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Actions of nitric oxide and expression of the mRNA encoding nitric oxide synthase in rat vagal afferent neurons

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Abstract

The present study has investigated whether nitric oxide (NO) is involved in neurotransmission of rat vagal afferent neurons. The diethylamine-NO complex (diethylamine-NO, 10–100 μM) and *S*-nitroso-*N*-acetylpenicillamine (3–100 μM) both elicited a concentration-dependent depolarisation of the isolated rat nodose ganglion preparation. Pre-treatment with 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ, 300 nM), 6-(phenylamino)-5,8-quinolinedione (LY83,583, 30 μM) and Methylene blue (100 μM) all caused a significant shift to the right in the concentration-response curve to diethylamine-NO. Incubation of rat nodose ganglion sections with a ^{35}S-labeled antisense oligonucleotide to neuronal NO synthase resulted in visualisation of the mRNA encoding NO synthase over vagal afferent perikarya. The anatomical findings, therefore, suggest that a number of rat vagal afferent perikarya possess the ability to produce the enzyme required for the biosynthesis of NO. Collectively, these data suggest that NO may be functionally important as a neuromodulator of rat vagal afferent neurons.

Keywords: Nitric oxide (NO); Nodose ganglion; Guanylate cyclase; Electrophysiology; Enzyme; mRNA

1. Introduction

Since the identification of nitric oxide (NO) as the endothelium-derived relaxing factor (Furchgott and Zawadzki, 1980; Palmer et al., 1987), this free radical has been recognised as an important messenger molecule within the central and peripheral nervous systems (Garthwaite et al., 1988; Bredt and Snyder, 1992; Garthwaite and Boulton, 1995). Endogenous NO is liberated during the conversion of arginine to citrulline, catalysed by the enzyme NO synthase (Palmer et al., 1988; Garthwaite et al., 1989).

The vagus, or Xth cranial nerve, largely comprises bipolar sensory afferent neurons with its central branches projecting to the medulla oblongata in the brain stem. Included in these central vagal projections are arterial baro- and chemoreceptor afferents and cardiopulmonary afferents, whose cell bodies are located in the nodose ganglia and all of which terminate in the nucleus tractus solitarius (Van Giersbergen et al., 1992; Dampney, 1994). Considerable evidence exists implicating NO as a neuromodulator of central cardiovascular control pathways within the nucleus tractus solitarius (Lawrence and Jarrott, 1996). Thus, microinjection of *S*-nitrosocysteine into the nucleus tractus solitarius of both anaesthetised (Lewis et al., 1991a) and conscious rats (Machado and Bonagamba, 1992) results in immediate hypotension and bradycardia which is sensitive to antagonism by an inhibitor of soluble guanylate cyclase, Methylene blue. In a similar manner, *S*-nitrosocysteine was also demonstrated to be involved in the processing of cardiopulmonary afferent input to the nucleus tractus solitarius (Lewis et al., 1991b). Furthermore, NO has an ability to modulate the release of endogenous glutamate in the rat nucleus tractus solitarius (Lawrence and Jarrott, 1993), the favoured candidate primary neurotransmitter at baroreceptor, chemoreceptor and cardiopulmonary afferent terminals (Talman et al., 1980; Andresen and Yang, 1990; Bonham et al., 1993; Lawrence and Jarrott, 1994; Colombari et al., 1994).

Therefore, while the involvement of NO as a neuromodulator of vagal afferent input to the nucleus tractus solitarius is well established, less is known about the role of NO in the nodose (inferior vagal) ganglion. The present study has addressed this issue by employing the isolated rat nodose ganglion preparation to characterise whether NO donors can evoke an electrophysiological response when applied to vagal soma. The rat nodose ganglion-vagus

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preparation has been routinely employed as a model to examine the properties of membrane-bound receptors on vagal perikarya that would be representative of such receptors on largely inaccessible, fine vagal terminals within the nucleus tractus solitarius (Jarrott, 1994). In the case of NO, the identity of an endogenous 'receptor' is still uncertain; however, the ability of NO donors to depolarise the preparation would indicate that a similar action could be expected in the nucleus tractus solitarius. Furthermore, the ability of NO to diffuse transsynaptically (Garthwaite et al., 1988, 1989) may indicate a role for the free radical as an interneuronal messenger within the nodose ganglion itself. The localisation of NO synthase in vagal afferent perikarya has previously been observed using the technique of NADPH-diaphorase staining (Morris et al., 1993); however, whether NADPH-diaphorase-positive cells are always NO synthase-positive has become a controversial issue (Vizzard et al., 1994). Therefore, in addition to the in vitro electrophysiology, in situ hybridisation histochemistry utilising ^{35}S-labeled oligonucleotides has been performed to establish whether cell bodies of the nodose ganglion express the mRNA encoding NO synthase.

