Electric field stimulation of precision-cut lung slices

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Schlepütz M, Uhlig S, Martin C. Electric field stimulation of precision-cut lung slices. J Appl Physiol 110: 545–554, 2011. First published November 25, 2010; doi:10.1152/japplphysiol.00409.2010.—The precision-cut lung slice (PCLS) technique is widely used to examine airway responses in different species. We developed a method to study nerve-dependent bronchoconstriction by the application of electric field stimulation (EFS) to PCLS. PCLS prepared from Wistar rats were placed between two platinum electrodes to apply serial rectangular impulses (5–100 Hz), and bronchoconstriction was studied by videomicroscopy. The extent of airway contractions increased with higher frequencies. Stable repeated airway contractions were obtained at a frequency of 50 Hz, a width of 1 ms, and an output of 200 mA for 2.5 s each minute. Larger airways showed stronger responses. The EFS-triggered contractions were increased by the acetylcholine esterase inhibitor neostigmine (10 μM) and reversed by the muscarinic antagonist atropine (10 μM), whereas the thromboxane protandim receptor antagonist SQ29548 (10 μM) had no effect. Magnesium ions (10 mM) antagonized airway contractions induced by EFS, but not by methacholine, indicating that nerve endings remain intact in PCLS. Our data further show that the electrically evoked airway contractions in PCLS are mediated by cholinergic nerves, independent of thromboxane and more prominent in larger airways. Taken together these findings show that nerve endings remain intact in PCLS, and they suggest that the present method is useful to study neurogenic responses in airways of different size.

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also of clinical relevance. In the present study, EFS was applied to PCLS for the first time. PCLS from rats responded to EFS indicating that nerve endings remained intact in this preparation. The electrically triggered airway contractions were of neural origin and mainly cholinergic. This novel method allowed us to study the functional consequences of nerve activation in airways of different size and revealed a significant correlation between airway size and neurally induced airway contraction. Moreover, we showed that thromboxane prostanoid (TP) receptors exert no effect on the muscarinic airway contractions induced by EFS or methacholine.

**MATERIALS AND METHODS**

*Animals.* Lungs were prepared from female Wistar rats weighing 200–300 g. Rats were obtained either from Harlan Winkelmann (Borchen, Germany) or Janvier (Le Genest, St. Isle, France) and were kept under controlled conditions in the animal house. Animal experiments were approved by the local ethic committee (Reference number 8.87–51.05.20.09.245).

*Chemicals.* Acetylcholine, atropine, methacholine, neostigmine, magnesium sulfate (MgSO₄), and standard laboratory chemicals were purchased from Sigma (Steinheim, Germany). SQ29548 and U46619 were provided by Biomol (Hamburg, Germany). Substances for cell culture were obtained from PAA laboratories (Colbe, Germany).

*Preparation of PCLS.* PCLS were prepared as described (43) and used 1 day after preparation. Briefly, the lungs were filled with low-melting agarose after tracheotomy and cooled on ice. The hardened lung was removed en bloc from the animal and lobes were separated. Tissue cores with a penetrating airway in its center were prepared from the left and right inferior lobe by a rotating sharpened metal tube (diameter 10 mm). Tissue slices were cut from the cores perpendicular to the airway by the aid of a Krumdieck tissue slicer (Alabama Research and Development, Munford, AL.). Only slices free of agarose, with beating cilia and an intact smooth muscle layer in a relaxed state, were used (Fig. 1C).

Airway area was monitored by videomicroscopy (SensiCam 365KL, Visitron Systems, Munich, Germany) at a frame rate of 0.36 s⁻¹ in experiments with EFS and 0.2 s⁻¹ in all other experiments. Camera control and image analysis were achieved by Optimas 6.5 software (Optimas, Bothell, WA). Airway area was defined as 100% area size before the first stimulation (Fig. 1D).

*Electrical field stimulation.* EFS of PCLS was carried out in cavities of standard 12-well plates in a reaction volume of 1 ml standard minimal essential medium [2 mM CaCl₂, 1 mM MgSO₄, 5 mM KCl, 116 mM NaCl, 1 mM NaH₂PO₄, 17 mM glucose, 26 mM NaHCO₃, 25 mM HEPES, 1 mM sodium pyruvate, 2 mM glutamine, amino acids (PAA Laboratories, M11-002, 1:50) and vitamins (PAA Laboratories, N11-002, 1:100)]. The PCLS (10 mm in diameter) were provided by Biomol (Hamburg, Germany). Our standard provocation protocol comprises a stimulation train lasting 3.3 min.

