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Soluble guanylate cyclase and neuronal nitric oxide synthase colocalize in rat nucleus tractus solitarii

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Abstract

Nitric oxide has been implicated in transmission of cardiovascular signals in the nucleus tractus solitarii (NTS). Pharmacological studies suggest that activation of neurons by nitric oxide in the NTS may involve soluble guanylate cyclase (sGC). However, anatomical data supporting this suggestion have not been available. In this study, we tested the hypothesis that neurons and fibers containing neuronal nitric oxide synthase (nNOS) lie in close proximity to those containing sGC and the two enzymes colocalize in some neurons and fibers in the NTS. We perfused six rats and obtained brain stem sections for double immunofluorescent staining utilizing antibodies selective for sGC and for nNOS combined with confocal microscopy. The distribution and staining intensity of nNOS-immunoreactivity (IR) was similar to our earlier reports. IR of sGC was present in cell bodies, proximal dendrites and fibers of many brain stem regions. Strong sGC-IR was noted in the hypoglossal, dorsal motor nucleus of vagus and gracilis nuclei. The NTS exhibited moderate sGC-IR. Superimposed images showed that many NTS neurons contained both nNOS-IR and sGC-IR. The percentage of sGC-IR positive cells that were also nNOS-IR positive differed among NTS subnuclei. Similarly, the percentage of nNOS-IR positive cells that were also sGC positive differed among NTS subnuclei. Fibers stained for both nNOS-IR and sGC-IR were also present in NTS subnuclei. In addition, we identified fibers that were stained for nNOS-IR or sGC-IR alone and often found such singly labeled fibers apposed to each other. These data support our hypothesis and provide anatomical support for the suggestion that nitroxidergic activation of the NTS involves sGC.

Keywords: nNOS; sGC; NTS; Confocal; Immunofluorescent

1. Introduction

The nucleus tractus solitarii (NTS) receives visceral inputs from arterial and cardiopulmonary baroreceptors and chemoreceptors and plays a pivotal role in modulating cardiovascular, respiratory, gastrointestinal and renal function (Feldman and Ellenberger, 1988; Lawrence and Jarrott, 1996; Rinaman et al., 1989). Located in the dorsomedial aspect of the medulla oblongata, the NTS contains synapses whose neuronal elements express one of the most diverse populations of neurotransmitters and neuropeptides found in the central nervous system (CNS) (Maley, 1996; Ruggiero et al., 1994). For example, among the many potential transmitters found in the NTS are acetylcholine, adenosine, angiotensin, glycine, carbon monoxide, nitric oxide, glutamate, substance P, neruopeptide Y, neurotensin, γ-

Abbreviations: 4V, fourth ventricle; AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; AP, area postrema; ce, central subnucleus; com, commissural subnucleus; DMV, dorsal motor nucleus of vagus; dl, dorsolateral subnucleus; GABA, γ-aminobutyric acid; ge, gelatinosus subnucleus; Gr, gracilis nucleus; im, intermediate subnucleus; IR, immunoreactivity; is, interstitial subnucleus; me, medial subnucleus; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase; NTS, nucleus tractus solitarii; PBS, phosphate buffered saline; SDS, sodium dodecyl sulphate; sGC, soluble guanylate cyclase; tr, tractus solitariu; vt, ventral subnucleus; XII, hypoglossal nucleus

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aminobutyric acid (GABA), serotonin, dopamine, noradrenaline, and adrenaline (Johnson et al., 1999; Lawrence and Jarrott, 1996; Maley, 1996; Misu et al., 2002; Ruggiero et al., 1994). Each of these agents is distinctly distributed within the NTS. Some may exist in terminals and fibers only while some may also be present in neuronal cell bodies of the NTS.

What neurotransmitter plays a role in regulation of the aforementioned physiological functions has been the topic of extensive investigation over the past few decades. A number of these neurotransmitters are implicated in regulation of specific functions. For example, glutamate, substance P, dopamine and GABA have been implicated in cardiovascular regulation by the NTS (Kubo et al., 1992; Lawrence and Jarrott, 1996; Maley, 1996; Riley et al., 2002; Ruggiero et al., 1994). Substance P and dopamine also may modulate ventilatory responses (Bauman et al., 2002; Goiny et al., 1991; Srinivasan et al., 1991), and recent reports suggest that nitric oxide (NO) may function as a neurotransmitter or as a second messenger in the NTS (Lewis et al., 1991; Ogawa et al., 1995). Supporting that suggestion, microinjection of an NO precursor or NO donors into the NTS elicits cardiovascular, respiratory, or gastric responses (Krowicki et al., 1997; Lewis et al., 1991; Lipton et al., 2001; Lo et al., 1997; Wu et al., 2002) and blockade of NO formation alters cardiovascular and gastric motor function (Krowicki et al., 1997; Lin et al., 1999a; Talman et al., 2001). The suggestion that NO may play a role in modulation of NTS functions is further strengthened by anatomical data that demonstrate the NO synthesizing enzyme neuronal nitric oxide synthase (nNOS) in cell bodies, terminals and fibers of the NTS (Krowicki et al., 1997; Lin et al., 1997).