2. Materials and methods

All of the experiments described here were performed in accordance with the Prevention of Cruelty to Animals Act 1986 under the guidelines of the NH & MRC Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia.

2.1. In vitro electrophysiology

Electrophysiological experiments were performed as previously described (Castillo-Meléndez et al., 1994; Lawrence et al., 1995). In brief, male Sprague-Dawley rats (250–350 g) were killed by cervical dislocation and the nodose ganglia with attached distal vagal trunk were removed and desheathed. The tissue was then placed in a twin-chambered Perspex bath with the nodose ganglion placed in one compartment of the bath and isolated from the vagal nerve trunk in an adjacent compartment by a silicone grease seal, as previously described (Widdop et al., 1990). The preparation was superfused with Krebs buffer (36°C, 2 ml/min) of the following composition (in mM): NaCl 118, $NaHCO_3$ 24.9, KH_2PO_4 1.3, KCl 4.7, $CaCl_2$ 2.6, glucose 11, $MgSO_4$ 1.2, gassed with 95% O_2/5% CO_2, pH approximately 7.4. The d.c. potential between the two compartments following drug administration to the nodose ganglion was recorded by calomel electrodes connected to the preparation through agar-KCl bridges. The potential changes were amplified and displayed on a Grass polygraph (model 79D). Drugs were applied non-cumulatively and remained in contact with the tissue until apparent equilibrium was reached. This was followed by a washout and recovery period (10–20 min) to allow full repolarization prior to another drug addition. When used, antagonists-enzyme inhibitors were added to the superfusate 15 min prior to agonist application. This method allowed stable responses to be measured over a 5–6-h period. The magnitude of an observed depolarization was measured from a projection of the baseline preceding the response to account for any drift in the preparation. At the beginning and end of each experiment a positive control of a previously determined maximal concentration of 5-hydroxytryptamine (5-HT, 3 μM; Widdop et al., 1992) was applied to the nodose ganglia in order to check the viability of the preparations.

2.2. In situ hybridisation histochemistry

2.2.1. Surgery and tissue preparation

Male rats (290–320 g) were anaesthetised with sodium pentobarbitone (60 mg/kg i.p.) and placed on their back. A midline incision was made in the neck and the nodose ganglia were isolated, removed and placed in cryoprotectant (OCT-embedding compound, Miles Diagnostics). Rats were then killed by decapitation. Sections of cryostat-cut nodose ganglia (12 μm) were thaw-mounted onto poly-L-lysine coated microscope slides and allowed to dry at room temperature. When dried, tissue slices were placed in ice-cold 4% depolymerized paraformaldehyde for 5 min, followed by phosphate-buffered saline at room temperature for 3 min. Sections were then dehydrated through serial alcohol (70, 95 and 100%), prior to delipidation in chloroform at room temperature for 20 min. Finally, sections were placed in 100% alcohol and stored at 0–4°C until use.

2.2.2. Oligonucleotide labeling and hybridisation procedure

An antisense 45-mer oligonucleotide probe to rat neuronal NO synthase (Bredt et al., 1991a) with the sequence 5′-GGC CTT GGG CAT GCT GAG GGC CAT TAC CCA GAC CTG TGA CTC TGT-3′ was purchased (Biotech International, Australia) and diluted to a working stock of 0.3 pmol/μl. Then, 2-μl aliquots were 3′-end-labeled with [^{35}S]dATPαS (1200 Ci/mmol; Amersham, UK) in the presence of terminal deoxynucleotidyl transferase (Boehringer Mannheim, Germany) yielding an activity of 90 000–100 000 dpm/μl. Unincorporated nucleotides were separated from the labeled probes through Sephadex G-25 columns spun at 2000 rpm.

