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Neurotransmitters in parasympathetic ganglionic neurons and nerves in mouse lower airway smooth muscle

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ABSTRACT

In most species, including humans, lower airway smooth muscle (ASM) contains nerve terminals from two distinct populations of parasympathetic ganglionic neurons based on neurotransmitter phenotype: cholinergic and non-adrenergic non-cholinergic (NANC), causing contraction and relaxation, respectively, of ASM. Using immunohistological staining, the density and distribution of NANC-associated neurotransmitters, vasoactive intestinal peptide (VIP) and nitric oxide synthase were 6% of total nerve profiles compared to 19% cholinergic nerves in ASM in mouse (C57BL/6) central airways. The location of the NANC parasympathetic neurons innervating the tracheal ASM, as determined by retrograde neuronal tracer from the trachealis muscle, was the myenteric plexus of the esophagus, closely associated with the outer striated longitudinal muscle layers; the majority of the retrograde-labeled neurons were VIP- and NOS-IR. The results of these experiments provide the first direct evidence that VIP-IR and NOS-IR neurons intrinsic to the mouse esophagus project axons to the adjacent trachealis muscle.

1. Introduction

Neurons in airway parasympathetic ganglia function to control and distribute the signals emanating from the central nervous system and are pivotal in regulating airway smooth muscle (ASM) tone and airway caliber. In most species, airway parasympathetic neurons display complex anatomical and electrophysiological characteristics that contribute to regulation of synaptic activity (Myers, 2001). Neuronal complexity and function is further increased as a result of two distinct populations of parasympathetic neurons, cholinergic and non-adrenergic non-cholinergic (NANC), mediating contraction and relaxation, respectively, of ASM; rarely do these nerve types overlap (Canning, 2006). These different parasympathetic nerve types also differentially affect airway mucous secretion which may be altered in the diseased airway (Choi et al., 2007). Lower ASM in most mammalian species, including humans, has sparse or no direct sympathetic innervation (Richardson and Beland, 1976; Canning and Fischer, 2001; Canning, 2006), making studies of the regulation of parasympathetic nerve pathways critical for understanding control of autonomic tone of ASM.

The lower airways of many mammalian species, including guinea pig, ferret, cat, chicken, cow, sheep, monkey, baboon, mice and human, have varying degrees of NANC parasympathetic relaxant innervation (Diamond and Richardson, 1982; Canning and Fischer, 2001). The neuroactive compounds most commonly released from NANC nerves causing ASM relaxation are vasoactive intestinal peptide (VIP) and nitric oxide (NO; Hasaneen et al., 2003). Interestingly, cholinergic and NANC nerve profiles rarely overlap in ASM or in ganglionic neurons and, in several species (guinea pigs, ferrets), cholinergic and NANC neurons are in anatomically distinct areas on the airway wall (Fischer et al., 1998; Dey et al., 1996) and are differentially activated (Mazzone and Canning, 2002; Canning, 2006).

In mice, the allergen-induced contraction of ASM has a well-defined cholinergic component (Eum et al., 1999) that is predominantly due to activation of cholinergic parasympathetic nerves (Weigand et al., 2009). Although functional studies indicate that ASM in mouse airways has NANC relaxant responses, (Hasaneen et al., 2003) the source of NANC efferent nerves is unknown. To address this issue, we designed a series of experiments to locate and characterize the parasympathetic neurons innervating the tracheal ASM and compare neurotransmitter phenotypes of the neurons and distribution of their nerve terminals. We combined immunohistochemical staining with tract tracing techniques to determine that the location of NANC airway ganglionic neurons that innervate the trachea are located in the ventral esophageal myenteric layer.

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2. Methods

Animal research was approved by the Johns Hopkins University Animal Care and Use Committee.

2.1. Tissue preparation for immunostaining

For immunostaining, adult male mice (C57BL/6, 20–22 g; Jackson Labs, Bar Harbor, ME) were overdosed with pentobarbital (75 mg/kg, IP), transcardially perfused with phosphate buffered saline (PBS, pH 7.4) containing 0.1% procaine, followed by perfusion with 4% formaldehyde in PBS containing 0.1% procaine, post-fixed for 24 h at RT, and rinsed in PBS. For sectioning, the tissue was cryoprotected with 18% sucrose in PBS for 24 h (4 °C), covered with O.C.T. mounting medium and frozen. Cross sections (10 μm thickness) of the trachea with esophagus attached or right primary bronchus were cut on a cryostat, collected on lysine-coated slides and air dried for 30 min. For whole mount immunostaining, the fixed tissue was dehydrated with an increasing series of ethanol concentrations (50–100%) to xylenes and rehydrated with decreasing concentrations of ethanol, the trachea and bronchi were cut longitudinally down the ventral length, and opened as a sheet. All tissue (whole or sections) were incubated with blocking solution containing 1% bovine serum albumin (BSA), 10% normal goat or donkey serum (depending on source of secondary antibody) and 0.1% Tween 20 in PBS for 60 min. Tissue sections were incubated for 24 h, whole mounts with rocking motion for 72 h, at 4 °C in a mixture of antibodies (below) in PBS containing 0.1% Triton-X 100 and 1% BSA (PBS-Tx + BSA). Slides and whole tissues were washed in PBS-Tx + BSA and incubated for 2 h at 22 °C with a mixture of appropriate anti-primary antibody labeled with either Alexa 647 or Alexa 488 (Life Sciences, Inc., Carlsbad, CA) diluted with PBS-Tx + BSA.

