Structure of slowly adapting pulmonary stretch receptors in the lung periphery

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Yu, J., Y. F. Wang, and J. W. Zhang. Structure of slowly adapting pulmonary stretch receptors in the lung periphery. J Appl Physiol 95: 385–393, 2003. First published March 28, 2003; 10.1152/japplphysiol.00137.2003.—Pulmonary sensory receptors are the initiating sites for lung reflexes; however, little is known about their structure, especially the relationship between the structure and function of these receptors. Using a novel approach (combining electrophysiological and morphological techniques), we examined the structures of the typical slowly adapting pulmonary stretch receptors (SARs) located in the lung periphery. We recorded SAR activities in the cervical vagus nerve, identified the receptive field, dissected the SARs in blocks, fixed and processed these blocks for immunohistochemical staining using anti-Na/K-ATPase, and examined the blocks under a confocal microscope. These SAR structures have multiple endings that have terminal knobs. Some structures that are located in the airway walls have terminal knobs buried in smooth muscle. Others are in the most peripheral part of the lung, and their terminal knobs have no obvious relation to smooth muscle, suggesting that muscle contraction may not be a direct factor for SAR activation.

METHODS

Experiments were conducted in 20 New Zealand White rabbits (1.5–2.2 kg). The protocol used conforms to ethics requirements and has been reviewed and approved by the International Animal Care and Use Committee of the University of Louisville. Rabbits were anesthetized with a mixture of 10% urethane and 1% α-chloralose (5 ml/kg iv). The trachea was cannulated low in the neck. The chest was opened in the midline, bleeding spots were cauterized, and a volume-cycled mechanical ventilator (model 683, Harvard Apparatus, South Natick, MA) provided a tidal volume set at 10 ml/kg of body weight and 3 cmH2O of positive end-expiratory pressure. Airway pressure was measured from the tracheal tube by a pressure transducer (model P23, Statham). The midline of the neck was sectioned for isolation of the cervical vagus nerve for electrical recording.

Electrical recording. The afferent activity of typical SARs was recorded and identified according to conventional methods (34). Briefly, the vagus nerve was separated from the carotid sheath, placed on a dissecting platform, and covered with mineral oil. A small afferent bundle was cut from the vagus nerve, dissected into thin filaments, and placed on the recording electrodes. The electrodes were connected to a high-impedance probe (model 511) from which the output is led to a Grass (model P511) amplifier. After suitable amplification, action potentials from single-unit activity of the vagus nerve were displayed on an oscilloscope and monitored by a loudspeaker (model AM8 audio monitor, Grass). In addition, a voltage analog of impulse frequency was produced.
by a ratemeter at a bin width of 0.1 s. Action potential signals together with airway pressure were thermorecorded (Dash IV, Astro-Med). SARs were identified by their characteristic firing patterns and their adaptation indexes.

Receptor sampling. One or two receptor blocks believed to contain a SAR were obtained from each rabbit. After action potentials from a single pulmonary afferent were recorded, the receptive field was first grossly identified by directly probing the lung with a cotton tip applicator. More accurate location was followed by probing the lung with a very fine, round-tipped rod to find the most sensitive point from which bursts of impulses were elicited. Tissue containing the receptor was carefully dissected under microscope while the animal was alive, by sectioning a small piece of lung tissue (a tissue block ~1 mm³ in size) at a time while SAR activity was recorded by the thermorecorder and monitored by the loud-speaker. Piecemeal dissection continued until a block containing the receptor was obtained. Successful sectioning was indicated by very high discharge frequency when the receptor was grabbed for dissection and by the disappearance of afferent activity in the loudspeaker and on the recorder (Fig. 1 and see Fig. 5). The section of the receptor was further confirmed by the failure to elicit activity from the recorded vagal afferent while touching the receptive field or by hyperinflation of the lung. Blocks that were in doubt of containing a recorded SAR were discarded without further investigation. Otherwise, receptor blocks were further processed for immunohistochemical staining. We also obtained 12 control blocks. Control blocks were dissected in the same way as the receptor blocks except that they were usually obtained at sites adjacent to the experimental blocks. An additional 12 control blocks were randomly dissected from the peripheral lung.

