Invited Review

HIGHLIGHTED TOPIC | Reflexes from the Lungs and Airways

Evidence for a role of neuroepithelial bodies as complex airway sensors: comparison with smooth muscle-associated airway receptors

Dirk Adriaensen, Inge Brouns, Isabel Pintelon, Ian De Proost, and Jean-Pierre Timmermans
Laboratory of Cell Biology and Histology, Department of Veterinary Sciences, University of Antwerp, Antwerp, Belgium

Adriaensen, Dirk, Inge Brouns, Isabel Pintelon, Ian De Proost, and Jean-Pierre Timmermans. Evidence for a role of neuroepithelial bodies as complex airway sensors: comparison with smooth muscle-associated airway receptors. J Appl Physiol 101: 960–970, 2006; doi:10.1152/japplphysiol.00267.2006.—The epithelium of intrapulmonary airways in many species harbors diffusely spread innervated groups of neuroendocrine cells, called neuroepithelial bodies (NEBs). Data on the location, morphology, and chemical coding of NEBs in mammalian lungs are abundant, but none of the proposed functions has so far been fully established. Besides C-fiber afferents, slowly adapting stretch receptors, and rapidly adapting stretch receptors, recent reviews have added NEBs to the list of presumed sensory receptors in intrapulmonary airways. Physiologically, the innervation of NEBs, however, remains enigmatic. This short overview summarizes our present understanding of the chemical coding and exact location of the receptor end organs of myelinated vagal airway afferents in intrapulmonary airways. The profuse populations that selectively contact complex pulmonary NEB receptors are compared with the much smaller group of smooth muscle-associated airway receptors. The main objective of our contribution was to stimulate the idea that the different populations of myelinated vagal afferents that selectively innervate intraepithelial pulmonary NEBs may represent subpopulations of the extensive group of known electrophysiologically characterized myelinated vagal airway receptors. Future efforts should be directed toward finding out which airway receptor groups are selectively coupled to the complex NEB receptors.

sensory airway receptors; innervation; lung

GENERAL ASPECTS OF PULMONARY NEBs

Pulmonary NEBs (38) may be defined as highly specialized and extensively innervated groups of pulmonary neuroendocrine cells (PNECs) that are normal components of the epithelium of intrapulmonary airways in humans, other mammals, and in all other air-breathing vertebrate groups studied.

During the last 25 years, detailed information has become available about the ontogenetic development, distribution, microscopic morphology, and chemical coding of NEBs (for reviews, see Refs. 2, 60, 63, 65).

Neuroendocrine cells of the airway epithelium have been included in the “diffuse neuroendocrine system” (50), members of which are known to have important functions in the local control of various organs. PNECs and NEBs harbor typical endocrine-like dense-cored vesicles that store ATP, serotonin (5-HT), and several neuropeptides, such as gastrin-releasing peptide (bombesin), calcitonin gene-related peptide (CGRP), calcitonin, enkephalin, somatostatin, cholecystokinin, and substance P (SP) (for reviews, see Refs. 1, 2, 61, 63).

NEBs are now believed to have different functions in the regulation of physiological processes during specific periods in prenatal, early postnatal, and adult life (2, 60, 63, 64), but their exact role is still poorly understood.

Potential oxygen-sensing and effector mechanisms have been identified in PNECs (20, 33, 51, 89). NADPH oxidase has been identified as a molecular oxygen sensor in both native NEB cells (29, 78) and PNEC cell line models (44, 78), but also other oxygen-sensing mechanisms may be involved (46). Evidence obtained from NEBs in situ suggests that hypoxia evokes both K+ channel inhibition (27) and release of 5-HT (28). The nature of the oxygen-sensitive K+ channels involved has been the subject of several elegant studies in both PNEC cell lines (30, 45, 47) and native NEBs (27).
Because of their undisputable vagal sensory innervation, NEBs have been added to the list of presumed afferent receptors in the lower airways, which, until recently, included slowly (SARs) and rapidly adapting stretch receptors (RARs) and C-fiber receptors only. In general, however, all studies about the effect of hypoxia on pulmonary vagal afferent fibers have yielded negative results, and physiologically their innervation remains an enigma (83).

SHORT HISTORICAL OVERVIEW OF CONCEPTS REGARDING THE INNERVATION OF PULMONARY NEBs

Long before the existence of pulmonary NEBs was known, several authors had described intraepithelial varicose nerve terminals that were concentrated in groups and irregularly distributed along the airways of different species, including humans (8, 23, 35, 37). Fröhlich (25) was the first to describe delicate nerve terminals that were intimately related to grouped neuroendocrine cells in the airways of rabbits and cats. Several groups have since then reported an indisputable innervation of NEBs in both light and electron microscopic investigations.