In preliminary experiments the following setting were identified as useful: train rhythm (TR) = 60 s, train width (TW) = 2.5 s, frequency (F) = 50 Hz, pulse duration (B) = 1 ms, current (A) = 200 mA (Fig. 1B). To systematically examine the best settings for frequency, pulse duration, current, and train width, PCLS were stimulated by modulation of one parameter, while the others were kept constant at the values noted above. Frequency, pulse duration, current, and train width were examined at 5–100 Hz, 0.1–5 ms, 1–200 mA, and 0.5–20 s, respectively. Recovery between the repetitive stimulations lasted until the airway had relaxed to its initial state (3–5 min).

*Cholinergic contribution to airway contraction in EFS.* Each PCLS was exposed to three stimulation trains. The first stimulation train was carried out in the absence of any additive, the effect of acetylcholine esterase inhibition (10 μM neostigmine) was examined in the second train, and the role of acetylcholine receptors (10 μM atropine) was studied in the third train. All drugs were added 15 min before stimulation.

*Neural contribution to airway contraction in EFS.* PCLS were repeatedly stimulated by EFS. The first stimulation train occurred at 10 μM neostigmine. Half an hour later the second stimulation occurred either in the presence or absence of 10 mM MgSO₄ to block neuronal calcium entry. MgSO₄ was present 30 min before stimulation. After the last stimulation, 10⁻⁶ M acetylcholine was added to examine airway contractility in the presence of 10 mM MgSO₄, and responses were monitored at a frame rate of 0.2 s⁻¹ for 5 min.

*Magnesium in exogenously evoked ASM contraction.* A dose-response curve was conducted with methacholine (10⁻⁸–10⁻⁴ M) in the presence or absence of 10 mM MgSO₄. PCLS were preincubated with MgSO₄ 30 min before addition of the first methacholine concentration. Methacholine was cumulatively added for 5 min each.

*Examination of large and small airways in EFS.* PCLS containing differently sized airways ranging from 3.8 × 10³ to 1.2 × 10⁶ μm² (0.07–1.2 mm in diameter) were stimulated by EFS. The first stimulation train was conducted without additive, and the second train was carried out in the presence of 10 μM neostigmine given 15 min in advance.

*Effect of TP receptor antagonist SQ29548 in EFS.* PCLS were repeatedly stimulated by EFS. The first stimulation train occurred at 10 μM neostigmine. Half an hour later the second stimulation occurred either in the absence or presence of 10 μM SQ29548 to block TP receptors. SQ29548 was applied 15 min before stimulation. After the second stimulation, 10⁻⁵ M U46619 was added to prove receptor blocking by SQ29548 and to verify a priming of acetylcholine release by U46619 in a following third EFS train. Airway responses during U46619 application were monitored at a frame rate of 0.1 s⁻¹ for 15 min.

*Atropine in U46619 evoked ASM contraction.* A dose-response curve was conducted with U46619 (10⁻⁸–10⁻⁴ M) in the presence or absence of 10 μM atropine. Preincubation of atropine occurred for 15 min before addition of the first U46619 concentration. U46619 was cumulatively added every 5 min.

*Data analysis.* Data are expressed as means ± SD. Nonlinear regression, Spearman correlation, unpaired t-test, t-test with Welch’s correction, Mann Whitney test, or Tukey’s multiple comparison tests were performed with GraphPad Prism 5 (GraphPad Software, La Jolla, CA).

**RESULTS**

PCLS were placed between two electrodes, and reduction in airway lumen area became apparent after electric stimulation (Fig. 2A). The bronchoconstriction was reversible as long as the Teflon ring was used (Fig. 2A). Contractions increased in a frequency-dependent manner up to 50 Hz, (Fig. 2B) with a half-maximal response (EF₅₀) at 16.7 ± 4.9 Hz. Bronchoconstriction became stronger with pulse duration, current, and train width. A plateau response for pulse duration was reached at pulses ≥ 1 ms (Fig. 2C). At 200 mA, the maximal current output of the stimulator, airways contracted about 35% (Fig. 2D). Maximal ASM contraction occurred if train width lasted 10 s or longer (Fig. 2E). However, the recovery phase to reach the initial airway area lasted longer at TW = 10 s, and contractions persisted in presence of 10 mM magnesium (data not shown), suggesting unspecific nonneural stimulation at
this train width. We therefore decided to carry out the following experiments with the Teflon ring in place, at $F = 50$ Hz, $B = 1$ ms pulse duration, $A = 200$ mA, and $TW = 2.5$ s. These conditions resulted in reproducible airway contractions (Fig. 3).

To examine the role of cholinergic nerves in the EFS-induced bronchoconstriction, PCLS were stimulated in the presence of neostigmine to enhance acetylcholine concentrations in the synaptic cleft and atropine to block postsynaptic acetylcholine receptors. In the presence of neostigmine (10 $\mu$M), EFS contracted airways to $49.2 \pm 11.0\%$ of the initial airway area compared with $84.1 \pm 5.3\%$ without neostigmine (Fig. 3). With neostigmine still present, atropine nearly completely abolished the EFS-induced airway contractions (Fig. 3; see supplementary video 1 online). Similar observations were made for pulse durations ranging from 0.5 to 2 ms (data not shown). These findings indicate that airway contractions in rat PCLS mainly depend on muscarinic receptors.

