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SCIENCE DIRECT

Regulatory Peptides 126 (2005) 189–194

REGULATORY PEPTIDES

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Expression of substance P and nitric oxide synthase in vagal sensory neurons innervating the mouse airways

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Received 6 September 2004; accepted 19 September 2004
Available online 12 October 2004

Abstract

Introduction: Airway sensory nerves have the capacity to release neuromediators such as substance P and nitric oxide to control airway functions. The aim of the present study was to investigate substance P and neuronal nitric oxide synthase (NOS-1) expression in airway-specific sensory neurons.
Methods: Airway-projecting neurons in the jugular-nodose ganglia were investigated for NOS-1 and substance P expression by neuronal tracing and double-labelling immunoreactivity.
Results: Of the Fast blue labelled neurons, 14.6±1.8% (mean±S.E.M.) were immunoreactive only for NOS-1, 3.0±0.3% for NOS-1 and substance P, 2.7±0.3% only for substance P, and 79.7±1.7% of the labelled neurons were nonimmunoreactive for substance P or NOS-1 but were partly positive for I-B4-lectin-binding. Fast blue labelled NOS and/or substance P-positive neurons were small to medium sized (<20μm).
Conclusion: Based on the expression of substance P and nitric oxide synthase in airway neurons, the present study suggests that there may be substance P and NO biosynthesis and release following a peripheral activation of the afferents, there could be a triggering of substance P and NO-mediated phenomena, including those related to airway inflammation, such as plasma extravasation and vasodilatation.

Keywords: Airway sensory innervation; Neuronal tracing; Double immunohistochemistry; Mice; Jugular and nodose ganglia

1. Introduction

Airway sensory nerves have the capacity to respond to a broad range of stimuli, including capsaicin, bradykinin, adenosine 5́-triphosphate (ATP), hyperosmolar saline, tobacco smoke and cold dry air with the release of substance P (SP) and/or nitric oxide (NO) [1–6]. The gas molecule NO and the peptide SP are reported to be important modulators of the respiratory system. In the periphery released from the afferent nerve terminals, SP has been demonstrated to mediate many effects such as neurogenic airway inflammation [7,8]. Neurogenic inflammation can be described as a complex response consisting of increased vascular permeability, plasma extravasation, glandular secretion and proinflammatory cells influx which are mediated by SP.

There is also increasing evidence that endogenous nitric oxide (NO) plays an important role in the physiological regulation of airway functions and may be involved in many

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0167-0115/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.regpep.2004.09.006

aspects of airway diseases [3,9,10]. NO is generated by NO synthase (NOS), of which three isoforms, the neuronal (nNOS, NOS-I), endothelial (eNOS, NOS III) and inducible (iNOS, NOS II) are known [11,12].

SP and NOS-I expression so far has been described only in the innervation of human and guinea pigs lower respiratory tract [13,14], the present study, therefore, aimed to examine the occurrence and distribution of SP and NOS-I in airway-specific jugular-nodose ganglia complex (JNC) neurons that innervate the lower respiratory tract of mice by using neuronal tracing techniques in combination with double immunohistochemistry.

The mouse has been chosen in this study as it serves increasingly as species for animal models to study airway diseases due to the advantages of genetically altered mice with targeted disruption or overexpression of genes [15]. Contrary to numerous existing reports on murine airway immunology [16,17] and airway neurobiology of species such as guinea pig [13,18,19], rat [20,21] and human [14,19], only little is known on the airway neurobiology of the mouse.

2. Materials and methods

2.1. Animals

In total, seven adult female FVB mice weighing 15–20 g were used. The animals were kept under standard laboratory conditions. All protocols were performed in accordance with the Animal Welfare Act and the National Institutes of Health "*Guidelines for the care and Use of Laboratory Animals*" (NIH publication 85-23, revised 1985) and approved by the state animal committee (0059/01).

