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Partial Colocalization of NADPH-Diaphorase and Acetylcholinesterase Positivity in Spinal Cord Neurons

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Summary

The freely diffusible radical, nitric oxide (NO), has been assumed to act as a retrograde signaling molecule that modulates transmitter release. Acetylcholine (ACh) is known to function as a typical neurotransmitter. In the present work we have examined the presence of both transmitters (NO and ACh) and their possible relations in the rabbit spinal cord. In our experiments we have used histochemical methods for the visualization of acetylcholinesterase (AChE) and NADPH diaphorase (NADPH-d) which label neurons that express nitric oxide synthase (NOS). Both histochemical methods were performed separately or together on the same sections of the thoracic spinal cord. NADPH-d positive dark blue stained neurons were seen mostly in superficial and deep layers of the dorsal horn, preganglionic autonomic neurons and pericentral area. The presence of AChE positive amber yellow neurons was confirmed mostly in motoneurons located in the ventral horns and in neurons of the pericentral and intermediate zone. Besides the above mentioned neurons, also double-labeled neurons were found which contained both the yellow and dark blue histochemical product. Their presence was confirmed in the intermediate zone and in the pericentral area. Thus, the co-existence of NADPH-d and AChE occurred in the location of interneurons. Our observations suggest that NO may play a role in the control of cholinergic neuronal activity and that NO can be involved in the modulation of synaptic transmission.

Key words
NADPH diaphorase • Acetylcholinesterase • Co-existence • Spinal cord

Introduction

In the central nervous system (CNS) various types of neural cells use different intercellular messengers. To understand the complex functioning of CNS, it is important to know which messengers are used by each type of neurons.

Acetylcholine (ACh) is a well-known classical neurotransmitter, which plays an important role in the circuitry of the spinal cord. Cholinergic neurons have been identified in the spinal cord by using acetylcholinesterase (AChE) histochemistry (Malátová and Maršala 1993). Although AChE is not considered as an exclusive marker for cholinergic neurons, it has been established that these cells are usually cholinoceptive in nature, providing valuable information on the neuronal systems using ACh (Butcher and Wolf 1984). In the spinal cord, at least five different types of cholinergic

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neurons have been found (Sheriff and Henderson 1994). These comprise: 1) somatic motor neurons, 2) preganglionic autonomic neurons at the thoracolumbar (sympathetic) and lumbosacral (parasympathetic) level of the spinal cord, 3) neurons of the intermediate zone (lamina VII), 4) central canal cells (lamina X), and 5) neurons in the dorsal horn (laminae III-V). The last three comprise only a small proportion of total population of neurons in the spinal cord. The functional role for acetylcholine was confirmed in the modulation of primary sensory transmission in the spinal cord, especially that involved in nociception (Gordh *et al.* 1989).

Another messenger is nitric oxide (NO), a free radical gas that diffuses unhindered from the cells of its origin with the potential to affect all cells in its immediate vicinity (Bredt and Snyder 1992). This property allows NO to play a number of different roles in the CNS. NO, generated by nitrergic neurons, can be identified by NADPH-diaphorase (NADPH-d) histochemistry (Dawson *et al.* 1991, Hope *et al.* 1991). In the spinal cord, there are five areas which contain NADPH-d positive neurons: 1) autonomic preganglionic neurons, 2) neurons of the intermediate zone (lamina VII), 3) pericentral neurons (lamina X), 4) neurons in the deep (lamina III-V), and 5) superficial (lamina II) part of the dorsal horn (Kluchová *et al.* 1997, 1998, Maršala *et al.* 1997). A large number of NADPH-d positive structures appear to be involved in spinal sensory processes and visceral regulation, suggesting that NO may play a role of a neurotransmitter in the spinal cord.

The aim of the present study was to elucidate which groups of neurons can co-express both transmitters and could be double-labeled or whether there is heterogeneity in this regard. In order to assess these two possibilities, both NADPH-d and AChE histochemistry were performed on the same sections of the rabbit spinal cord.

Material and Methods

Adult rabbits of both sexes (250-350 g) were anesthetized with pentobarbital (30 mg/kg, i.v.) and perfused transcardially with saline followed by freshly prepared 4 % paraformaldehyde + 0.1 % glutaraldehyde buffered with 1 M sodium phosphate, pH 7.4. Following perfusion fixation, spinal cords were carefully dissected out and stored in the same fixative for 3-4 h. Specimens were then cryoprotected in ascending concentrations of sucrose (15-30 %) with the same phosphate buffer and stored overnight. The fixation procedure, sectioning and storage of the sections, as well as NADPH-d histochemical detection were performed as reported in our previous studies (Maršala *et al.* 1997, Kluchová *et al.* 1997). In brief, dissected parts of the spinal cord were frozen after fixation, and transverse sections (45 μm) were cut from thoracic spinal cord segments and processed for NADPH-d activity according to the previously published protocol (Maršala *et al.* 1997, Kluchová *et al.* 1997).