To verify that the EFS-evoked airway contractions were due to neural stimulation, we examined the effects of magnesium
concentrations high enough to block voltage-gated calcium entry in neuronal cells (12, 16). MgSO₄ (10 mM) totally abolished the EFS-induced contractions in the presence of 10 μM neostigmine (Fig. 4, A–C; see supplementary video 2 online). In contrast, airway contractions by exogenously added acetylcholine were not affected by MgSO₄ (Fig. 4, D), and 10 mM MgSO₄ did not alter the concentration-response curve of methacholine-induced bronchoconstriction (Fig. 4 D).

Since small airways respond stronger to methacholine than larger ones (25), we analyzed the response of small and large airways to EFS (Fig. 5). EFS were studied in PCLS with airway sizes ranging from 3.8 × 10³ to 1.2 × 10⁶ μm² in the presence or absence of neostigmine. Our findings show that larger airways were more responsive than smaller ones (Fig. 5). Additionally, the amplification of airway contraction by neostigmine was more pronounced in larger airways.

An interaction between TP receptor signaling and parasympathetic cholinergic acetylcholine release has been shown for the dog, guinea pig, and mouse (2, 3, 37, 39), whereas this mechanism has not yet been characterized in rat lungs. We therefore examined the influence of the TP receptor antagonist SQ29548 in EFS of rat PCLS. No difference was found for electrically evoked cholinergic airway responses in the presence and absence of a TP receptor antagonist (Fig. 6). A statistical power calculation based on these results (Fig. 6D) indicated that at least 32 experiments were needed to possibly reach significance, indicating that any role of TP receptors would be rather small. The addition of U46619 demonstrated that SQ29548 was capable of blocking TP receptors in our system (Fig. 6D). Moreover, a subsequent EFS train in the presence of U46619 did not increase the response (Fig. 7). Vice versa, the influence of TP receptor agonist U46619 and its
possible interactions with muscarinic receptors was studied by the use of atropine. The airway responses for cumulative concentration-response curves with U46619 were the same in the presence and absence of atropine (Fig. 8).

**DISCUSSION**

Viable PCLS have been established as a useful tool to study airway tone, pulmonary vascular responses, ciliary beating, as well as immunological and toxicological properties of the lungs (11, 18, 25, 26, 42, 43). Here we show that neural stimulation of PCLS is feasible and that the EFS-induced bronchoconstriction is consistent, reproducible, sensitive to atropine, and enhanced by an acetylcholine esterase inhibitor in rats. The effect of the EFS was strongest in the larger airways and appeared to be mediated by synaptic release of acetylcholine. This model will be useful to further study mechanisms of neural airway control and cholinergic airway hyperreactivity in rats and other species.

**General EFS setup in PCLS.** The extent of the EFS-induced contractions of 15% in large airways roughly corresponds to concentrations elicited by 330 nM of the metabolically stable acetylcholine derivative methacholine [Martin et al. (25), Fig. 8B], suggesting that this may be the effective acetylcholine-concentration in the synaptic cleft following presynaptic discharge. These findings propose that neurally induced bronchoconstriction can regulate airway tone but is unlikely to lead to complete airway closure as shown, for instance, for allergen provocation (43). When comparing the present findings with other studies, it should be noted that we did not normalize our data to contractions induced by KCl, a procedure that may seemingly increase the magnitude of the effects. Normalization to receptor agonists (e.g., methacholine) was omitted throughout the study as we have previously shown stronger responsiveness of small airways to methacholine and other stimuli (25, 26, 42, 43). In such a situation, normalization would distort the results. The EFS-induced contraction by ~15% was strongly potentiated by inhibition of the acetylcholine esterase (Fig. 3). These findings indicate that in PCLS cholinergic synapses remain intact, as they respond to electrical currents, possess active acetylcholine esterase (see experiments with neostigmine) and acetylcholine-receptors (see...
experiments with atropine), and recycle acetylcholine in a normal fashion (otherwise repetitive stimulations were not possible).

EFS was applied to stimulate neuronal cells, but depending on the frequency and duration of the electric stimulus, direct contraction of smooth muscle cells may also occur (13). To exclude such an unspecific direct stimulation of voltage-gated channels on the airway smooth muscle, we examined the effect of magnesium. At high concentrations, magnesium prevents voltage-induced calcium entry and thereby the release of neurotransmitters from nerve terminals (12, 16). Since magnesium completely prevented the bronchoconstriction induced by EFS but not by methacholine, we conclude that in our setup the EFS acts only on nerve endings and not on airway smooth muscle (Fig. 4).