In the brain, NO is synthesized from L-arginine by one of three different isoforms of nitric oxide synthase (NOS): nNOS (or NOS-I), inducible NOS (iNOS or NOS-II) and endothelial NOS (eNOS or NOS-III) (Bredt and Snyder, 1994; Dawson et al., 1994; Llorens et al., 2002; Moncada et al., 1991). Once NO is formed and released it may react with soluble guanylate cyclase (sGC) resulting in activation of the enzyme and production of cyclic guanycine monophosphate (cGMP) (Chinkers and Garbers, 1991). The cGMP then stimulates cGMP-dependent protein kinase, which phosphorylates substrate proteins to effect a number of actions (Linden et al., 1995). However, the NO signaling pathway in the NTS is only partially understood. It is known that hemoglobin, which may scavenge NO, and methylene blue, an inhibitor of sGC, block L-arginine-induced neuronal activity in cultured NTS neurons (Tagawa et al., 1994). In whole animal studies, microinjection of methylene blue significantly diminishes the hypotensive and bradycardiac effects of the NO donor, *S*-nitrosocysteine (Lewis et al., 1991). Furthermore, methylene blue may attenuate cardiovascular responses to agonists that act at receptors for glutamate, itself a transmitter of cardiovascular reflex signals in NTS (Talman et al., 1980; Talman, 1997). More recent studies have shown that more specific sGC inhibitors, LY83583 and 1*H*-[1,2,4]-oxadiazolo-[4,3,−1]-quinoxalin-1-one (ODQ), suppressed depressor and bradycardiac effects induced by injection of L-arginine into NTS (Lin et al., 1999a) and also attenuated responses to activation of ionotropic glutamate receptors in NTS (Chianca Jr. et al., 2004). Thus, pharmacological data indicate that sGC may be involved in NO-directed functional regulation in the NTS and could provide a link between NO and glutamate signal transduction in NTS. However, despite the demonstration that nNOS is present in the NTS, the distribution of sGC in the NTS and its anatomical relationship with nNOS-containing neurons in the NTS has not been reported. Therefore, we performed an immunofluorescent study for both sGC and nNOS in NTS. We combined immunofluorescent staining with confocal microscopy to examine the distribution of sGC in the NTS and to test the hypothesis that nNOS and sGC are found in the same or closely apposed neurons and fibers in NTS. Through these studies we sought to provide anatomical support for the suggestion that NO may exert its effect via sGC in the NTS.

2. Materials and methods

All procedures adhered to standards established in the National Institute of Health Guide for Care and Use of Laboratory Animals (National Academy Press, Washington, DC, USA, 1996). The Institutional Animal Care and Use Committees of the University of Iowa and Department of Veterans Affairs Medical Center, Iowa City reviewed and approved all protocols. We made every effort to minimize the number of animals used and to militate against causing the animals any pain or distress.

2.1. Confirmation of the specificity of sGC antibody by Western blot analysis

A commercial rabbit anti-sGC antibody (catalogue number 210-724-1, Alexis Biochemical, San Diego, USA) was used for immunofluorescent staining of sGC. This antibody was developed using synthetic peptides from α1 and β1 subunits of sGC (Nakane et al., 1988, 1990). Although the specificity of this antibody had been tested (Gibb and Garthwaite, 2001) and the antibody had been used in a number of studies (Fathian-Sabet et al., 2001; Gibb and Garthwaite, 2001; Hess et al., 1999; Michel et al., 2000), we performed Western blotting of sGC to confirm the specificity of this antibody in NTS tissue. Fresh rat brains ($n = 3$) were frozen immediately with dry ice and cut with a cryostat at −6 °C to obtain 180 μm coronal sections. The NTS was dissected from surrounding tissue with the aid of a dissecting microscope and NTS tissue was pooled. Tissues were homogenized in homogenization buffer (2% sodium dodecyl sulphate or SDS, 1 mM phenyl methyl sulfonyl floride, 1 mM dithiothreitol and 1 mM EDTA in Tris-buffered saline, pH 7.4). Protein concentration was determined using