2.2. Neuronal tracing and tissue preparation

Animals were anaesthetised with intraperitoneal injections of ketamine hydrochloride (Ketanest, Parke-Davies, Freiburg, Germany; 10 mg/kg, body weight) and xylazine hydrochloride (Rompun, Bayer, Leverkusen, Germany; 10 mg/kg). The midcervical trachea of the animals was surgically exposed, and 5 μl Fast blue tracing dye (2% aqueous solution containing 1% dimethyl sulphoxide (DMSO); Sigma, St Louis, MO, USA) was injected between the fourth and fifth tracheal cartilage, using the tip of a 10-μl syringe (Hamilton, Bonaduz, Switzerland) into the right main stem bronchus. The cut between the two cartilages was sutured with a 7–0 Vicryl suture, and the skin sutures were made with 4–0 Vicryl suture (Ethicon, Norderstedt, Germany). Major bleeding was not observed during and after the operation. After the injection procedure, animals were placed with elevated head for about 5 min. With this technique, we have previously demonstrated that this small amount of the tracer dye is limited to the lower trachea and the lung [18,22]. All animals recovered undisturbed for a postoperative period of 7 days until they were killed by CO_2 asphyxiation.

Directly after asphyxiation, the animals were perfusion-fixed retrograde through the abdominal aorta with freshly prepared Zamboni's solution (2% paraformaldehyde, 15% picrinic acid, 0.1 M PBS, pH 7.4 for 5 min), as described previously [23]. This routine protocol was performed to ensure optimal conditions for immunohistochemistry and published previously for immunohistochemical analysis of ganglionic neuropeptide expression [18,22]. Dissection of the Zamboni-fixed JNC and lung samples was followed by rinses in 0.1 M phosphate buffer (pH 7.4) and cryoprotection with 18% sucrose in 0.1 M phosphate buffer overnight. Serial sections of the ganglia and lung were cut on a cryostat (Leica, CM 1900, Bensheim, Germany) to 8 μm and air dried for 30 min.

2.3. Immunohistochemistry for SP-, NOS-1- and I-B4-lectin binding

Ganglia and lung sections were first incubated for 1 h with phosphate-buffered saline (PBS) containing 10% normal swine serum to block nonspecific protein binding sites and 0.5% Tween 20 to enhance penetration of immunoreagents. Ganglia and lung sections were then incubated with a combination of a monoclonal antibody from rat that recognises SP (Boehringer Ingelheim Heidelberg, Germany; dilution 1:400) with a polyclonal antiserum to NOS (Dr. B. Mayer, Graz, Austria; dilution 1:1000). In separate studies, biotinylated I-B4 lectin (Sigma, Deisenhofen, Germany; dilution 1:50) was used to identify nonpeptidergic C-fiber neurons. After rinsing in phosphate-buffered saline (PBS), a biotinylated sheep antirat immunoglobulin (IgG) (Amersham; dilution 1:100) was applied for 1 h. Following several washing steps, all

Table 1
Colocalisation of SP and NOS-1 immunoreactivities in sensory neurons in the jugular-nodose ganglia innervating the mouse airways

FB+/NOS-1−/SP−[a] (10–38±2 μm)	FB+/NOS-1+/SP−[b] (10–25±2 μm)	FB+/ NOS-1+/SP+[c] (10–20±2 μm)	FB+/NOS-1−/SP+[d] (10–20±2 μm)
JNC 79.7±1.7%	14.6±1.8%	3±0.3%	2.7±0.3%

Colocalisation of SP and NOS-1 immunoreactivities in JNC neurons innervating the mouse airways. Nearly 79.7% of the Fast blue labelled neurons express neither SP nor NOS-1, 14.6% of Fast blue labelled neurons express NOS-1, only 3% of Fast blue labelled neurons is reactive for NOS-1 and SP, and 2.7% is solely positive for SP-immunoreactivity. Percentages of Fast blue labelled neurons which express [a]FB+/NOS-1−/SP− were neurons with diameters ranging from 10 to 40 μm; [b]FB+/NOS-1+/SP− were neurons with diameters shorter than 20 μm; [c]FB+/NOS-1+/SP+ were neurons with diameters shorter than 20 μm; and [d]FB+/NOS-1−/SP+ were neurons with diameters shorter than 20 μm Fast blue (FB).

sections were incubated with a mixture of a streptavidin–texas red conjugate (Amersham, Freiburg, Germany; dilution 1:200) and FITC-conjugated goat antirabbit IgG (Cappel, ICN Pharmaceuticals, OH, USA; dilution 1:400) for 1 h. Finally, the sections were washed again in PBS and mounted in carbonate-buffered glycerol at pH 8.6.