The labeled oligonucleotide probe to NO synthase (1 pg/μl, 100 μl) was applied to adjacent sections of rat nodose ganglia in a hybridisation buffer containing 50% formamide, 4 × saline sodium citrate (SSC; 0.6 M sodium chloride, 0.06 M sodium citrate, pH 7.0), 10% dextran sulphate and 10 mM dithiothreitol (Wisden et al., 1991). Slide-mounted sections were allowed to hybridise overnight in a humidified atmosphere at 42°C. The following day,

sections incubated with the NO synthase probe were washed in 1 × SSC for 1 h at 55°C and then rinsed in 1 × SSC and 0.1 × SSC at room temperature. Finally, sections were dehydrated through serial alcohol and dried. When dry, slides were dipped in photographic emulsion (LM-1, Amersham) and left for 100 days.

Following photographic development, tissue slices were stained with thionin (0.1%), differentiated, cleared and coverslipped. Individual sections were then examined under a light microscope (Olympus BH-2) under both light- and dark-field condensers and photographed.

2.2.3. Specificity controls

Hybridisation signals obtained for NO synthase were inhibited by coincubation with a 100-fold molar excess of the unlabeled antisense oligonucleotide. In addition, the labeled antisense oligonucleotide yielded consistent, topographically distinct profiles of gene expression on adjacent tissue sections from different rats.

2.3. Materials

Diethylamine-NO, *S*-nitroso-*N*-acetylpenicillamine, 6-(phenylamino)-5,8-quinolinedione (LY83,583), arginine and indomethacin were purchased from Research Biochemicals (USA). 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) was purchased from Tocris Cookson (UK). Methylene blue was purchased from Sigma Chemicals (USA). All other chemicals were either analytical or laboratory grade from various suppliers.

2.4. Statistical analysis

Concentration-response curves generated from the in vitro electrophysiology experiments were analysed by a two-way analysis of variance followed by post-hoc Student-Newman-Keuls comparisons of drug concentrations before and after the addition of enzyme inhibitors. In all cases, $P < 0.05$ was considered significant.

3. Results

3.1. In vitro electrophysiology

Addition of either diethylamine-NO or *S*-nitroso-*N*-acetylpenicillamine to the superfusate bathing the isolated nodose ganglia resulted in a concentration-dependent depolarisation (Fig. 1). On the other hand, L-arginine (10 μM–1 mM) had no effect on the isolated rat nodose ganglia. Diethylamine-NO was chosen to study in further detail and Fig. 1 shows a representative series of polygraph traces from an individual experiment involving diethylamine-NO. Due to difficulties consistently obtaining an apparent maximal response, as has previously been reported for the characterisation of dopamine D_2 receptors using this preparation (Lawrence et al., 1995), concentration-response curves have been analysed by two-way analysis of variance. The specific inhibitor of NO-stimulated guanylate cyclase, ODQ (300 nM) shifted the concentration response curve for diethylamine-NO to the right ($P < 0.05$ at all concentrations of diethylamine-NO; $n = 5$; Fig. 2C). At a much higher concentration, LY83,583 (30 μM, $P < 0.05$ at all concentrations of diethylamine-NO; $n = 6$) caused a greater shift in the diethylamine-NO concentration-response curve (Fig. 2A). In a similar manner, Methylene blue (100 μM; $P < 0.05$ at 30 and 100 μM of diethylamine-NO; $n = 3$) also caused a rightward shift in the concentration-response curve to diethylamine-NO (Fig. 2B).

Pre-incubation with the cyclo-oxygenase inhibitor, indomethacin (1 μM), resulted in a slight shift to the right in the concentration response curve of diethylamine-NO ($n = 4$, Fig. 2D). None of the enzyme inhibitors employed were capable of evoking an electrophysiological response during the pre-incubation period and, following washout of inhibitors, the ability of diethylamine-NO to depolarise the isolated rat nodose ganglion was restored.