A number of methods have been used to visualize nerve fibers that contact mammalian pulmonary NEBs. An unambiguous and simultaneous identification of PNECs and nerves appeared to be essential. For an extensive review and references, we refer to Adriaensen and coworkers (1). Among others, frequently used techniques were silver staining, the histochemical demonstration of acetycholinesterase, formaldehyde-induced fluorescence, immunocytochemistry using antisera against general neuronal and neuroendocrine markers and/or more selective markers for specific nerve fiber populations, and transmission electron microscopy (TEM). Different morphological types of nerve terminals have been described using TEM. Most often reported in contact with NEBs in many species are nerve endings packed with mitochondria and some small clear vesicles, penetrating deep between the NEB cells, often situated close to the luminal surface. These nerve terminals reveal asymmetric synaptic contacts, with an accumulation of dense-cored vesicles near electron-dense, cone-shaped thickenings of the surface membrane of NEB cells and are believed to be afferent. TEM offered a good morphological characterization of the direct innervation of a limited number of pulmonary NEBs but unfortunately left another important question unanswered, i.e., that of the origin of the nerve fiber population(s) that selectively innervate(s) NEBs.

Until the early 1990s, studies dealing with the latter question combined TEM, for the evaluation of nerve terminals contacting NEBs, and experimental vagotomy [rabbits (39, 40); rats (75)]. Infranodosal vagotomy in several species strongly reduced the number of nerve terminals in NEBs, while the innervation appeared to be intact after supranodosal vagotomy. Based on these findings, it was suggested that NEBs are predominantly innervated by sensory nerve fibers originating from neurons located in the vagal nodose ganglia.

As a conclusion of this short historical perspective, and to come back to the main point of the present review, i.e., the potential role of pulmonary NEBs as airway receptors, it is useful to explain the origin of the general belief that no physiologist has ever measured specifically NEB-related activity in pulmonary vagal afferents. Based on the above-outlined electron microscopic literature data about the organization of NEBs and directly related nerve terminals, and on the strongly promoted suggestion by several investigators that NEBs are airway hypoxia sensors, many reviews on pulmonary NEBs came to the same conclusion, i.e., NEB innervation has a mainly vagal nodose origin, and NEBs may act as hypoxia sensors via this vagal afferent pathway (2, 20, 74). Moreover, the proposed scheme (see Ref. 2) was considered representative of pulmonary NEBs in a 2001 review on airway receptors (83). The belief in this concept has recently been strengthened by the characterization of a carotid body-like oxygen-sensing mechanism in NEBs, which implicates exocytosis of transmitters induced by hypoxia (for reviews, see Refs. 33, 51). As a result, many researchers in the field today believe that hypoxia may cause, besides local reflex actions (39), an afferent signal to travel toward the central nervous system via the vagus nerve. This, however, leaves us with the essential question of why lung physiologists have been unable to confirm this apparently rather simple mechanism. Are NEBs really the lung receptors we would like them to be? If so, did we overlook something? Did we pay sufficient attention to the published physiological data, and/or did the lung physiologists use the available morphological data in an optimal way?

Further on, we will try to find out if it is really that simple by focusing on the innervation of rat lung NEBs. If NEBs are indeed airway receptors, the best way to understand them would be to have a really good look at their nervous connections. Neurochemical coding combined with denervation experiments was used to identify the origin of different nerve fiber populations with receptor-like terminals in the airways, and, because conduction velocity is a key feature for differentiating functional classes of airway receptors, myelin sheaths were visualized.

SHORT LITERATURE SURVEY ON THE MORPHOLOGICAL IDENTITY OF ELECTROPHYSIOLOGICALLY IDENTIFIED MYELINATED MECHANOSENSORS IN THE LOWER AIRWAYS

Physiologically, myelinated vagal mechanosensors in the lower airways are classically subdivided in two groups, i.e., RARs and SARs, that are considered to harbor several subtypes.

RARs are characterized by their fast adaptation rate to a maintained stimulus, their mechanosensitivity, and discharge related to some chemical stimuli. It is, however, difficult to define the “true” stimulus, and the adaptation appears to show a wide range of variation that may overlap with certain populations of SARs. Except for the fact that they are undoubtedly myelinated (mainly A6 range), the location and morphology of RARs have so far not been determined with certainty in most species (56, 57, 82). Considerable efforts have been made to identify RAR-like sensors in guinea pig airways (for reviews, see Refs. 24, 71).

Although it has always been somewhat controversial (for reviews, see Refs. 56, 58, 83), it is generally believed that the predominantly mechanosensory SARs are located in airway smooth muscle (83). The latter was apparently confirmed by a recent elegantly combined morphological and electrophysiological study (92). Especially for the trachea and extrapulmonary bronchi, evidence based on direct dissection of tissues has provided convincing evidence for the location of SARs in the smooth muscle layer (6). There is general consensus that SARs...
structurally correspond to myelinated fibers (mainly Aβ and Aγ ranges), as clearly reflected by the conduction velocity (56, 82), which averages ~30 m/s in rats (7). SARs, however, reveal a large variation in distribution and discharge patterns that so far have only been poorly related to variable reflex responses, including control of breathing (58).

Morphologically defined mechanoreceptor-like structures have been described at locations believed to represent the airway smooth muscle layer in different animal species. Early work using classic light microscopic methylene blue, silver, or osmium tetroxide staining revealed nerve fibers that give rise to complex terminals, considered sensory, in airway smooth muscle bands (5, 23, 36, 37). Myelinated afferents with terminals integrated in the “myoelastic system” of bronchi were also seen in a combined conventional light and electron microscopic study in rats (77). More recent immunohistochemical studies have reported branching receptor-like nerve complexes in the airway wall of different animal species. Calretinin immunoreactivity (IR) was reported to be expressed in extensively branching nerve endings in apparently the smooth muscle layer of rat airways (87), and very recently Na+/K+-ATPase α3 immunostaining in rat and rabbit lungs resulted in the visualization of nerve terminals with multiple branches, presumably embedded in airway smooth muscle or in the lamina propria (92, 93). Neurofilament protein and protein gene-product 9.5 (PGP9.5) IR (86, 88) were seen in treelike nerve endings in dog airways. Different names have been given to the sensory receptor-like terminals supposed to be associated with the airway smooth muscle, based on their location, appearance, and presumed relationship to physiologically characterized receptors: “smooth muscle nerve spindles” (36), “pulmonary stretch receptors” (77), and SARs (87, 93).

