Innervation pattern of guinea pig pulmonary vasculature depends on vascular diameter

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Haberberger, Rainer, Michael Schemann, Holger Sann, and Wolfgang Kummer. Innervation pattern of guinea pig pulmonary vasculature depends on vascular diameter. J. Appl. Physiol. 82(2): 426–434, 1997.—The pulmonary vasculature is supplied by various neurochemically distinct types of nerve fibers, including sensory substance P-containing and autonomic noradrenergic, nitrergic, and cholinergic axons. Pharmacological experiments have suggested that various segments of the pulmonary vascular tree respond differently to the respective neuromediators. We, therefore, aimed to determine histochemically and immunohistochemically for each of these neurochemically distinct perivascular axons their quantitative distribution along the vascular tree from the extrapulmonary trunks to the smallest intraparenchymal ramifications in control guinea pigs (n = 5). Generally, arterial innervation was more developed than that of veins. Along the arterial tree, noradrenergic and substance P-containing axons were ubiquitously from the pulmonary trunk to smallest intraparenchymal vessels, whereas nitrergic axons were practically restricted to large (>700-µm) extrapulmonary arteries. Cholinergic axons were regularly present at arteries down to 100 µm in diameter and innervated two-thirds of small arteries (50–100 µm). The results demonstrate that the noradrenergic vasoconstritor innervation extends throughout the pulmonary vascular system whereas the innervation pattern with various types of vasodilator fibers changes with vascular diameter, parallel to known pharmacological differences in cholinergic and nitrergic vasodilator effects.

Material and Methods

Specimens

Specimens for immunostaining were obtained from five adult female guinea pigs. The animals were perfused transcardially with polyvinylpyrrolidone- and procainamide HCl-containing rinsing solution (13), followed by 4% buffered paraformaldehyde (pH 7.4). The thoracic viscera were removed en bloc, washed repeatedly in 0.1 M phosphate buffer, cryoprotected in the same buffer with 18% sucrose added, snap frozen in liquid nitrogen, and stored at −30°C until required for immunostaining. They were serially sectioned at a thickness of 12–14 µm with a cryostat (model HM 500, Microm, Heidelberg, Germany).

Immunohistochemistry

The sections were first covered for 1 h with a blocking medium consisting of 10% normal swine serum, 0.1% bovine serum albumin, and 0.5% Tween 20 in 0.1 M phosphate-buffered saline (PBS). Primary antibodies were applied overnight at room temperature. Sections were washed and incubated for 1 h either with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody or with a biotinylated secondary antibody, washed again, and incubated for 1 h with streptavidin-conjugated Texas red to label the biotinylated secondary antibody (see Table 1). After incubation, sections were rinsed in PBS and coverslipped in carbonate-buffered...
glycerol at pH 8.6. The slides were evaluated by epifluorescence microscopy by using a band-pass 470- to 490-nm excitation filter and band-pass 515- to 550-nm barrier filter for FITC-labeled antibodies, and a band-pass 545- to 580-nm excitation filter and a long-pass 610-nm barrier filter for fluorescence of Texas red (Fluorescence microscope BX 60, Olympus, Hamburg, Germany).

Preincubation with antigen (ChAT: amino acids 168–189, NOS purified from porcine cerebellum) at a concentration of 20 µg/ml antiserum diluted to working concentration resulted in a complete absence of staining with the corresponding antisera in the lung (Fig. 1). Specificity of the other antisera for immunohistochemistry of guineapig lung was established by preabsorption controls in a preceding study (20).

**NADPHd Histochemistry**

Sections were incubated in 1 mM NADPH (Biomol, Hamburg, Germany) and 0.3% Triton X-100 (Sigma Chemical, Deisenhofen, Germany) in 0.1 M phosphate buffer (pH 7.4) for 30–45 min at 37°C, rinsed in 0.1 M phosphate buffer, and coverslipped in carbonate-buffered glycerol (pH 8.6).

**Quantification of Vascular Innervation**

Pulmonary arteries and veins were classified into four groups according to the size of their luminal, i.e., inner diameter: 1) vessels >700 µm in diameter (located between the heart and lung hilum), 2) vessels 350–700 µm in diameter (located at the hilum), 3) intraparenchymal vessels with a diameter between 100 µm and 350 µm, and 4) intraparenchymal vessels with a diameter between 50 µm and 100 µm.

