present in the crayfish tail.

MATERIALS AND METHODS

We used NADPH-d histochemistry to determine whether NO is present in the superficial extensor muscle in the crayfish tail.

Preparing the Sample

We isolated sections of the superficial extensor tail muscle of the crayfish (*Procambaris clarkii;* Carolina Biological Company), which we pinned in a dish with crayfish saline composed of 5.4 mM KCl, 196 mM NaCl, 2.6 mM MgCl2.6H2O, 10.0 mM Na Hepes Buffer, and 13.5 mM CaCl2.2H20 (pH 7.4). Then we performed the NADPH histochemistry procedure (see below). Finally, we placed the preparation onto a microscope slide to assess the extent of the NADPH-d staining.

NADPH-d histochemistry

We used NADPH-d histochemistry to look for indications of NADP+ which suggest the NOS reaction occurred and provide evidence for the presence of NO.

Immediately after isolation, we fixed the muscle for 15 min at 4°C in 3% paraformaldehyde Ringer solution (pH 7.3), rinsed for 30 min (switching solution every 10 minutes) in Ringer solution (pH 8), permeablized at 37°C in Ringer solution (pH 8) containing 0.3% Triton X-100, and incubated for 75 min at 37°C in Ringer solution (pH 8) containing 1 mg/mL β-NADPH and 1 mg/mL NBT. Then we rinsed the preparation for 30 min in Ringer solution (pH 7.3), mounted it onto a glass slide with Antifade Buffer and let cure for 24 h before sealing. Finally, examination of the prepared muscle using brightfield microscopy with a 100x oil immersion lens revealed the extent of NADPH-d staining (adapted from Graves et al. 2004).

We performed a control experiment using NG-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NOS (Graves et al. 2004). Adding L-NAME to the solution inhibits the NADPH-d staining if it is caused by NOS. As another control experiment, we performed histochemistry on the neuromuscular junction located in the *Ceratomandibularis* muscle, isolated from the lower jaw of the American Anole (*Anolis carolinensis;* Carolina Biological Company).

RESULTS

We used NADPH-d histochemistry to determine whether NOS was present in the superficial extensor muscle of the crayfish tail. NOS uses NADPH as a co-factor to oxidize arginine and create NO along with $NADP^+$, and with histochemistry we investigated the presence of $NADP^+$ as evidence this reaction had occurred. We added nitro blue tetrazolium (NBT) to the solution, which changes color as an indication of the presence of $NADP^+$. Brightfield microscopy with a 100x oil immersion lens revealed extensive staining (Figure 1). The staining was evenly distributed over the entire sample, suggesting that NOS is diffuse throughout the muscle in the crayfish tail.

Our control experiment in which we added L-NAME resulted in no staining of the sample (Figure 2). This provides further evidence that the NADPH-d staining was indeed caused by NOS.

Figure 1. NADPH-d Staining of Crayfish Superficial Extensor Tail Muscle. All images were visualized using brightfield microscopy.

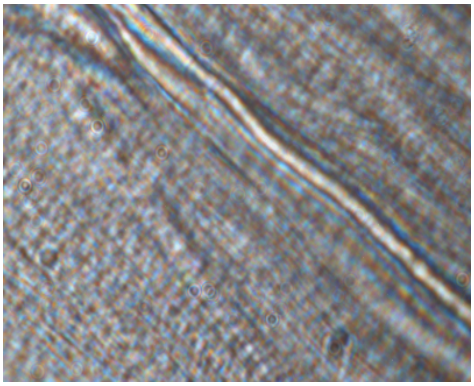

Figure 2. Absence of NADPH-d Staining of Crayfish Superficial Extensor Tail Muscle in the presence of L-NAME.