The distribution and verification of neuronal cell bodies or nerve fibers in ASM were determined after immunostaining with a rabbit polyclonal antibody that binds to protein gene product 9.5 (PGP9.5; Ultraclone, Isle of Wight, UK, 1:400). To determine ASM delineation and area measurements, Alexa 488-labeled phalloidin (Life Sciences) was used. Nerve quantification was based on area and not by the total length of axons in a given reference volume (i.e. unlike Muhlfeld et al., 2010). For percentage of neurotransmitter subtypes in ASM nerves, separate sections were immunostained with either rabbit anti-PGP9.5 antibody (UltraClone) or chicken anti-PGP9.5 antibody (Abcam, Cambridge, MA, 1:100) combined with either goat anti-ChAT (Chemicon-Millipore, Billerica, MA; 1:100), sheep anti-nNOS and, unless otherwise stated and to improve the staining of iNANC nerves, sheep anti-VIP (both from Millipore-Chemicon, 1:200), rabbit anti-tyrosine hydroxylase (Abcam, Cambridge, MA, 1:1000), rat anti-substance P (Millipore-Chemicon, 1:500), or sheep anti-neuropeptide Y (NPY; Millipore-Chemicon, 1:1000). To evaluate non-specific staining, separate sections were also processed similarly but species-specific immunoglobulin replaced the primary antibody. For all immunofluorescent (IF) staining, the slides were evaluated with the appropriate filter sets to allow separate visualization of the Alexa fluorochromes (e.g. donkey anti-goat Alexa 488 with donkey anti-sheep Alexa 647). Quantitative analysis of digitized images of IF stained areas was determined by threshold intensity segmentation using iVision software (Biovision Technologies, Exton, PA, USA). Measurements were performed on five sections, 100 μm apart, from each area (upper and lower trachea; middle of each right primary bronchus) of airway. All images for analysis were taken with a UPlanFL 20× objective with a 7.0 μm depth of focus; stained sections were routinely 7.5 μm thick, based on *z*-plane measurement (iVision) of phalloidin-stained smooth muscle, i.e. there was approximately 25% shrinkage from the 10 μm frozen section and most of the section was within the depth of focus.

2.2. Tissue preparation for retrograde tracing

In order to determine if neurons in the esophageal myenteric plexus (EMP) ganglia project axons to the airway, retrograde tracing techniques were used: mice ($n = 6$) were anesthetized, the ventral trachea exposed, and 2 μl of hydroxystilbamidine dye (similar to Fluorogold dye, 5%, Life Sciences) was injected from a Hamilton syringe needle, pushed through the ventral trachea into the dorsal lumen wall, among 4–6 sites into the trachealis muscle in dorsal wall of the thoracic and cervical trachea. The mice were allowed to recover for seven days, overdosed with pentobarbital, and perfused with formaldehyde (as above). EMP ganglionic neurons that project axons to the trachea were identified by the accumulation of hydroxystilbamidine in their cell bodies in complete series of cryostat sections (20 μm thick sections) throughout the entire length (4–5 mm) of esophagus associated with the injected tracheal region or in whole mount esophagus using a fluorescent microscope equipped with a filter set appropriate for visualizing hydroxystilbamidine dye (excitation 330–385 nm, dichroic 400 nm, barrier 420 nm). In another group of animals, the dye was injected into the trachealis muscle just before the cervical trachea and esophagus were separated by blunt dissection, presumably cutting the nerves between the EMP ganglia and cervical trachealis muscle and either the quantity of nerves (VIP/NOS- and PGP9.5-IF) in tracheal ASM was quantified ($n = 4$), or the uptake of dye by EMP neurons was determined as above ($n = 4$); in both cases, the animals were allowed to recover for seven days prior to perfusion fixation for immunostaining, or check for dye, respectively.

2.3. Materials

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.4. Data analysis

Results are presented as mean ± one standard error of the mean. Sample mean values were analyzed by analysis of variance, and the values comparing staining areas of two types of neurotransmitters or two different areas were compared using Student's t statistics for two means. Means were considered to differ significantly if P values were <0.05, and n represents the number of animals studied in each group.

3. Results

3.1. Nerve density in ASM

The overall high density of nerves in tracheal airways smooth muscle (ASM) is obvious in whole mount staining using PGP9.5 immunofluorescence (IF). On a 4 mm length of dorsal thoracic trachea (Fig. 1A), there were many large nerve bundles and ganglia between the cartilaginous rings, smaller nerves can be seen throughout the ASM, especially in a cross section at a higher magnification where single nerve fibers and terminals can be seen in and around the ASM (Fig. 1B–D). In the upper (cervical) trachea (Fig. 1B), lower (intrathoracic) trachea (Fig. 1C), or primary bronchus (Fig. 1D; $n = 6$ for all regions) there was no difference in density of PGP9.5 immunostained nerve fibers, relative to ASM area (Fig. 1E). Using double IF staining for PGP9.5 and ChAT in whole mount tissue, the only obvious ChAT IF staining was the ganglionic neurons (not shown) and, due to the low levels of ChAT nerves in the ASM, these nerve profile were more obvious in sections (below). There was even lower VIP IF nerves in whole mount ASM but could be quantified in sections (below).