Great efforts have been done to characterize guinea pig airway afferents, using elegant combinations of physiological recording and visualization of nerve cell bodies in vagal sensory ganglia and afferent terminals in the airways. Today, guinea pig airways are suggested to harbor at least three subtypes of myelinated vagal mechanosensitive afferents, i.e., SARs, RARs, and “cough receptors” (17, 18, 42).

It is clear that data on the morphology and especially the neurochemical characteristics of putative airway mechanoreceptors remain limited, hampering a scientifically justified correlation between the multiplicity of physiologically identified receptors and the rare morphologically well-defined lung receptors. One reason might be that many of the sensory receptors have a so far poorly identified morphology; another reason is that they may be integrated in more complex receptor “end organs” that are able to combine various sensory activities.

**MORPHOLOGICAL AND NEUROCHEMICAL CHARACTERIZATION OF THE SELECTIVE SENSORY INNERVATION OF NEBs IN RAT LUNGS**

For a summary, see Fig. 4.

**CGRP-Immunoreactive Component of the Innervation of Pulmonary NEBs**

For many years now, literature mentions a population of sensory CGRP-immunoreactive (ir) nerve fibers that contacts NEBs (62, 65, 69). Retrograde tracing from the lungs and denervation studies (Ref. 68 and unpublished observations) imply that the CGRP-ir nerve fibers that selectively contact NEBs in rat lungs belong to a spinal sensory population that originates from dorsal root ganglia T1 to T6.

Clearly, rat airways also harbor a vagal CGRP/SP+ nerve fiber population that, in contrast to the vagal nodose fibers that are described to contact NEBs, originates from the jugular ganglia (Fig. 4). These vagal C-fiber-like nerve terminals can be found in the epithelium of large-diameter bronchi only, apparently without any specific relationship to NEBs (unpublished observations).

A few years ago, our laboratory further characterized the CGRP-ir nerve fiber population that selectively contacts rat NEBs (13). The varicose fibers invariably appeared to colocalize SP, and CGRP/SP double labeling, therefore, allowed the differentiation of individual nerve terminals at the level of CGRP-ir NEB cells. It was shown that the spinal sensory CGRP+/SP+ nerve terminals do not penetrate the epithelium, as will further be demonstrated for the vagal sensory endings in NEBs, but that they form a plexus at the basal pole of NEBs (Fig. 4). After capsaicin treatment, the percentage of NEBs contacted by CGRP/SP-positive nerve terminals was dramatically reduced compared with control lungs, while the numbers of CGRP-ir NEBs revealed no significant changes (13). All CGRP-ir nerve fibers in the vicinity of and contacting NEBs expressed transient receptor potential vanilloid 1 (TRPV1) receptors (capsaicin receptors) and may therefore be considered capsaicin sensitive, while NEBs themselves appeared to be TRPV1 negative (13).

All available data on the spinal sensory component of the selective innervation of NEBs are summarized in the scheme of Fig. 4 (nerve fibers shown in dark blue). Briefly, the sensory nerve fiber population concerned forms a mainly basal plexus, most likely has its origin in the dorsal root ganglia T1-T6, can be marked by its CGRP/SP IR, is capsaicin sensitive and expresses TRPV1 receptors, and therefore presents obvious C-fiber characteristics.

**Vagal Nodose Connections of Pulmonary NEBs**

Considering what was known in the late 1990s, it was clear that a determining factor in the recognition of NEBs as sensory airway receptors would be the full confirmation and characterization of a vagal nodose innervation. When we started to address this hypothesis a few years ago, the following questions needed an answer: Is the vagal nodose innervation of NEBs, which was suggested based on TEM data (see higher), really there? How can it unambiguously be identified in the light microscope? What are the neurochemical characteristics? How does this vagal nodose connection relate to what lung physiologists have reported?

Because literature data indicated that, in rat lungs, NEBs are contacted by CGRP-positive nerve fibers (16, 62, 65, 69), it was generally believed that the latter represented the predicted vagal sensory connection of NEBs. However, as explained above, our vagotomy and other experiments unambiguously showed that this CGRP-ir nerve fiber population is nonvaginal.

We then injected the red fluorescent neuronal tracer DiI into the rat nodose ganglia and visualized traced fibers in combination with NEBs in lung cryosections (4). Extensive intraepithelial terminal arborizations of DiI-labeled vagal nodose af-
different appeared to be associated with the presence of NEBs and were shown to be different from the CGRP-ir nerve fibers innervating NEBs. This was the first conclusive evidence demonstrating at the light microscopic level that vagal nodose sensory nerve terminals indeed reveal selective contacts with pulmonary NEBs in rats.