For immunohistochemical analysis, 20 randomly chosen sections were selected from the serial sections of every ganglia (14 ganglia of seven animals), and all neurons with an apparent nucleus were analysed by using an epifluorescence microscopy (Leica, DMRA2, Bensheim, Germany) and appropriate filter combinations. Fast blue was detected with the filter module U1 (excitation filter: band pass 330–380 nm, barrier filter long pass 418), Texas red with the filter G1 module (band pass 546/10 nm, long pass 590 nm) and FITC with the B2 module (band pass 455–490 nm, long pass 515–560 nm). The section was photographed with the camera (Visitron-System, Imagine microscopy, Puchheim, Germany).

The immunohistochemical analysis was carried out by two investigators. Next to the analysis of the expression pattern of SP, NOS-I and I-B4-lectin, the neuronal diameter was calculated as the mean of the maximum and minimum diameters of the cell bodies by using a calibrated ocular grid (Software program Spot-Advance, Visitron, Germany). To verify the immunoreactive specificity, preabsorption studies was undertaken resulting in an absence of the labelling. Specific immunohistochemical signals also disappeared

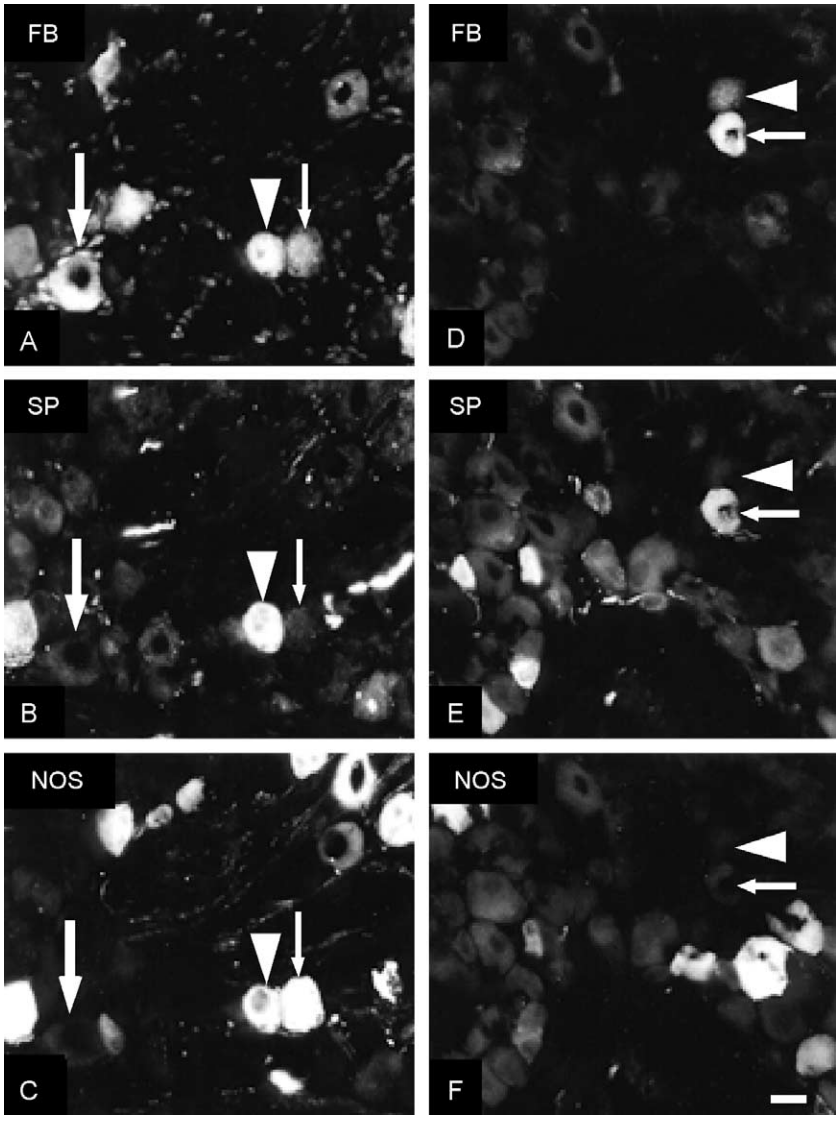

Fig. 1. Correlation of SP and NOS-1 immunoreactivities in Fast blue labelled neurons of JNC complex of mice. (A–C) Fast blue labelled neuron is strongly immunoreactive to SP and NOS-1 (arrowheads). The neighbouring Fast blue labelled neuron displays NOS-1 but not SP immunoreactivities (arrows). This Fast blue labelled neuron expresses neither SP nor NOS-1 immunoreactivities (large arrows). (D–F) Two neighbouring Fast blue labelled neurons, one of these neurons expresses SP immunoreactivity but not NOS-1 immunoreactivity (arrows). The other neuron is neither immunoreactive for SP nor for NOS-1 immunoreactivity (arrowheads). Scale bar=20 μm.

after replacement of the primary antisera by PBS or preimmune serum.