Sections were then processed for the AChE histochemical method using the classical technique of El Badawi and Schenk (1967).

As a control for NADPH-d we carried out the processing with the omission of the substrate β-NADPH and for the AChE histochemistry the omission of the substrate acetylthiocholine iodide (Alonso *et al.* 1995). No residual activity was observed.

After the reaction, the sections were rinsed in 0.1 M phosphate buffer (pH 7.4), mounted on slides, air-dried overnight and coverslipped with Entellan.

Results

The observation of spinal cord sections stained for NADPH-d and AChE confirmed that a large portion of neurons simultaneously exhibited both neurochemical markers (Fig. 1). In a detailed view through lamina VI, three types of stained neurons could be seen (Fig. 2). The amber yellow color confirmed the presence of AChE positive neurons. Dark blue stained neurons expressed the presence of NADPH-d. Finally, double-labeled neurons containing both the yellow and the dark blue histochemical products were found (Fig. 2). The violet color of these neurons demonstrated the presence of both investigated enzymes (AChE and NADPH-d).

Sections through the thoracic spinal cord revealed neurons with all three colors but their presence was different in various areas. The ventral horn contained only AChE positive neurons and no NADPH-d staining was seen in this location.

Preganglionic autonomic neurons were intensively stained. For detailed investigation of both enzymes in this area, we used longitudinal sections with separate NADPH-d and AChE histochemistry and also sections with double staining. All three types of neurons were seen (Fig. 3).