Neuronal tracing and vagal denervation experiments, combined with multiple immunolabeling, revealed that this vagal innervation of NEBs, but also the NEBs themselves, express the calcium-binding protein calbindin D28k (CB) (9), and hence that CB is an interesting routine marker for NEBs in rat lungs. However, because both NEBs and contacting vagal nodose nerve fibers are stained, this marker does not allow a clear evaluation of the intraepithelial terminals. Furthermore, it was revealed that CB and CGRP IR mark different nerve fiber populations, although often contacting the same NEBs, that the CB-ir population is insensitive to capsaicin treatment, and that it does not express TRPV1 capsaicin receptors (13).

A functionally very important question regarding the vagal sensory component of the innervation of pulmonary NEBs, especially in light of the present efforts for characterizing NEBs as sensory airway receptors, was that of myelination. We therefore used antibodies against the myelin basic protein (MBP) to visualize myelinated nerve fibers in the lung. These experiments showed that the CB-ir vagal nodose fibers contacting NEBs are invariably myelinated (13). Although myelinated nerve fibers had been observed in the vicinity of NEBs using TEM (75), this was the first evidence for a direct link between the myelinated fibers and vagal nodose intraepithelial nerve terminals in NEBs.

Immunostaining for P2X3 purinoreceptors (ATP receptors) revealed intraepithelial arborizations of P2X3 receptor-ir nerve terminals that always colocalized with the presence of NEBs in rat airways, while NEB cells did not express P2X3 receptors (9). P2X3 receptor and CB IR revealed a complete overlap in the vagal nodose innervation of NEBs and were clearly different from the CGRP-ir fibers. Infranodosal vagal denervation further supported our hypothesis that the P2X3 receptor-expressing nerve fibers contacting NEBs have their origin in the vagal nodose ganglia. Combination of MBP and P2X3 receptor immunostaining confirmed that this nerve fiber population was myelinated (13). Furthermore, combined quinacrine histochernistry, applied to selectively visualize high concentrations of ATP in secretory granules, and P2X3 receptor staining revealed that the ATP receptor-expressing nerve terminals in rat lungs are exclusively associated with quinacrine-stained NEBs (9). It was therefore suggested that ATP may be a neurotransmitter in the vagal sensory innervation of NEBs and that, given the extensive date obtained in other systems (14, 15), at least part of the NEBs might be involved in vagal afferent mechanosensory and/or nociceptice transduction from the airways.

Over the past few years, several immunohistochemical markers for the visualization of selective populations of sensory nerve terminals have been reported. Antibodies against the plasma membrane sodium/potassium exchanging protein, Na\(^{+}\)-K\(^{+}\)-ATPase \(\alpha\)3 (21, 80, 93), and against proteins that load glutamate into synaptic vesicles, vesicular glutamate transporter 1 (VGLUT1) (85) and VGLUT2 (55), have been used to identify mecanoreceptor terminals in other organs. We very recently performed multiple immunostaining using the above panel of modern markers. It was found that antibodies against VGLUTs (selective markers for so-called glutamatergic neurons) were excellent key markers for many different populations of sensory nerve terminals in rat lungs (10–12). The resulting new findings are summarized below.

Multiple immunocytochemical staining for VGLUTs and CB (as a marker for NEBs and their contacting vagal nodose nerve fibers) revealed that all intraepithelial vagal nodose nerve terminals in NEBs express VGLUTs (Figs. 1 and 3). The above-mentioned myelinated P2X3-ir nerve fibers that terminate in NEBs (13) were shown to invariably coexpress VGLUTs (12). Furthermore, we were able to establish that all myelinated nerve fibers that selectively contact NEBs had diameters ranging from 1 to 3.5 \(\mu\)m (10, 11).

Na\(^{+}\)-K\(^{+}\)-ATPase \(\alpha\)3-ir nerve fibers, seen to approach the rat airway epithelium, branch and form basketlike terminals that completely surround part of the neuroendocrine cells in NEBs (Fig. 3). All Na\(^{+}\)-K\(^{+}\)-ATPase \(\alpha\)3-ir nerve fibers that contact NEBs appear to coexpress CB and VGLUTs. Extensive double labeling of VGLUTs and Na\(^{+}\)-K\(^{+}\)-ATPase \(\alpha\)3, however, revealed that only part of the VGLUT-ir nerve fibers also show Na\(^{+}\)-K\(^{+}\)-ATPase \(\alpha\)3 IR (Fig. 3). The latter could be
explained by the observation that pulmonary NEBs receiving a Na\(^{+}\)-K\(^{+}\)-ATPase \(\alpha_3\)-positive terminal were often additionally innervated by separate strongly P2X\(_3\) receptor-expressing nerve endings that may occupy remarkably distinct areas in the same NEBs. P2X\(_3\) receptor-ir intraepithelial nerve terminals in NEBs apparently never express Na\(^{+}\)-K\(^{+}\)-ATPase \(\alpha_3\) IR and vice versa (10, 11).

All available data on the neurochemical coding of the vagal nodose sensory component of the innervation of NEBs are summarized in the scheme of Fig. 4 (nerve fibers shown in red and light blue). Briefly, two different myelinated nodose nerve fiber populations are concerned, which both reveal extensive intraepithelial terminals in NEBs and are CB IR, glutamatergic, and insensitive to capsaicin: 1) additionally expresses P2X\(_3\) receptors; 2) additionally expresses Na\(^{+}\)-K\(^{+}\)-ATPase \(\alpha_3\).