3. Results

3.1. Neuronal tracing

Fast blue dye was found in the epithelium of the main stem bronchi but not in the nasal mucosa or in the epithelium of the proximal trachea of the animals. The dye was detected in the basement membrane and underneath the lamina propria of the bronchial epithelium. JNC neurons of all cell sizes were labelled with the tracer injected in the right main stem bronchus. Fast blue labelled neurons were found on the right and the left side (bilateral) of the JNC.

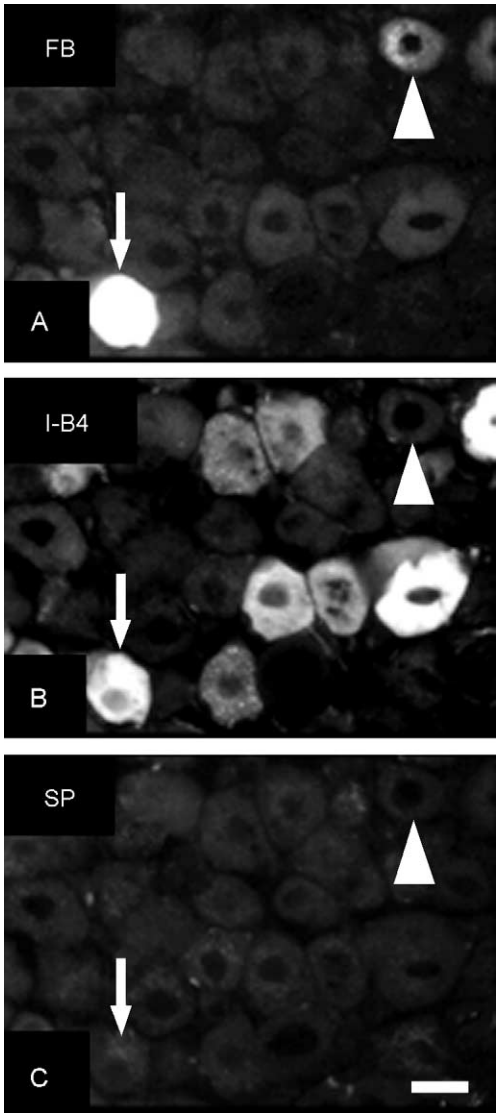

Fig. 2. SP and I-B4 lectin reactivity in Fast blue labelled neurons of JNC of mice. Airway-projecting neuron that binds to I-B4 lectin (arrow in panel [B]) and negative for SP (arrow in panel [C]). A Fast blue labelled neuron which is negative for both I-B4 lectin (arrowhead in B) and SP immunoreactivities (arrowhead in panel [C]). Scale bar=20 μm.

3.2. Immunohistochemistry for SP, NOS-1 and I-B4 lectin reactivity in Fast blue labelled sensory neurons of the JNC neurons innervating the lower airways

In total, 2548 neurons labelled with a neuronal tracer Fast blue from the lower airway were investigated in the present study. All combinations of mediator profiles were found in Fast blue labelled neurons. Fast blue labelled neurons, which were SP positive, were predominantly located to a region in the immunohistochemical section, whereas Fast blue labelled and NOS-1 positive neurons were evenly distributed on the section. Of the Fast blue labelled neurons, 14.6±1.8% (mean±S.E.M.) were immunoreactive only for NOS-1, 3.0±0.3% for NOS-1 and SP, 2.7±0.3% only for SP. Fast blue labelled NOS- and/or SP-positive neurons were found to be small- to medium-sized neuronal profiles (<20 μm). A large proportion (79.7±1.7%) of Fast blue labelled neurons was nonimmunoreactive for SP or NOS-1 (Table 1, Fig. 1).

Further characterisation of NOS-1- and SP-negative neuronal population showed that most of them were found to be I-B4-lectin positive. These neurons had diameters ranging from 10 to 40 μm. Also, Fast blue negative neurons positive for I-B4-lectin binding were found to be abundantly present. (Fig. 2).