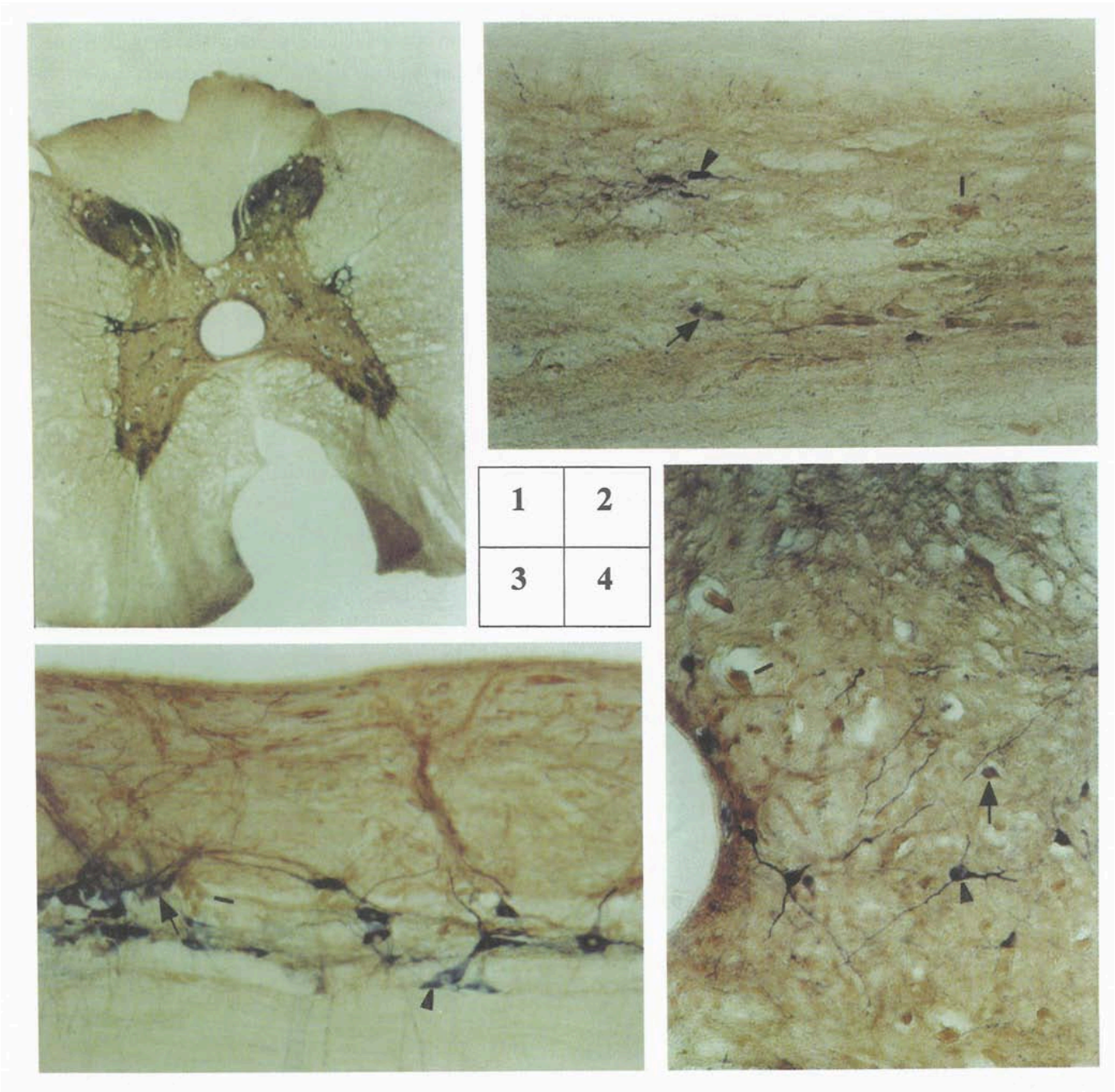

Fig. 1. *Transverse section through the rabbit thoracic spinal cord. Stained both for AChE and NADPH-d. (3,2x3,2).*

Fig. 2. *AChE positive neurons were seen as yellow colored cells (bar), dark blue stained neurons expressed the presence of NADPH-d (arrowhead). Double-labeled neurons containing both yellow and dark blue histochemical product were found (arrow). (25x4).*

Fig. 3. *All three types of neurons were seen in the area of preganglionic autonomic neurons (bar – AChE, arrowhead – NADPH-d, arrow – double stained cells). (10x4)*

Fig. 4. *Pericentral and intermediate zone revealed the presence of single AchE positive yellow neurons (bar), single NADPH-d positive dark blue neurons (arrowhead) and number of cells expressing both co-localized enzymes (arrow). (25x2)*

The pericentral and intermediate zone revealed the presence of single AChE positive yellow neurons, single NADPH-d positive dark blue neurons and a number of cells expressing both co-localized enzymes (Fig. 4).

In the dorsal horn, single AChE and single NADPH-d positive structures were again found, but double-labeled neurons were seen only rarely in this location. This co-existence of enzymes was not found in the superficial layers of the dorsal horn.

Discussion

Using NADPH-diaphorase and acetylcholinesterase histochemistry we have confirmed the co-existence of NADPH-d and AChE in neurons of the thoracic rabbit spinal cord. This co-expression is only partial. The number of co-localized neurons varied in different areas of the spinal cord, ranging from the absence of co-localization in the ventral horn and superficial layer of the dorsal horn up to the highest density in the intermediate and pericentral zone (lamina VII). Similarly, co-localization of NADPH-d and choline acetyltransferase was found in spinal cord neurons of the rat (Wetts and Vaughn 1994) and in the lumbosacral spinal cord of the cat (Vizzard *et al.* 1994).

In the central nervous system, multiple interactions have been studied between the production of NO and ACh (Crespo *et al.* 1998). NO may modulate the cholinergic transmission at the prejunctional and/or postjunctional level in the neuromuscular pathway (Baccari *et al.* 1994). It appears to exert a marked inhibitory effect on ACh release in different regions such as the trachea (Belvisi *et al.* 1991) and tenia coli (Knudsen and Tottrup 1992).

Neural transmission is a complex process which can be explained in the spinal cord as follows: NO as a gas can diffuse freely across membranes and affect all cells within its immediate vicinity (Bredt and Snyder 1992). It can function as an inhibitory neurotransmitter on interneurons and influence the release of their mediator which can be of quite different chemical origin from that of ACh and NO. The inhibitory effect of NO on interneurons would exert an inhibitory effect on ACh release and in this way NO could function as a messenger in such a complex system of neurotransmission. This hypothesis can be supported by the fact that most of the co-localized neurons were seen in the intermediate and pericentral zone which are known to possess the highest incidence of spinal interneurons. It can thus be suggested that neurotransmission is a complex process in which a group of neurons providing classical neurotransmission could be composed of a multiple, functionally distinct neuronal subsets. Future studies should confirm this hypothesis which could be useful in clinical practice, suggesting novel strategies during the therapy of various neurological diseases.

It can thus be concluded that NADPH-d and AChE histochemistry used in the same sections of the rabbit spinal cord has revealed the presence of single- and double-labeled neurons. This difference in their staining can reflect the utilization of different intercellular messengers which could perform different functions within the central nervous system.

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