Consequently, it is now beyond discussion that most pulmonary NEBs are supplied by extensive populations of myelinated vagal nodose afferents, which, according to our quantification, may account for more than 3,000 receptor sites in rat lungs (73). The latter observation makes it hard to imagine that lung physiologists have so far been unable to link these NEB-related populations to measurable activities in vagal afferents.

**FUNCTIONAL MORPHOLOGICAL AND NEUROCHEMICAL CHARACTERIZATION OF SMOOTH MUSCLE-ASSOCIATED AIRWAY RECEPTORS IN RAT LUNGS**

For a summary, see Fig. 4. Very recently, we started to further characterize receptor terminals that have been described in the wall of rat bronchi (87, 93). To achieve that goal, an extensive panel of more or less selective markers forafferent nerve fiber populations, identical to the one that has been used to establish the neurochemical coding of NEB afferents (described in the previous paragraph), was used (10, 11). The data obtained by combining neurochemical coding and vagal denervation experiments enabled us to identify the nature and origin of nerve fibers with complex receptor-like terminals that were selectively located in airway smooth muscle bundles, further referred to as “smooth muscle-associated airway receptors (SMARs)” (10, 11).

Branching nerve terminals with well-delineated laminar end organs, located just beneath but never within the epithelium of intrapulmonary airways, sometimes very close to NEBs, were seen to express Na\(^{+}\)-K\(^{+}\)-ATPase \(\alpha_3\) (Figs. 2 and 3) and VGLUTs (Fig. 3). VGLUTs revealed a cytoplasmic location in the nerve endings of SMARs, while Na\(^{+}\)-K\(^{+}\)-ATPase \(\alpha_3\) appeared to predominantly label the surface membrane, accentuating the laminar appearance of the terminals (Fig. 3). Three days following a unilateral cervical vagal denervation, SMARs could no longer be observed in ipsilateral intrapulmonary airways.

The subepithelial nerve endings invariably colocalized with airway smooth muscle bundles, as clearly revealed by additional labeling of rat lung sections that were processed for VGLUTs or Na\(^{+}\)-K\(^{+}\)-ATPase \(\alpha_3\), with antibodies against the smooth muscle marker \(\alpha\)-smooth muscle actin (Fig. 2).

MBP immunostaining proved that SMARs, identified by their Na\(^{+}\)-K\(^{+}\)-ATPase \(\alpha_3\) or VGLUT IR, are myelinated (diameters ranging between 1 and 3.5 \(\mu\)m), the myelin sheath being lost just before branching of the fibers into the smooth muscle bundles (Fig. 2).

A differential expression of purinergic P2X\(_3\) receptors could be demonstrated in SMARs. While some of the laminar end organs exhibited a strong P2X\(_3\) receptor IR, expression could hardly be visualized in other SMARs.

Multiple immunostaining for Na\(^{+}\)-K\(^{+}\)-ATPase \(\alpha_3\) and several calcium-binding proteins revealed a strong cytoplasmic calretinin IR in most SMARs, confirming the observations of Yamamoto and coworkers (87), while an unambiguous CB IR was only seen in a smaller subpopulation.

SMARs did not express CGRP, and thin varicose CGRP-ir nerve fibers that were often seen to cross smooth muscle bundles in rat airways apparently did not reveal a specific relation to the SMAR endings.

All available data on the sensory receptors described in airway smooth muscle bundles of rat airways are summarized in the scheme of Fig. 4 (nerve fibers shown in green). Briefly, the nerve fiber population concerned has its origin in the vagal nodose ganglia, is myelinated, forms laminar end organs (SMARs) between airway smooth muscle cells, is glutamatergic, and can be marked by its expression of calcium-binding proteins, Na\(^{+}\)-K\(^{+}\)-ATPase \(\alpha_3\), and P2X\(_3\) ATP receptors. Although a detailed quantification is not available so far, it is
clear that the number of SMARs is much lower than that of NEBs.

IN SITU LIVE CELL IMAGING OF DIFFERENT POPULATIONS OF MYELINATED LUNG SENSORS IN AIRWAY WHOLE MOUNTS AND LUNG SLICE PREPARATIONS

Recently, we have developed in our laboratory different models for the in situ visualization of NEBs (53), and/or SMARs (De Proost et al., unpublished observations), in both airway whole mount preparations and vibratome slices of whole lungs. We are now evaluating the possibilities of these models for further physiological characterization of the neurochemically identified “sensors.”