Morphological examination. For identifying the structure of the typical SARs, 24 receptor blocks and 24 control blocks were immunohistochemically stained by using a newly developed method (27). Immunohistochemical staining by using the antibody against Na⁺-K⁺-ATPase as a marker for identifying the SARs was carried out by an indirect immunofluorescence method. First, tissue blocks were fixed overnight in a 0.1 M phosphate-buffered (PB) solution containing 4% paraformaldehyde (at a pH of 7.4). They were then thoroughly washed with a solution containing 0.4% Triton X-100, incubated in a serum containing blocking solution for 2 h, and then incubated overnight at 4°C with a monoclonal antibody (anti-Na⁺-K⁺-ATPase, α₃-subunit; Biomol Research Laboratories, Plymouth Meeting, PA) diluted 1:100 to 1:200. The antiserum was washed from the preparations by using PB solution, and the blocks were incubated overnight with Cy3-labeled donkey anti-mouse immunoglobulin G (Jackson Immuno Research Laboratories, Westgrove, PA) diluted at 1:100. After being washed with PB solution, blocks were mounted on a glass slide with 80% glycerol in 0.1 M PB solution. The blocks were viewed by laser scanning confocal microscopy (model 510, Zeiss) equipped with a HeNe1 laser (543 nm) and filtered for the detection of Cy3. Images in optimal focus were recorded and saved. These images of optical slices were reconstructed as a projection to give a total view of the structure.

METHODS

Forty-eight tissue blocks were processed for immunohistochemical staining using anti-Na⁺-K⁺-ATPase and examined under a confocal microscope following the same protocols. Twenty-four of the 48 blocks were controls. The other 24 were receptor blocks that presumably contained SARs located in the lung periphery. These SARs were first identified by electrical recording of single-unit activity (Fig. 1A). Then, tissue containing the receptive field was dissected in blocks. The receptor blocks are the ones in which a section can silence the afferent discharge (Fig. 1B), and no activity occurred in response to lung inflation or touching the surrounding tissue of the receptor field afterward. These receptor blocks were then processed and examined under a confocal microscope for receptor structures (Fig. 2). The structure in Fig. 2 is observed from the block containing the receptor recorded in Fig. 1. All 24 receptors are typical SARs (32), having an adaptation index of <15%. Some presented tonic activity (low threshold), and the others displayed phasic activity (high threshold) (33). Among them, 5 were low threshold (21%), which discharged continuously, peaking at the peak of airway pressure, and 19 were high threshold (79%) with phasic discharge only during inspiration.

All 24 control blocks were negative. In other words, no receptor structures were found in those blocks under the microscope. On the other hand, 15 of the 24 receptor blocks (62.5%) were positive (showing structures). Among them, 3 were low threshold and 12 were high threshold. These SARs were found in bronchioles of different diameters. One was located in a 1.2-mm airway, which had cardiac rhythm. One was in 0.5-mm bronchioles, and the other was in 0.6-mm bronchioles. Three SARs were identified in airways 0.12–0.18 mm in diameter. The other nine SARs were in the most peripheral regions, surrounded by alveoli. Axon diameters were in the range of small myelinated fibers,

**RESULTS**

**Fig. 1.** Activities of a typical pulmonary slowly adapting receptor (SAR) located in periphery of the right lower lobe. The activity was recorded from the contralateral (left) vagus nerve of an anesthetized, open-chest, mechanically ventilated rabbit. Traces are receptor activity expressed as impulses per second (IMPs), receptor activity (IMP), and airway pressure (Paw). The SAR activity decreased during positive end-expiratory pressure removal (indicated by 2 arrows in A) and had a very slow adaptation rate during constant pressure inflation (A). The receptor discharged rapidly during dissection and ceased firing after the tissue block containing the SAR was removed (arrow in B).
ranging from 1.4 to 4.9 μm. The main axon diameter of nine SARs averaged 2.83 ± 0.35 μm. Because of their orientation, we could not measure the main axon diameter in another six receptors. We took the largest value from the axon branches in the image, which was 2.58 ± 0.28 μm, ranging from 1.7 to 3.6 μm. The number of knobs was 11.3 ± 2.9 in low-threshold SARs (n = 3) and 10 ± 0.8 in high-threshold SARs (n = 12).

Essentially similar receptor structures were observed. They had multiple endings with terminal knobs (Fig. 2). The axon innervating the receptor structure had many twigs and gave off many endings. Each ending swelled to form a knoblike device, which was 10–30 μm in size. In four blocks, receptor structures could be clearly identified in the airway walls, and, in the others, the structures were more peripheral down to alveolar regions where no clear airway walls can be identified. Figures 3 and 4 illustrate the details of two typical high-threshold SARs, one located in a 180-μm airway (Fig. 3) and the other in a terminal bronchiole with a diameter of ~120 μm (Fig. 4). In Fig. 3, the axon fiber runs outside the bronchiole wall and has many twigs. The axon gradually penetrates the wall, and then it branches and forms knobs (at least 6 knobs in this structure). The knoblike terminal endings are buried in the wall, which includes a thin layer of smooth muscle (Fig. 3C). It is noteworthy that a branch in the lower left corner of Fig. 3E comes off the main axon and extends into a branching point of the airway.