For each group the number of innervated section profiles was counted and expressed as percentage of the total number of vascular section profiles. In each animal, five different levels, each being at least 200 µm apart in craniocaudal direction, were investigated, and at each level at least five sections were quantitatively evaluated. Differences in the percentages of innervated section profiles between 1) different vessel sizes in a given neurochemical class and 2) different neurochemically defined types of axons in a given vascular size class were evaluated by F- and t-tests. Differences were considered as significant for P values ≤ 0.05.

**RESULTS**

**ChAT-Immunoreactive (irChAT) Nerve Fibers at Pulmonary Vessels**

Pulmonary arteries and veins of the guinea pig were supplied by a sparse-to-moderately dense plexus of perivascular nerve fibers with immunoreactivity to ChAT. The varicose irChAT axons were located at the adventitial-medial border of the vascular wall (Figs. 2 and 3).

Arteries. Pulmonary arteries with diameters ranging from >700 µm to <100 µm were innervated by irChAT axons (Fig. 2). Approximately two-thirds of the smallest intraparenchymal vessels (50–100 µm) showed irChAT perivascular axons, whereas, for larger pulmonary arteries, irChAT nerve fibers occurred on almost all vessels (Table 2). The percentage of innervated section profile-

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**Table 1. Characteristics of immunoreagents**

<table>
<thead>
<tr>
<th>Code</th>
<th>Host Species</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>P3</td>
<td>Rabbit</td>
<td>1:800–1:1,500</td>
<td>Own (31)</td>
</tr>
<tr>
<td>NC 1/34 HL</td>
<td>Rat monoclonal</td>
<td>1:600</td>
<td>Serva (Heidelberg, Germany)</td>
</tr>
<tr>
<td>NOS1</td>
<td>Rabbit</td>
<td>1:3,000</td>
<td>Dr. B. Mayer (Graz, Austria) (6)</td>
</tr>
<tr>
<td>TH 2/40/15</td>
<td>Mouse monoclonal</td>
<td>1:40</td>
<td>Boehringer (Mannheim, Germany)</td>
</tr>
</tbody>
</table>

ChAT, choline acetyltransferase; SP, substance P; NOS, nitric oxide synthase; TH, tyrosine hydroxylase; IgG, immunoglobulin G; FITC, fluorescein isothiocyanate.
in the size class of 50–100 µm was significantly lower than for TH-immunoreactive (irTH) \( (P \leq 0.01) \) and SP-immunoreactive (irSP) axons \( (P \leq 0.05) \). On the other hand, it was significantly higher \( (P \leq 0.001) \) than for NADPHd-positive axons in all vessels <700 µm in luminal diameter.

Veins. The percentage of section profiles of pulmonary veins supplied by irChAT axons depended on the diameter of the veins, with the larger blood vessels being more frequently innervated than the smaller (Table 2). Veins >700 µm in diameter, surrounded for the most part by myocardium, were innervated with irChAT nerve fibers. The axons were distributed within the myocardial coat (Fig. 3a) and could also be observed at the border between heart muscle and smooth muscle. Sixty-one percent of veins with a diameter between 350 and 700 µm showed perivascular irChAT axons, whereas vessels <350 µm were only occasionally supplied by irChAT nerve fibers (Table 2, Fig. 3, b–d). The percentage of innervated section profiles in the size class of 50–100 µm was significantly lower \( (P \leq 0.01) \) than for irTH and irSP axons and significantly higher \( (P \leq 0.05) \) than for NADPHd-positive axons.