DISCUSSION, CONCLUDING REMARKS, AND FUTURE PROSPECTS

The present short review has been focused on recent data exploring the location, morphology, and neurochemical coding of both subepithelial and intraepithelial sensory receptor-like structures in the airways. Newly identified subepithelial lamellar terminals appeared to be consistently associated with the airway smooth muscle layer and were therefore named SMARs (10, 11). The intraepithelial receptor end organs were invariably colocalized with the well-known pulmonary NEBs. SMARs, neurochemically characterized by their IR for a selected panel of markers for myelinated (mechano)sensory nerve fibers (Na<sup>+</sup>-K<sup>-</sup>-ATPase α3; VGLUTs; P2X<sub>3</sub> ATP receptors; calcium-binding proteins; MBP), likely represent structures identical to those described earlier in rat airways (77, 87, 93). In rat lungs, however, SMARs were often found very close to pulmonary NEBs that appeared to harbor intraepithelial vagal nodose sensory fibers with a nearly identical neurochemical coding. As a consequence, none of these markers may be regarded as “selective” for SMARs. On the other hand, the combination of Na<sup>+</sup>-K<sup>-</sup>-ATPase α3, VGLUTs, and/or P2X<sub>3</sub> ATP receptor, with α-smooth muscle actin immunostaining, finally provided indisputable evidence for the specific location of subepithelial receptor-like terminals within airway smooth muscle bundles (10, 11). The expression of P2X receptors on subpopulations of the vagal airway receptors may reveal a role of ATP in sensory transduction from the airways.

In this respect, the very recent observation that several populations of vagal airway receptors in the guinea pig are sensitive to ATP agonists via P2X receptors (34) is very interesting, because it confirms the neurochemical coding and may open new possibilities for the functional identification of neurochemically identified sensory receptors in the airways. In contrast to the rat, NEB cells in hamster airways were reported to express P2X receptors (26), but nothing was said about connecting sensory nerve terminals, and no vagal fiber recordings are available.

Today, it is not clear whether the rather frequent close association of NEBs and SMARs represents a coincidence or a specific functional colocalization. The epithelial layer of the rat airway tree harbors several thousands of NEBs (73), and the smooth muscle layer is invariably located very close to the mucosa. Considering the rather extensive receptive fields occupied by SMAR terminals (93), it is not surprising to find sections of some of them close to NEBs.

Our finding that Na<sup>+</sup>-K<sup>-</sup>-ATPase α3 is expressed in myelinated vagal nodose fibers that terminate intraepithelially in rat NEBs, combined with the fact that NEBs are often closely associated with Na<sup>+</sup>-K<sup>-</sup>-ATPase α3-ir SMARs (10, 11), is not in agreement with a recent study of SARs in rat lungs (93). In the latter study, receptive fields were identified by probing the lung surface, while recording SAR activity in the cervical vagus nerve. Tissue blocks containing “SAR activity” were removed and immunostained for Na<sup>+</sup>-K<sup>-</sup>-ATPase α3 (using the same antibody as in our study) to label so-called SARs in rat lungs. In that way, these investigators, per definition, used peripheral lung blocs only. Na<sup>+</sup>-K<sup>-</sup>-ATPase α3-ir receptor-like terminals, very similar to the SMARs described above,
however, were observed in only 8 out of 15 identified “blocs.” Earlier physiological studies suggest that the majority of SARs is located in extrapulmonary and large-diameter intrapulmonary airways (59, 83), but this is certainly not the case for all species studied (32), and no clear data are available for rats. NEBs, on the other hand, are present in all intrapulmonary airways in the rat lung and abundant in peripheral airways (73), making it even more strange that intraepithelial Na⁺-K⁺-ATPase 3-ir terminals were explicitly reported to be absent in all blocs studied by Yu and colleagues (93). In our opinion, a problem of staining sensitivity is the most likely explanation for the discrepancy observed between the number of physiologically and morphologically identified “mechanoreceptors” and the detection of SMARs but not NEBs in peripheral rat lung blocs (93). Furthermore, the present observation (10, 11) of an almost identical chemical coding of SMARs and nearby populations of vagal sensory terminals in NEBs clearly points out that electrophysiological data based on “local” stimuli should be interpreted with great caution.

Although SMARs and NEBs indeed reveal a generally different morphology and location in smooth muscle and epithelium, respectively, their neurochemical coding and receptor-like characteristics appear to be very similar. Functionally very important was the observation that both SMARs (10, 11) and intraepithelial nerve terminals in NEBs (10–13) originate from myelinated vagal nodose afferents that have similar diameters, ranging from 1 to 3.5 μm.

Because the used panel of markers (Na⁺-K⁺-ATPase α3, VGLUT1, VGLUT2, P2X3 ATP receptors, calcium-binding proteins) (10, 11) has been described to rather selectively label mechanoreceptor terminals in other organs (21, 22, 55, 81, 85), both SMARs and vagal nodose nerve terminals in NEBs seem to be good candidates to represent the morphological counterparts of at least subsets of the physiologically identified myelinated vagal airway mechanoreceptors. The nearly identical

---

**Fig. 4.** Schematic representation of the main innervation of airway smooth muscle and of the sensory innervation of complex NEB receptors in rat airways. Only nerve fiber populations that are important for the present short review were added (color coded). Known characteristics of the represented neuronal populations and the NEB are included in the scheme in the same color as the respective structures. The central part of the scheme shows airway smooth muscle that receives nerve terminals from postganglionic parasympathetic neurons located in an airway ganglion (bottom part; cholinergic neurons = purple). As summarized in the present work, laminar nerve terminals of a SMAR (colored green) intercalate between the smooth muscle cells. The top center part of the scheme represents a pulmonary NEB (colored yellow) and its extensive interactions with sensory nerve terminals. The top left part shows the myelinated vagal nodose afferent connections (red and light blue neurons = innervate the NEB; green neuron = gives rise to the SMAR), and C-fiber afferents that originate from the vagal jugular ganglion (orange neuron = innervates the nonendocrine epithelium of large-diameter airways). The top right part represents dorsal root C-fiber afferents (dark blue neuron = innervates NEB). CALC, calcitonin; CRT, calretinin; TRPV1, transient receptor potential vanilloid 1; SP, substance P; DRG, dorsal root ganglia; VACHT, vesicular acetylcholine transporter; ð, diameter.
neurochemical and morphological characteristics of both SMARs and vagal nodose nerve terminals in pulmonary NEBs, however, obviously complicate the straightforward correlation between so-called “SAR activity” and the presently neurochemically identified SMARs, which has been suggested for many years based on inconclusive knowledge of sensory airway receptor morphology (56, 58, 83, 92, 93).