Because previous experiments have used histochemistry to locate NOS at the lizard NMJ and have shown it is present, we performed a control experiment on this muscle to confirm our procedure. We isolated the *Ceratomandibularis* muscle of the lizard and performed the histochemistry using the same procedure as performed above for the crayfish muscle. Figure 3 shows that NADPH-d staining is prevalent at the NMJ, confirming the results that have previously been found (Graves et al, 2004) and providing further support that our procedures are correct. As a final control, the histochemistry was performed on the *Ceratomandibularis* muscle of the lizard with the addition of L-NAME. Careful examination of the sample revealed no NADPH-d staining (Figure 4), which is consistent with the results of previous experiments (Graves et al, 2004).

We refined our methods through repeated trial and error, and were only able to generate reproducible results in our final experiment. Therefore our findings are not representative of a large sample size and should be corroborated by further experimentation.

DISCUSSION

Histochemistry procedures with crayfish superficial extensor muscles and lizard *Ceratomandibularis* muscles yielded results consistent with our hypothesis. The staining of the crayfish sample was evenly distributed over the entire muscle, suggesting that NOS is diffuse throughout the superficial extensor muscle in the crayfish tail. To support our results and confirm that we were indeed seeing staining caused by NOS, we performed a control experiment using L-NAME, an inhibitor of NOS. No staining was evident in this control sample (Figure 2).

A parallel control experiment was done with the lizard, since previous experiments have used equivalent histochemistry to illustrate the presence of NOS at the lizard's neuromuscular junction. The staining pattern in the lizard muscle has been confirmed by immunoflourescence and by physiological observation (Graves et al, 2004). The histochemistry resulted in NADPH-d staining at the NMJ (Figure 3), confirming the previous results and providing further support that our procedures were correct. L-NAME was added to the lizard preparation in another control that did not result in any apparent NADPH-d staining (Figure 4), which is consistent with the results of previous experiments.

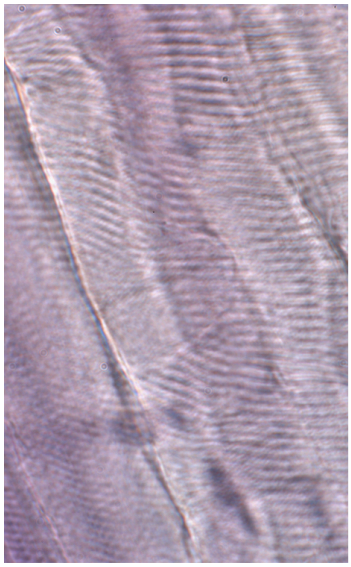

Figure 3. NADPH-d Staining of lizard NMJ.

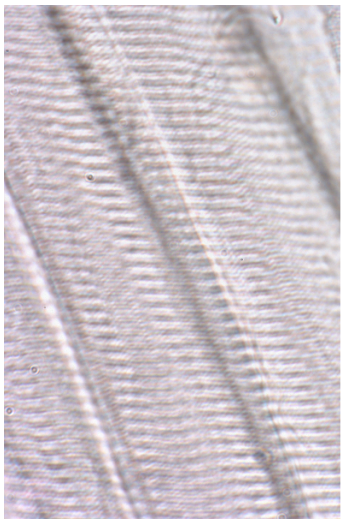

Figure 4. Absence of NADPH-d Staining of lizard NMJ.

Previous studies have shown that NO is active at the neuromuscular junction (NMJ) of a variety of animals, including rabbits, lizards, rats and cows (Palmer *et al.* 1987; Graves *et al*. 2004; Kusner & Kaminski 1996; Ignarro *et al.* 1987). It has been assumed that NO plays an equivalent role in invertebrates such as crayfish, but to our knowledge the assumption has not been tested until now. We have demonstrated the presence of NOS in the crayfish superficial extensor muscle by using histochemistry.

Since histochemistry showed diffuse staining in the superficial extensor muscle it did not shed light upon the specific locations of NOS in the tail. Immunofluorescence would detail exactly where the NOS is located and whether it colocalizes with the synapses (Kusner & Kaminski 1996). Overlap of NOS and the synapses would more definitively suggest a signaling role for NO at the crayfish NMJ.

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