3.2. In situ hybridisation histochemistry

Light microscopic examination of rat nodose ganglion sections incubated with the NO synthase antisense oligonucleotide revealed that a proportion of vagal afferent

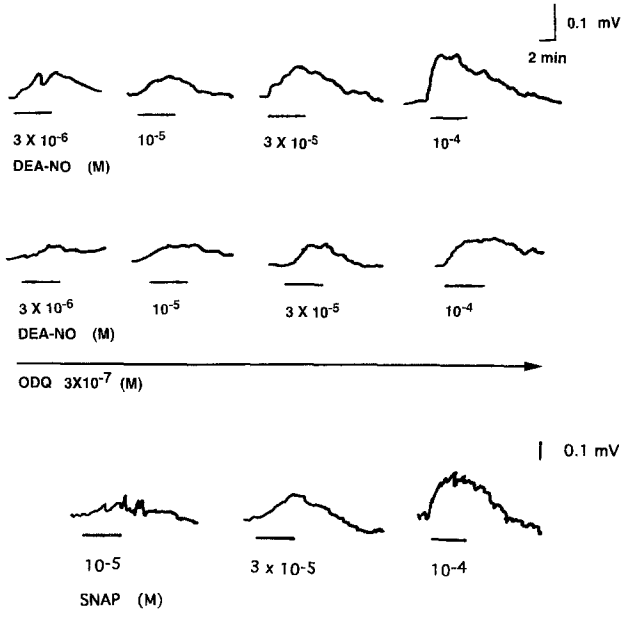

Fig. 1. Polygraph trace showing a representative series of responses to different concentrations of diethylamine-NO (DEA-NO) applied to the rat isolated nodose ganglion preparation, in the absence (top line) or presence (middle line) of ODQ (300 nM). Bottom line shows a representative concentration-response series to *S*-nitroso-*N*-acetylpenicillamine (SNAP). Horizontal bars represent periods of drug superfusion.

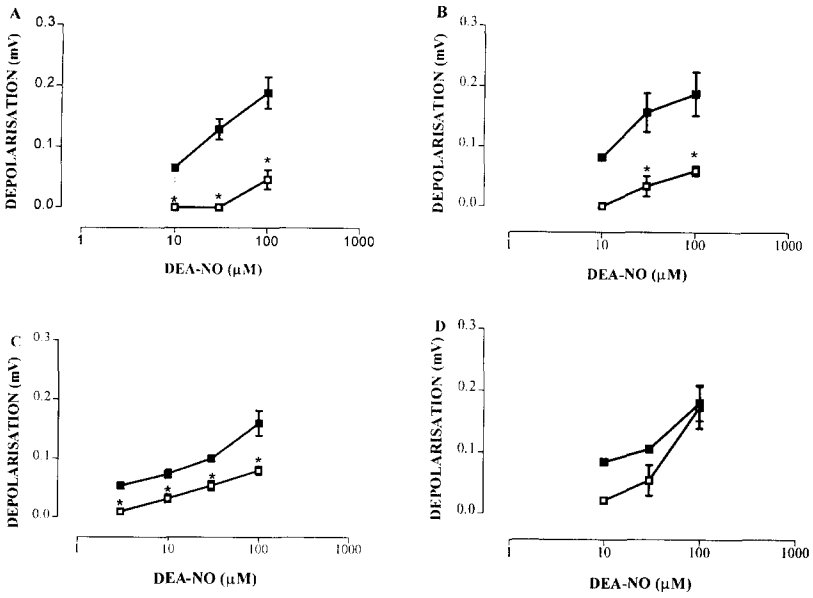

Fig. 2. Concentration-response curves of diethylamine-NO on the rat isolated nodose ganglion preparation, in the absence (closed symbols) or presence (open symbols) of: (a) LY 83,583 (30 μM, $n = 6$); (b) Methylene blue (100 μM, $n = 3$); (c) ODQ (300 nM, $n = 5$); and (d) indomethacin (1 μM, $n = 4$). Data points are mean ± S.E.M. * $P < 0.05$ (two-way anaylsis of variance followed by a Student-Newman-Keuls post-hoc comparison).