Historically, the largest number of fiber recordings of vagal airway afferents has been obtained from dogs, cats, and rabbits, but more recently also an increasing number of studies have used guinea pigs and rats (for recent review, see Ref. 41). Considerable species differences appear to exist regarding the number and distribution of the physiologically characterized subtypes of sensory airway receptors, and in particular of SARs (for review, see Ref. 58), but corresponding morphological data are not available. Data regarding species differences in the characteristics of pulmonary NEBs are substantial (2, 61, 63, 76). However, detailed location, origin, and neurochemical information for the various nerve fiber populations that contact pulmonary NEBs is at present available for rats only. Unfortunately, the substantial number of fiber recordings performed in rats (7, 31, 70) have resulted in conflicting interpretations, especially with regard to RARs. According to some authors, notable differences may exist between vagal pulmonary receptors in rats and those known in other species (7), while other reported data for rats in general do not appear to be all that different from other species (31, 83). Although direct experimental evidence is not yet available, our own observation that by far the largest number of myelinated vagal nodose afferents in rat intrapulmonary airways selectively innervates NEBs strongly suggests that discharges from NEB-related myelinated vagal afferent fibers may be part of the already characterized activity of vagal myelinated receptors in rat lower airways. Efforts for matching the available physiological and morphological data may, however, be hampered by potentially important species differences.

Support for a possible mechanosensory and/or nociceptive role of NEB receptor complexes in the airways has emerged from the very recent publication of evidence that mechanical stretch is an important physiological stimulus for the release of 5-HT via mechanosensitive (gadolinium chloride sensitive) channels in NEB cells that were isolated from rabbit lungs (49). Furthermore, a population of so-called “high-threshold A-fiber receptors” (HTARs; Ref. 90; see below) in rabbit airways, suggested to represent myelinated nociceptors that are potentially connected with NEBs, has also very recently been reported to be activated by 5-HT (91). In guinea pigs, on the other hand, an airway-related nodose C-fiber population has been shown to be 5-HT sensitive due to the expression of 5-HT3 receptors (19), while the other groups of vagal airway sensors did not express 5-HT3 receptors (34). Although species differences prevent further detailed interpretation for the moment, the apparent involvement of mechanosensitive channels, 5-HT, and 5-HT receptors may hold some clues for the further characterization of NEBs as airway sensors.

As suggested by Widdicombe and Nadel (84), SARs may play a role in the negative feedback mechanism that acts to limit increases in parasympathetic tone to the airway, and in that way optimize the reciprocal relationship between dead space and airway resistance. Evidently, airway receptors with terminals located between and parallel with the smooth muscle fibers, such as the SMARs described above, seem to be perfectly positioned to perform such a function. On the other hand, evidence that a receptor location in the muscle would be the optimal site for sensing an increasing transmural airway pressure, one of the most prominent activators of SAR activity (56), is not at all that convincing. An increasing intraluminal pressure would potentially stimulate mucosal sensors at least as effectively as receptors located in the muscle layer, which would essentially not “stretch” for most of the inflation period.

A similar discussion about the correlation between electrophysiologically and morphologically characterized mechanoceptors has been going on for many years in the gastrointestinal tract (52). It was generally believed for almost one-half a century that mechanosensors in the smooth muscle wall of the gastrointestinal tract consist of a single population of nerves with free endings between the muscle fibers. More recently, however, it has become increasingly clear that at least two types of vagal mechanosensors are involved: 1) a limited number presents as nerve endings located between the muscle cells, the “intradamalicular lamina endings,” and most likely represent “stretch” receptors; and 2) a much larger population presents as laminar nerve endings located in ganglia of the myenteric plexus, the “intramural lamina endings,” and are today regarded as “tension” receptors (52, 94). The latter clearly indicates that tension sensors in tubulike visceral organs are not necessarily located in the muscle layer.

Several groups have now published evidence for the involvement of more than just three types of sensors (SARs, RARs, and C-fiber receptors) in vagal afferent signaling from the airways. Nociceptor-like myelinated HTARs were reported as a separate group of myelinated sensors in rabbit airways because of obvious differences with SARs and RARs, such as low resting discharge frequency, extensive chemosensitivity, and relative insensitivity to mechanical stimulation (90). HTARs were even suggested to be good candidates for the populations of myelinated vagal afferents connected to NEBs (90, 91), but vagal pulmonary fibers with similar characteristics have so far not been reported for rat airways. In guinea pigs, airways are suggested to harbor at least three subtypes of vagal low-threshold myelinated mechanosensitive afferents (SARs, RARs, “cough receptors”) and three subtypes of vagal chemosensitive afferents (nodose C fibers, jugular C fibers, myelinated “A-fiber nociceptors”), which all are believed to be present in intrapulmonary airways (34, 42, 72), but unfortunately NEBs were not looked for or described. Moreover, a clear allergen-induced plasticity has been observed in the phenotype of some of the described subtypes of guinea pig myelinated airway afferents (43). The additional myelinated populations, i.e., “cough receptors” and A-fiber nociceptors, are apparently most abundant in extrapulmonary airways (42) and do not express P2X receptors. Clearly, the recognition of many more functional groups of airway afferents in several species complicates the efforts for matching structural/neurochemical and physiological information.