4. Discussion

Using this technique of neuronal tracing in combination with double immunohistochemistry, the present study aimed to investigate SP and NOS-1 expression in JNC neurons that innervate the vessels and the epithelium of the lower airways. Sensory neurons innervating the lower airways of mice were largely found to be located at JNC as reported for rats and guinea pigs by previous studies [18,20].

The present study showed that only 5.7% of the total amount of Fast blue labelled sensory neurons exhibit SP immunoreactivity. The percentage of SP immunoreactivity in neurons projecting to the airways of the mouse is relatively low if compared to the high percentage (60–67%) of SP-positive neurons innervating the respiratory tract of the species guinea pigs, as published previously [18]. This discrepancy in the innervation pattern may arise from the species-specific difference as reported before for the neurochemical basis of the sensory primary neurons in trigeminal ganglia of different species such as the rat, guinea pig and mouse [24,25] and may also arise from the different location where the tracing substances were injected in the present and the previous studies. In the present experiments, Fast Blue was injected in the right stem bronchus, whereas in previous study, Fast Blue was injected in the upper guinea pigs trachea [18].

Fast blue labelled neurons displaying SP immunoreactivity belonged to the subgroups of neurons with small diameters < 20 μm. These neurons are most probably capsaicin-sensitive C-fibers which are known to be mechanically insensitive and have low conduction velocities averaging about ~0.5 ms^{-1} [5]. Furthermore, we have provided information on diameters of airway-specific neurons, which may be useful information for the study of neuronal plasticity which has been already described for guinea pigs large-diameter neurons after allergen and NGF exposition [26–28]. The present study revealed that only 3% of the Fast blue labelled neurons displayed SP and NOS-1 IR. These neurons are likely to be cell bodies of the SP /NOS-1 IR nerves fibres found in the airways of many species including human [14,18]. Multimodal stimulation of the vagus nerve of rodents was demonstrated to cause microvascular leakage, bronchoconstriction and vasodilatation, which could be prevented by prior treatment with capsaicin or tachykinin antagonists indicating the tachykinin release from sensory nerves [7,18,29–32]. SP has also been reported to stimulate mucus secretion from submucosal glands and goblet cells [33]. Endogenous tachykinins have been shown to enhance cholinergic neurotransmission and capsaicin pretreatment to reduce significantly cholinergic neural response both in vitro and in vivo [34,35].

In contrast to the large body of evidence for the functional role of the tachykinins in the airways, however, only little is known about their cellular source of origin. Here, we directly show airway-specific neurons of vagal sensory ganglia of mice containing SP, which may release SP from their terminal endings to mediate neurogenic airway inflammation.

The costorage of NO and SP in the same vagal sensory airway-specific neurons suggests an interaction of these two mediators in their effects. Numerous studies have been carried out to examine the pharmacological effects of NO in the airways and have revealed that NO appears to be an important bronchodilator of human and rodents airways [6]. NO has been shown to enhance tachykinergic activity by modulating NK receptors [36]. In accordance with previous findings on guinea pig trachea [22], we found that 14.6% of the vagal sensory-labelled neurons expressed NOS-1 IR but not tachykinin IR, and that they were mainly medium-sized neurons. NOS-1 has also been localised to human airway-nonspecific vagal sensory neurons [13]. The physiological role of this NOS-1-positive neuronal population has not been investigated so far and therefore remains to be investigated in future experiments.

Out of the airway-projecting neurons, more than 79.7% displayed neither SP nor NOS-1 IR. To further characterise these neurons, we have examined their binding to the lectin I-B4, which is a marker for nonpeptidergic C-fiber neuronal population. These neurons were found to be partly binding to I-B4 lectin indicating that they belong to the group of nonpeptidergic C-fibers [37,38]. The findings that Fast blue labelled (airway-specific)-, SP- and NOS-1-negative neurons were different in their cell size (10 to 40 μm diameters) suggest that these neurons may belong to different neuronal subpopulations. Most of them are probably Aδ-fibres [39]. However, the exact physiological role of these murine airway neurons remains to be investigated in future studies.

In conclusion, based on the expression of SP and NOS-1 in airway sensory neurons, the present study suggests that, due to SP and NO release following a peripheral activation of the nerve endings, there may be a triggering of SP- and NO-mediated phenomena such as plasma extravasation and vasodilatation.

Acknowledgements

This project was supported by BMBF and DFG (SFB 547). The authors thank W. Kummer and R. Haberberger for the helpful discussions, M. Strozynski for his expert technical assistance and M. Scheibner for the editorial work.

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