In 2 of the 15 positive blocks, the results suggest that a parent axon may supply >1 receptor structure. Figures 5 and 6 illustrate this point. Figure 5 shows receptor activity from a high-threshold SAR. This SAR is located in a small bronchiole, with a diameter of ~500 μm. The structures of this SAR appear in Fig. 6. In Fig. 6A, three branches coming off the parent axon...
Some receptors appear in bronchiolar walls and in close relationship with airway smooth muscle (Figs. 7–9). Figure 8 illustrates the receptor structure identified in Fig. 7A, which is located in an airway 600 μm in diameter. The receptor endings extend into the muscular wall (Fig. 8E). Note that a thick trunk of the vagus nerve courses through the view outside the airway wall (8A). This receptor structure is innervated by an axon branch coming from the upper left corner (8B). The receptor endings (knobs) are embedded in the smooth muscle layer of the airway (Fig. 8C), extending to the inner layer close to the airway lumen (8D). This receptor shows the activity of a low-threshold SAR (Fig. 7A). Another receptor is also located in the bronchiolar wall (Figs. 7B and 9); however, it discharged with cardiac rhythm during the expiratory phase of the ventilator (Fig. 7B). During dissection, we noticed that this SAR appeared in the airway close to the hilum, with nearby blood vessels. Under microscope, this receptor was found in muscle walls of a bronchiole 1.2 mm in diameter. Morphologically, this receptor structure seems different from the other SARs illustrated. The whole structure was platelike in shape. Its terminal endings were leaflike (Fig. 9) instead of knoblike.

Figure 10 depicts four examples of receptor structures identified in the lung parenchyma viewed from different angles. In A, the structure was located in a deep portion of the left lower lobe. In B, the receptor structure was in the right lower lobe and close to the surface; its receptor activity was recorded from the contralateral (left) vagus nerve. The structure in C was located in a deep portion of the lung close to the hilum but still in the alveolar region. The structure in D was located immediately below the surface of left lower lobe.

DISCUSSION

This study introduces a novel approach used to investigate the relationship between the structure and...
function of a given airway receptor. In addition, we describe the detailed SAR located in the lung periphery. The use of the confocal microscope in combination with immunohistochemical labeling greatly enhances receptor image quality. To this end, we isolated and dissected receptors using our laboratory’s recently developed technique to locate SARs in the lung periphery (36). Also, we developed a method for immunohistochemical staining of airway mechanoreceptors with anti-Na\(^+\)-K\(^+\)-ATPase antibody (27).

What is known about the structures of SARs is far from comprehensive. Significant differences exist in how this “best known” type of pulmonary receptors (10, 13, 30) is described by various reviewers (6, 28, 29).

Fig. 6. Structures of the SAR (in an airway of 500 \(\mu m\)) identified in Fig. 5. A: composite of 2 projection images with a low magnification. The parent axon gives off 3 branches (indicated by arrows). Two of the branches (indicated by letters b and c) clearly innervate a receptor structure, enlarged in the projection images of B and C. The third branch ends in an area with numerous dots, forming no clear structure. B was located at the branching point of the bronchiole. Note that there were many knoblike endings in both structures in B and C. Bar in A = 50 \(\mu m\). Bars in B and C = 20 \(\mu m\).

Fig. 7. Electrical activities of 2 different SARs recorded from different rabbits. The structures of the receptors in A and B are illustrated in Figs. 8 and 9, respectively. A is typical of the low-threshold SAR. B is a typical SAR with cardiac rhythm during expiratory phase of the ventilator.
Critical analysis of the published data about the morphology of SARs is difficult, because in most early studies and even at present data are often presented as camera lucida tracings instead of photomicrographs. Under the ordinary light microscope, it is difficult to judge structures in relation to their surroundings, especially if there are few specimens in one plane of orientation. Because of advances in immunohistochem-