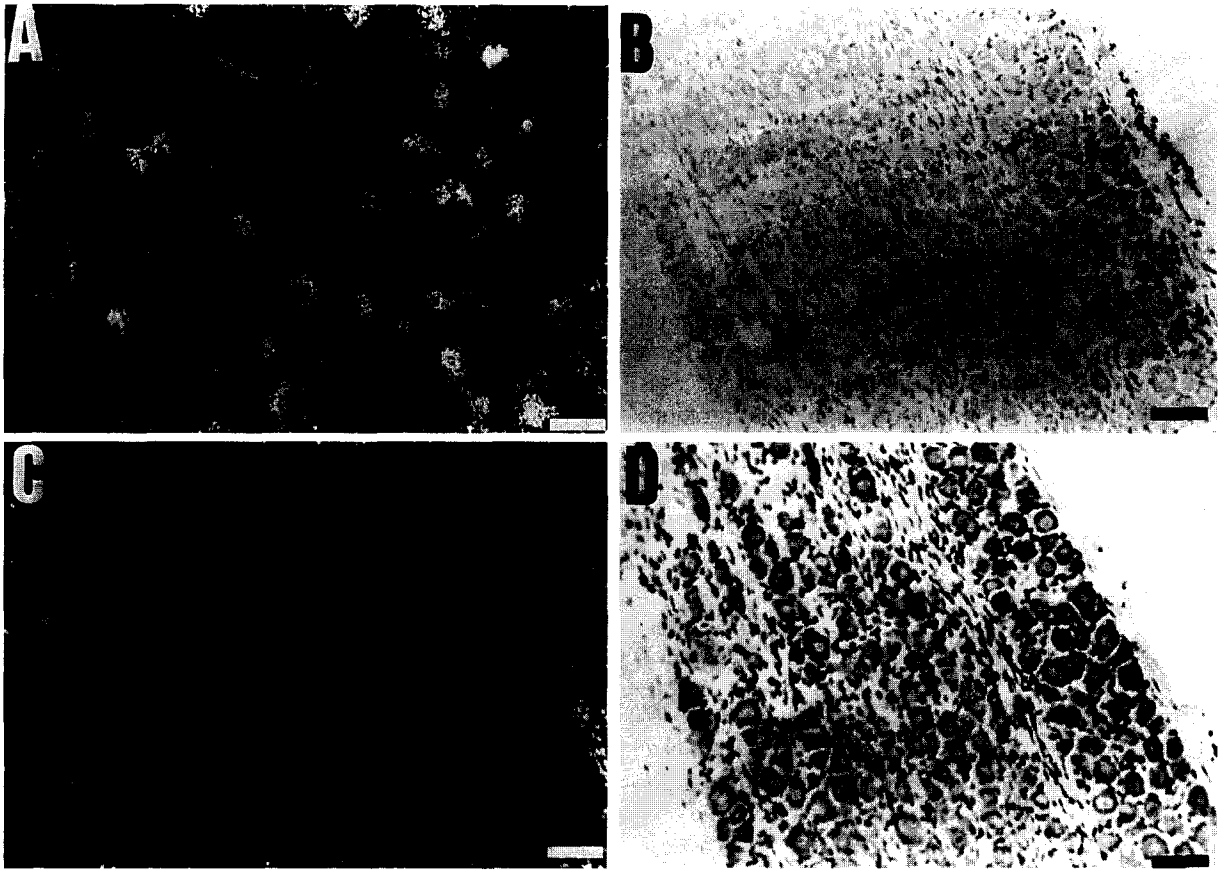

Fig. 3. Dark- and light-field photomicrographs demonstrating expression of the mRNA encoding the enzymes NOS and glutaminase in sections of rat nodose ganglia. (A) NOS, dark-field, demonstrating characteristic groupings of silver grains. Scale bar, 111 μm. (B) Light-field photomicrograph corresponding A, confirming that silver grains representing NOS gene expression are associated with vagal afferent perikarya. Scale bar, 111 μm. (C) Control hybridization of NOS antisense probe in the presence of a 100-fold molar excess of unlabelled oligonucleotide. Silver grains persist only over the external neural sheath of the ganglia. Scale bar, 111 μm. (D) Light-field photomicrograph corresponding to C. Scale bar, 111 μm.

perikarya express the mRNA encoding the production of the enzyme NO synthase (Fig. 3A,B). The characteristic groups of silver grains over neuronal cell bodies (Fig. 3A), representing hybridised mRNA encoding NO synthase, are inhibited by coincubation of tissue sections with a 100-fold molar excess of unlabeled antisense oligonucleotide, the only signal remaining occurring over the external neural sheath of the ganglia and, therefore, representing non-specific binding of the labeled probe to the sheath rather than hybridised mRNA (Fig. 3C,D).

4. Discussion

The present study has provided both functional and anatomical evidence for the potential involvement of NO in neurotransmission of rat vagal afferent neurons, acting via the guanylate cyclase effector pathway. Moreover, the isolated rat nodose ganglion-vagus preparation has been developed as a method to indicate the likely presence and properties of receptors on inaccessible central vagal terminals (Round and Wallis, 1986) and, therefore, the data presented herein may, by analogy, represent evidence for a physiological 'receptor' for NO on rat vagal afferent neurons.