Very recently, ionotropic and metabotropic receptors that are potentially involved in transduction of airway-related sensory information in vagal afferents have been identified for the guinea pig (for review, see Ref. 34), the knowledge of which may eventually lead to information that allows the selective association of physiologically and neurochemically identified subtypes of airway receptors.
To achieve a better understanding of the sensory interactions between the periphery (lung and airways) and the central nervous system, it will be unavoidable to pay proper attention to the increasing amount of structural and neurochemical information regarding sensory airway receptors that has recently become available. Clearly, future combined morphological and electrophysiological studies in whole animal, lung slice, and whole mount preparations will be essential to further unravel the complex maze of electrophysiologically and neurochemically identified airway mechanoreceptors. Recently, Yu and coworkers (92) introduced an experimental setup for such a combined approach.

For many years, evidence has accumulated, suggesting that vagotomy abolishes cardiorespiratory reflexes from the lungs. Several recent publications, however, provide evidence for a sympathetic afferent component in the airway reflexes and/or cardiorespiratory responses to intrapulmonary chemical stimulation in different species (48, 66, 67, 79). The selective C-fiber afferent spinal CGRP/SP innervation of NEBs in rat lungs may be involved in similar pathways. Very recently, Plato and coworkers (54) published a combined neuronal tracing and (immuno)cytochemical study in rats, comparing the different populations of vagal and spinal pulmonary afferents, but without identification of the terminals in the lungs. The latter study strongly suggests that also the sympathetic sensory component of lung innervation harbors various, likely functionally distinct, subpopulations.

In addition to their potential receptor properties, NEBs may exert many other functions during prenatal, perinatal, early neonatal, and adult life (for review, see Ref. 64). Since the vagal nodose sensory component of the selective innervation of NEBs appears to be differentiated well before birth (3), it may be essential for neonatal respiratory adaptation. It needs no further explanation that the receptosecretory pulmonary NEBs are excellent candidates for registering properties of the airway environment. Unfortunately, the in vivo physiological stimuli for NEBs are still unknown. So far, there is no hard evidence that, e.g., hypoxia activates NEBs in the airways of live animals, and certainly not for the suggestion (for review, see Ref. 20) that this activation would cause stimulation of the connected vagal afferents. Considering the spinal origin of the CGRP/SP-ir C-fiber-like component of the NEB innervation, a possible central transduction of hypoxic stimuli may be mediated by spinal instead of vagal afferents in rat lungs. On the other hand, NEBs harbor evident possibilities to act as local regulators of airway function(s) that do not necessarily require signaling to the central nervous system. In that respect, the main sensor/effector action to hypoxia could be local. These aspects, however, have been extensively reviewed before (1) and will not be discussed further here.

In conclusion, the main aim of this contribution was to provide evidence for the idea that the different populations of myelinated vagal afferents that comprise part of the very complex intraepithelial innervation of pulmonary NEBs may represent subpopulations of the extensive group of known electrophysiologically characterized myelinated airway receptors. Although direct functional data are not available so far, the nerve terminals on their own seem to have everything for performing a mechanosensory function, but, if so, the question remains as to what may be the meaning and possible input of the neuroendocrine cell groups in the NEB complexes. The presented data on the neurochemical coding and specific targets of myelinated vagal afferents in rat airways may provide clues for future physiological experiments, taking into account that only a minority of these fibers appears to be connected to airway smooth muscle receptors, while a much higher number is connected to different populations of NEB-associated receptor endings. Obviously, it will be necessary to validate the data, summarized for rats in the present review, in other species. Although it has been established that the vagal mechanisms that participate in the control of human breathing are not essentially different from those described in experimental animals, little or nothing is known so far about the presence, origin, and neurochemical coding of the nerve fiber populations that connect to potential SMAR-like structures and NEBs in human airways.

Finally, conclusive functional evidence for the nature of the information carried by any of the multiple populations of afferents that selectively connect to pulmonary NEBs is still lacking today.

ACKNOWLEDGMENTS

We especially thank D. De Rijk, J. Van Daele, D. Vindevogel, and H. De Pauw for expert assistance. We are indebted to G. Burnstock (Autonomic Neuroscience Institute, Royal Free and University College Medical School, London, UK) for invaluable input in the ATP receptor studies.

REFERENCES

11. Brouns I, Pintelon I, De Proost I, Alewaters R, Timmermans JP, and Adriaensen D. Neurochemical characterisation of sensory receptors in...


Invited Review

NEUROEPITHELIAL BODIES: POTENTIAL VAGAL AIRWAY SENSORS

Shimosegawa T and Said SI.