![Fig. 8. Images of the receptor structure identified in Fig. 7A. The structure is in an airway 600 μm in diameter in the lower lobe of the left lung. A–D: representative sections from outside (A) to inside (D) of the bronchiole wall. E: projection of the image stack (9 optical sections with 4.4 μm for each section). Smooth muscle bands can be clearly seen in C, where most knobs are located. Structurally, this low-threshold SAR is similar to the high-threshold SAR in the small airways and in the lung parenchyma. Arrow in A indicates the nerve fiber giving off the axon (identified by the arrow in B) that innervates the receptor structure. Bars = 50 μm.](image1)

![Fig. 9. Images of the receptor structure identified in Fig. 7B. The receptor is in an airway 1.2 mm in diameter, located close to the hilum of the left lower lobe. A–D: representative images. The whole structure covers a depth of 27 μm. E: projection of the images. The receptor structure has a close relationship with smooth muscle. This structure is platelike in shape, and the terminals end with 2 elongated leaflike extensions (indicated by the arrows). The morphology of this receptor, which has a discharge with cardiac rhythm, seems different from the others. Bars = 20 μm.](image2)
istry and in the technology of microscopy (the confocal microscope), observation of the structures has become easier and data interpretation less subjective. Our study took advantage of these advances.

Immunohistochemical techniques permit the study of airway innervation (9). Some receptor structures in the trachea and large bronchi are immunoreactive to neural filament protein, calretinin, and protein gene product (30, 31). Antibodies that work against these neural markers were either unavailable or did not work in rabbits. Therefore, we had to identify a new marker for SARs in the rabbit. Na\(^{+}\)/K\(^{+}\)-ATPase is an integral membrane enzyme that is widely distributed in cells. This transmembrane enzyme regulates intra-cellular Na\(^{+}\) and K\(^{+}\) concentrations [reviewed by Blanco and Mercer (4) and Therien and Blostein (26)], and should be very active in cells that require the redistribution of ions. SARs discharge at a high frequency during inspiration. On average, SAR activity during an entire respiratory cycle is \(\sim 40\) impulses/s in rabbits (34).

It should not be surprising that the SARs contain abundant Na\(^{+}\)/K\(^{+}\)-ATPase. ATPase is active in SARs because intravenous injection of ouabain (a Na\(^{+}\)/K\(^{+}\)-ATPase antagonist) stimulates SARs (18). In addition, the topical application of ouabain on the receptive field stimulates SARs (37), suggesting that this enzyme is very active in the sensory receptor. Thus it is reasonable to use Na\(^{+}\)/K\(^{+}\)-ATPase as a marker for identifying SARs. In fact, Na\(^{+}\)/K\(^{+}\)-ATPase (\(\alpha_3\)-subunit) proves an excellent marker for airway sensory receptors (27), especially for the myelinated mechanoreceptors. In the present study as well as in a previous report (27), we did not observe sensory fibers in the form believed to be airway C fibers (33). In addition, we did not encounter axons in the C-fiber range, that is, \(< 1 \mu m\). Immunoreactivity to the \(\alpha_3\)-subunit of Na\(^{+}\)/K\(^{+}\)-ATPase is sensory nerve terminal specific (25) but not the nerve terminals of motoneurons.

The terminology used for the SAR in our study needs emphasis before morphology is discussed. We define SARs according to electrophysiological classification (on the basis of the single-fiber recording) and not according to morphology. In this sense, we call the structures in Fig. 6, B or C, receptor structures instead of receptors. SAR activity recorded from a parent axon (Fig. 5) may supply many such receptor structures (Fig. 6). We define an ending as the terminal of a branch, these were knoblike in form in most of the figures and a leaflike extension in Fig. 9.

The structures of presumptive SARs reported so far are limited to the large airways. In general, we observed receptor structures in peripheral airways similar to those found in central airways in early studies [described as smooth muscle spindles (15, 16)] as well as in a recent report (27). However, structures are...
simpler in the lung periphery than in the central airways (27). The parent axon may give off many branches to innervate more than one receptor structure, and the axon to a receptor structure further divides into many endings, forming knoblike or leaflike extensions. In our study, some receptors have a close relationship with smooth muscle. Their endings are buried in the muscle layer (Figs. 3, 8, and 9), whereas others are in the alveolar regions and do not show a relationship with smooth muscle (Figs. 2, 4, and 10). Interestingly, smooth muscle-related and -unrelated SARs have the same electrophysiological properties and morphological structure. According to the electrophysiological classification, they belong to the same class: typical SARs (32).