NADPHd-Positive Nerve Fibers at Pulmonary Vessels

NOS immunohistochemistry and NADPHd reaction gave identical results with regard to vascular innervation. The quantitative analysis was performed at NADPHd-labeled sections.
Arteries. Nerve fibers labeled by NADPHd reaction were seen at large vessels. All section profiles of pulmonary arteries >700 µm in diameter received NADPHd-positive axons. At the hilar region, 62% of section profiles were innervated. For parenchymal vessels >100 µm in diameter, 10% were supplied, and the smallest intraparenchymal vessels were only occasionally innervated with NADPHd-positive nerve fibers (Table 2, Fig. 4). The percentage of innervated section profiles in vessels <700 µm was significantly lower (P < 0.05) than for all other neurochemically defined types of axons, except for NADPHd-positive fibers in the size class of 100–350 µm, in which a P value of 0.055 was evaluated.

Veins. NADPHd-positive nerve fibers were present at all pulmonary veins from their exit from the lung to their entrance into the left atrium. Only one-fifth of smaller vessels 350–700 µm in diameter received NADPHd-positive axons (Table 2, Fig. 5b). No nerve fibers could be labeled at pulmonary veins <350 µm in diameter (Fig. 5, c and d). The percentage of innervated section profiles in vessels <350 µm in diameter was significantly lower (P ≤ 0.05) than for all other neurochemically defined types of axons, except for NADPHd-positive fibers in the size class of 100–350 µm, in which a P value of 0.055 was evaluated.

### irSP Nerve Fibers at Pulmonary Vessels

Arteries. irSP fibers were found from the main arteries, which were invariably innervated, down to small arteries, which still received irSP axons by 90% (Table 2). The vessels showed a moderate-to-dense perivascular nerve plexus that did not vary considerably in density along the vascular tree (Fig. 6, a–d). The percentage of innervated section profiles was practically identical to irTH axons; differences compared with irChAT- and NADPHd-positive axons are reported in irCHAT-Immunoreactive (irCHAT) Nerve Fibers at Pulmonary Vessels.

Veins. irSP nerve fibers supplied all large pulmonary veins (Fig. 7a). Axons with irSP were noted at 62% of all investigated veins from 350 to 700 µm in diameter (Fig. 7b). About 20% of pulmonary veins <350 µm in diameter received irSP nerve fibers (Table 2, Fig. 7, c and d). The percentage of innervated section profiles of the smallest veins (50–100 µm) was significantly (P < 0.01) higher than for any other neurochemical class.

### irTH Nerve Fibers at Pulmonary Vessels

Arteries. irTH was shown in nerve fibers at virtually all pulmonary arteries from >700 µm in diameter down to arteries 100–350 µm in diameter (Fig. 8). Ninety-two percent of section profiles of smallest intraparenchymal

### Table 2. Innervation of pulmonary vessels related to vascular size

<table>
<thead>
<tr>
<th>Axonal Enzyme/Peptide</th>
<th>Luminal Diameter, µm</th>
<th>50–100</th>
<th>100–350</th>
<th>350–700</th>
<th>&gt;700</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pulmonary arteries</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ChAT</td>
<td>69±4.5 (199)*</td>
<td>90±4.1 (122)</td>
<td>98±1.5 (44)</td>
<td>100±0 (27)</td>
<td></td>
</tr>
<tr>
<td>TH</td>
<td>92±0.8 (169)*</td>
<td>98±1.5 (75)</td>
<td>100±0 (27)</td>
<td>100±0 (16)</td>
<td></td>
</tr>
<tr>
<td>SP</td>
<td>90±1.3 (207)*</td>
<td>98±0 (92)</td>
<td>100±0 (35)</td>
<td>100±0 (10)</td>
<td></td>
</tr>
<tr>
<td>NOS</td>
<td>1±0.3 (149)#</td>
<td>10±1.2 (104)#</td>
<td>63±1.2 (25)#</td>
<td>100±0 (15)</td>
<td></td>
</tr>
<tr>
<td><strong>Pulmonary veins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ChAT</td>
<td>4±1.3 (187)</td>
<td>3±3.6 (81)#</td>
<td>61±14.8 (30)</td>
<td>90±10 (25)</td>
<td></td>
</tr>
<tr>
<td>TH</td>
<td>11±0.8 (157)</td>
<td>14±5.0 (54)#</td>
<td>70±11.2 (33)#</td>
<td>100±0 (10)</td>
<td></td>
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<tr>
<td>SP</td>
<td>25±1.1 (229)</td>
<td>16±2.1 (73)#</td>
<td>62±5.0 (26)#</td>
<td>100±0 (12)</td>
<td></td>
</tr>
<tr>
<td>NOS</td>
<td>0±0 (138)</td>
<td>0±0 (89)#</td>
<td>21±3.9 (25)#</td>
<td>100±0 (10)</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE calculated from 5 animals given in percentage of innervated vascular section profiles by corresponding neurochemically defined type of axons; nos. in parentheses, total nos. of vascular section profiles. Significantly different compared with next bigger vessel class: *P ≤ 0.05, †P ≤ 0.01; ‡P ≤ 0.001. Differences among neurochemically distinct classes within a given size class are indicated in the text.