LY83,583 and Methylene blue have both been extensively employed as inhibitors of soluble guanylate cyclase; indeed, both compounds do in fact inhibit soluble guanylate cyclase activity, although by an indirect mechanism via the formation of hydroxyl and oxygen radicals (Kontos and Wei, 1993; Lee and Wurster, 1995). In addition, however, both compounds have also been demonstrated to prevent interleukin-1 induced increases in cAMP in human vascular smooth muscle cells (Beasley and McGuiggin, 1995) and inhibit neuronal NO synthase activity in rat cerebellum (Luo et al., 1995). Furthermore, LY83,583 has also been shown to inhibit glutathione reductase (Luond et al., 1993) and consume NADPH (Sundqvist and Axelsson, 1993). Such confounding properties of these two compounds, therefore, make data difficult to interpret; however, until recently, no other tools have been available to work with. The use of the recently available, selective inhibitor of NO-stimulated guanylate cyclase, ODQ (Garthwaite et al., 1995; Boulton et al., 1995), confirms the specificity of the current observations as being mediated by NO released in a controlled fashion from the nucleophile donor diethylamine-NO (Maragos et al., 1991) and acting via the soluble guanylate cyclase effector pathway.

The nodose ganglion contains the cell bodies of multiple sensory inputs to the central nervous system, particularly pathways governing key homeostatic processes at the medullary level. Included in these centrally-projecting vagal neurons are the primary afferents mediating the baroreceptor, chemoreceptor and cardiopulmonary reflexes (Van Giersbergen et al., 1992). While the electrophysiological data obtained from the isolated rat nodose ganglion preparation represents a population response lacking identification of neuron-type, these data suggest that NO is functionally active at vagal soma and also that NO synthase is synthesised within the nodose ganglion from where the enzyme could be axonally transported to vagal afferent terminals in the nucleus tractus solitarius, thereby providing the ability to produce NO as a cotransmitter-signal transducer for neurons involved for example in central cardiovascular control pathways. Whether endogenous NO is released from neurons within the nucleus tractus solitarius remains to be elucidated. It must also be remembered that while these data may represent evidence for an NO 'receptor' on vagal perikarya and central vagal afferent terminals within the rat nucleus tractus solitarius, the electrophysiological properties of NO donors on the isolated rat nodose ganglion-vagus preparation may also be explained by NO acting as an inter- and intraneuronal messenger within the nodose ganglion itself.

The ability of indomethacin to attenuate the electrophysiological responses of diethylamine-NO and *S*-nitroso-*N*-acetylpenicillamine, albeit to a far lesser extent than the guanylate cyclase inhibitors, suggests that NO may interact with eicosanoids in vagal afferent neurons. Similar interactions between NO and arachidonic acid products have previously been observed in the kidney (Zhang and Sassard, 1993; Zhang et al., 1993), uterus (Franchi et al., 1994), esophagus (Saha et al., 1993) and also NIH3T3 cells (Kelner and Uglik, 1994). Despite the greatly reduced effect of indomethacin compared to ODQ, Methylene blue or LY83,583 on the NO-mediated depolarisation of the rat nodose ganglion, higher concentrations of the cyclooxygenase inhibitor were not employed due to the possibility of non-prostaglandin related effects of indomethacin (Anderson et al., 1983) which would complicate interpretation of the data.

Of interest was the observation that while diethylamine-NO and *S*-nitroso-*N*-acetylpenicillamine were capable of producing electrophysiological repsonses on the rat nodose ganglion, the putative endogenous source of NO, L-arginine, had no effect even on prolonged superfusion. It is possible that the endogenous tissue level of arginine is already in a saturating concentration. Alternatively, the lack of effect of L-arginine may simply reflect that NO is not tonically active in this preparation, a hypothesis strengthened by the lack of electrophysiological effect of the guanylate cyclase inhibitors per se.

In conclusion, the data presented herein clearly indicate a role for NO, acting via the guanylate cyclase effector mechanism, in the processing of rat vagal afferent neurons. In addition, there is evidence to suggest that NO may also interact with eicosanoids derived from arachidonic acid. The presence of the mRNA encoding NO synthase in cell bodies of the nodose ganglion fulfills a requirement necesssary for NO to be released from vagal afferent terminals within the nucleus tractus solitarius.

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