The importance of SARs in relation to smooth muscle comes from the observation that airflow constriction can activate them (8). In general, airflow constriction alters SAR activity, which often increases. However, SARs behave differently in response to bronchoconstrictors and in a manner that is location dependent (8). This phenomenon is not well explained by direct smooth muscle contraction. The heterogeneous responses together with the present results seem to indicate that although smooth muscle constriction may activate the SAR, it may not be a direct factor. In addition, a receptor having a close relationship with airway smooth muscle is not necessarily a SAR. For example, RARs also activate by contraction of airway smooth muscles (19). Therefore, the relationship with airway smooth muscle, which morphologists used to claim as SAR, is not a gold standard.

A few points arise from these results. First, the separation of high- and low-threshold SARs as peripheral vs. central in location is inaccurate, because both can be found in peripheral airways, although more high-threshold SARs are found there. Second, on the basis of the limited number of SARs examined, there is no clear distinction between the morphology of high- and low-threshold SARs. It will be interesting to discover whether the threshold relates to the number of terminal knobs in a given SAR. For example, a low-threshold SAR may have more knobs than a high-threshold SAR. Although the present results did not indicate so, our method to count the number of knobs has limitations (see below), and the number of observations was small. This issue requires further study. Alternatively, the threshold may be established by the membrane properties of the receptor. Third, the structure of the SAR with the cardiac rhythm shown in Fig. 9 seems different from the others. These may represent different types of receptors. However, it is premature to make any conclusion about this difference by observing only one receptor.

The density of SARs in peripheral airways is low. In rough estimation, cats possess ~10,000 vagal afferent fibers distributed in the lungs and lower airways (1). Among these, one-fifth (2,000 fibers) are myelinated. We assume that 60% of the myelinated fibers are SARs (1,200) and 40% are RARs (12). In general, 50% (22) to 65% (21) of SARs in rabbits are in intrapulmonary airways, giving some 800 intrapulmonary SARs. The lung volume of a 2-kg rabbit is ~40 ml after excision and passive deflation by exposure to air (35). The 40-ml volume occupies a space of 40,000 mm3, giving 50 mm3 per SAR, assuming even receptor distribution. Our tissue blocks are ~1 mm3 in size, indicating that the chance to obtain a receptor by random section is 1 in 50. Even if we count all the myelinated afferents (SARs and RARs) together, the probability will be ~1 in 30. This yields a three-dimensional receptor density estimation; however, examination of receptor density under microscope and mounted on a slice would be different. It would be two dimensional. If we consider all cells are tightly packed together, the calculation corrected for tissue volume (~7% of the total lung capacity, excluding the blood volume; or 12.5% of the deflated lung volume) will give 5 ml. The probability instead of 1 of 30 would be one-fourth. This is the highest possible estimated density. It is known that SARs are not evenly distributed in the lung tissue, having a much lower density in small airways (3). Therefore, the actual probability will be much smaller than our estimations. Although our numerical estimation is rough, it assists interpretation. It is clear that the density of SARs in the peripheral airway is extremely low. Thus there is a slim chance to observe a SAR in the peripheral airway through random sectioning. This estimation explains the lack of information on receptor structures in the lung periphery and our negative results in the control blocks. The low density of SARs in the peripheral airway lends credibility to our positive results of SAR structures observed in the receptor blocks.

Here we described a new approach to investigate airway receptors. However, as with any technique, there are limitations. In our study, we identify SAR structures in ~60% of the receptor blocks. It is unknown why the other 40% of blocks were negative. Some negative blocks might be caused by loss of receptor structures during the preparation process (as we suspected in Fig. 6, an axon fiber ended in numerous dots without a structure). Others may be false-positives. That is, the receptors were not actually dissected in the blocks. The disappearance of recorded activity was caused by cutting the axon innervating the receptors. Although our method provides a clear total structure of a receptor, lacking is a detailed and exact subcellular relation to its environment, which needs resolution by electron microscopy. In addition, in our present study, the optical slices were thick (usually at 3.3–6.5 μm), which prevents accurate count of number of twigs and the volume of the terminals. The number of knobs given in the present study can provide only a general sense. To make a quantitative analysis, especially for the number of twigs and the volume of the terminals, the optical slices should be kept at <1 μm. Such quantitative characterization requires further investigation.

In conclusion, we have developed a novel approach that combines electrophysiological recording with morphological examination to study pulmonary receptors.
This provides detailed structures of a pulmonary receptor and allows exploration of the relationship between the structure and function of a given pulmonary receptor. The first pictures of electrophysiologically characterized SARs located in the lung periphery are shown.

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