![Fig. 4. Pulmonary arteries. NADPH diaphorase (NADPHd)-positive axons (arrows) at a section profile of a large artery (a) and at arterial vessels 350–700 µm in diameter (b). Smaller vessels lack NADPHd-positive fibers (c, d). Endothelium shows positive NADPHd reaction (arrowheads). See Fig. 2 for luminal diameter for a–d. Bar, 20 µm.](http://jap.physiology.org/Downloadedfrom)
vessels (50–100 µm) were still supplied with irTH axons (Table 2). Differences in the percentages of innervated section profiles compared with other axons are reported in ChAT-immunoreactive (irChAT) Nerve Fibers at Pulmonary Vessels and NADPH\textsubscript{d}-Positive Nerve Fibers at Pulmonary Vessels.

**DISCUSSION**

The present study demonstrates a caliber-specific distribution of four functionally and neurochemically distinct classes of perivascular axons along the pulmonary vascular tree of the guinea pig. In general, innervation of pulmonary veins was much less developed than that of pulmonary arteries and was mainly restricted to the veins at the lung hilum close to their entrance into the left atrium. Although not quantita-
tively investigated, a predominant arterial vs. venous innervation has been reported for acetylcholinesterase-positive (5, 10) and catecholamine-fluorescent axons (4, 28) in the lungs of several mammals. The different morphologies of pulmonary arterial and venous innervations are accompanied by heterogeneities in the pharmacological responsiveness of these vessels to neural and endothelial mediators. In full-term fetal lambs, acetylcholine induces endothelial-dependent relaxation of fourth-generation pulmonary veins but not arteries (15). On the other hand, data obtained in the isolated blood-perfused canine left lower lung lobe suggest that acetylcholine constricts the venous segments while moderately relaxing the arterial segment under conditions of elevated tone (11). The close neighborhood of irCHAT axons to both cardiac and smooth muscle cells in the venous wall observed in this study suggests that both types of myocytes are targets of neurally released acetylcholine. In line with this assumption, M2 muscarinic receptors have been pharmacologically identified on cardiomyocytes from guinea pig left atria (24), and binding sites of the muscarinic agonist, [3H]quinuclidinyl benzilate, have been reported on the smooth muscle of rat pulmonary vein (8). However, systematic studies on the differential responses of pulmonary veins of various diameters and comparison with corresponding arterial segments are missing, because due to its clinical relevance, e.g., in
pulmonary hypertension, the arterial innervation and regulation of arterial tone have attracted much more interest.

In accordance with previous reports based on aldehyde-induced catecholamine fluorescence (4, 28), sympathetic noradrenergic axons as identified by immunoreactivity to the rate-limiting enzyme of catecholamine synthesis, TH, innervated all pulmonary arterial ramifications from the pulmonary trunk down to arteriolar level. This distribution is shared with irSP axons, which, in the guinea pig, invariably also contain calcitonin gene-related peptide (CGRP) and are derived from sensory neurons (21). Thus the entire pulmonary arteriolar level is addressed by both vasoconstrictor (noradrenergic via $\alpha_1$-adrenoreceptors; Refs. 19, 23) and vasodilator (SP- and CGRP-containing fibers via CGRP release; Ref. 3) axons. Interactions between these separate but closely neighboring fiber types are likely to occur because it has been demonstrated that CGRP-induced relaxation of guinea pig pulmonary arteries in response to electrical field stimulation is attenuated by $\alpha_2$-adrenoreceptor agonists (3).

This ubiquitous distribution along the arterial tree is in sharp contrast to that of perivascular axons of presumably parasympathetic origin, i.e., those containing NOS and/or ChAT. Nitrergic axons are regularly present at the pulmonary trunk and the extraparenchymal right and left pulmonary arteries (12) but invade the lung only minimally and are absent at arteriolar level. This differential distribution of nitrergic fibers explains the inhomogenous pharmacological results obtained for guinea pig pulmonary arteries of different calibers: endogenous NO counteracts the $\alpha$-adrenoreceptor-mediated pulmonary vasoconstriction (6, 22, 23). In the main pulmonary artery, this endogenous NO is of neuronal origin because, after endothelium removal, an inhibitor of NOS is still effective in reducing vasorelaxation in response to electrical field stimulation (23). In contrast, endothelium removal largely reduces nonadrenergic noncholinergic vasorelaxation in preparations of branches of the guinea pig pulmonary artery (23) so that neurally generated NO does not contribute to regulation of vascular tone at this arterial segment. Thus pharmacological and immunohistochemical data coincide in that extrapulmonary but not intrapulmonary segments of the guinea pig pulmonary artery are controlled by vasodilatory nitrergic axons. Hence, in contrast to other vascular beds such as the cerebral arteries (1), NO cannot be regarded as the primary mediator of neurally induced relaxation of guinea pig intrapulmonary arteries. This may be different at the main pulmonary artery. At this vascular segment, there is a cholinergic component of neurogenic vasodilation, but the dominant part is mediated by other mechanisms because atropine reduces relaxation induced by electrical field stimulation only by $\sim$30% (19). These pharmacological data correspond well with the presence of both peptide-containing sensory and nitrergic nerve fibers in addition to irChAT perivascular axons at this vascular segment, as demonstrated in the present study. It has to be stressed, however, that the nitrergic axons at the main pulmonary artery might well be a subpopulation of the cholinergic nerve fibers so that individual axons at this vascular segment contain both ChAT and NOS, as it is highly likely in the innervation of cerebral vessels (27).

The distribution of cholinergic axons as identified by immunoreactivity against the acetylcholine synthesizing enzyme ChAT holds an intermediate position between that of noradrenergic and peptide-containing sensory axons on the one side and that of nitrergic axons on the other, in that these fibers do enter the parenchymal regions of the lung but are observed at only approximately two-thirds of arteriolar section...
profiles. This immunohistochemical observation suggests a heterogenous influence of neurally released acetylcholine on arteriolar vasomotion. Direct data obtained at guinea pig pulmonary small arteries are not available, but observations made on guinea pig intestinal small arteries correspond well with the incomplete cholinergic innervation pattern demonstrated here. Recording simultaneously small-artery diameter and smooth muscle membrane potential, Kotaecha and Neild (18) observed vasodilation induced by acetylcholine accompanied by rapidly developing hyperpolarization in 79% of small arteries but no involvement of acetylcholine in arteriolar vasomotion in 21%. These figures are remarkably similar to our quantitative immunohistochemical data demonstrating 69% ChAT-innervated vs. 31% noninnervated arteriolar section profiles in the lung. Thus, in contrast to the ubiquitous adrenergic regulation of arteriolar tone, there is morphological and functional evidence for a mosaic pattern of cholinergic arteriolar regulation. Physiological and pharmacological data in the cat suggest that axonally released acetylcholine diffuses through the thin muscular coat of small pulmonary arteries and small arteries to the endothelium where stimulation of muscarinic receptors results in the release of NO that activates soluble guanylyl cyclase in smooth muscle cells (25, 26). In favor of this assumption are 1) the presence of nerve terminals with the ultrastructural characteristics of cholinergic axons at the media/adventitial border of these vessels in the cat (17) and of irChAT axons in the guinea pig (this study), 2) the ability of perivascularly applied [3H]acetylcholine to permeate the vascular wall as demonstrated for rabbit central ear arteries (16), and 3) the immunohistochemical demonstration of muscarinic receptors on guinea pig pulmonary arterial endothelium (20). This does not exclude, however, the presence of other pathways of cholinergic vasodilation either involving the endothelium also or being endothelium independent.

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