Role of frequency, pulse duration, current density, and train width in EFS. For organ bath experiments, Wang and colleagues (40) systematically studied the influence of pulse duration (0.5–3 ms), muscle preload (2–20 g), voltage (5–20 V), and frequency (0.5–16 Hz) on the release of acetylcholine from equine airway cholinergic nerves, to provide guidelines for selecting EFS parameters in future studies. Because they observed an increasing acetylcholine release from 2.1 to 18.6 pmol·g tissue-wet wt⁻¹·min⁻¹ with increasing frequencies (2–16 Hz), we analyzed this frequency range while the stimulation period was kept constant. This resulted in a sigmoidal frequency-response curve for the airway area change (Fig. 2B) plateauing at F = 50 Hz, somewhat above the frequency observed by Wang and colleagues (40) (9.8 Hz). The most likely explanation for these differences is interspecies variability, as PCLS from other species (e.g., sheep) responded already at lower frequencies (data not shown), and our EF₅₀ of 16.7 ± 4.9 Hz is almost identical to the 19.3 ± 4.3 Hz reported by Gonzalez and Santana (17) on isometric tension studies of rat tracheal smooth muscle. In addition they also found a plateau phase above 40 Hz (17), whereas the EF₅₀ of 4.4 Hz in Wang’s and colleagues (40) study of horse airways was markedly lower. To put these figures into proportion, the maximal frequency that allows motoneurons to depolarize during repetitive stimulation is 500–1,000 Hz (21). It should also be noted that Mitchell and colleagues (30) showed relatively high frequencies of action potentials (peak frequency of 10–13 Hz) for feline tracheal

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**Fig. 4. Effect of magnesium on airway contractions by EFS and acetylcholine derivatives.**

A: first and second train: 4 × EFS (F = 50 Hz, B = 1 ms, A = 200 mA, TW = 2.5 s, TR = 60 s) in 3.3 min in the presence of 10 μM neostigmine; third train: addition of 100 μM acetylcholine; n = 6. The entire time course for a single experiment with one slice is shown on the abscissa. B: same experiments as in A with the addition of 10 mM MgSO₄ 30 min before the second train; n = 5. C: summary of the minimal airway area obtained in the experiments shown in A and B as bar chart. EFS, electric field stimulation; ex. ACh, exogenous acetylcholine; **P < 0.01; data are shown as means ± SD (A, B) or means ± SD (C). D: cumulative concentration-response curves of methacholine-induced airway contraction in PCLS were recorded in the absence or presence of 10 mM MgSO₄. MgSO₄ was added 30 min before the first addition of methacholine. Data are shown as means ± SD of 8 PCLS for each condition from 5 animals. A shared concentration-response curve was suitable to fit the data, because there was no statistical difference at P < 0.05 in any parameter of the 4-parameter logistic equation between the two data sets (GraphPad Prism).
parasympathetic ganglion cells during normal breathing. Also
other factors such as electrode geometry and the ionic strength
of our cell culture medium have to be taken into account, as the
inner electric field strength depends on the conductivity of the
medium and on the current density, which in turn depends on
a shape factor, the frequency, and the external electric field
strength. Taken together, the frequencies that were applied in
rat PCLS appear reasonable.

With respect to pulse duration we found, similar to Wang
and colleagues (40), that durations $B \geq 1$ ms were needed to
reach a plateau response. In all our experiments (except Fig.
2D) we used a current density of $A = 200$ mA, which was the
only current that gave a reproducible contraction; since $200$
 mA is the maximum current output of the HSE-stimulator II,
higher currencies could not be studied. Another important
factor influencing ASM contraction is train width: here maxi-
mal contraction was achieved at TW $\geq 10$ s, similar to Bosse
and colleagues (8, 9), who used a maximum airway contraction
at TW $= 9$ s when examining the influence of increased tone
(by acetylcholine) on the mechanical properties of ASM.
However, at TW $= 10$ s, airway contractions took longer to
reverse and, more importantly, could not be blocked com-
pletely by magnesium, indicating that the electrical stimulation
was unspecific at this train width and activated both nerve
endings and airway smooth muscle. Therefore, we decided to
use TW $= 2.5$ s to avoid direct activation of airway smooth
muscle, but a TW $= 5$ s would also seem appropriate. Since we
used $F = 50$ Hz, a TW $= 2.5$ s implies a series of 125 single
pulses that were separated by a recovery phase of about 1 min
(exactly 57.5 s). The duration of this phase allowed recovery
under normal conditions (Fig. 2A; Fig. 3, top) but was too short

Fig. 5. Correlation of airway size with EFS-triggered airway contractions.
PCLS were first stimulated in the absence (○) and then in the presence (●) of
10 μM neostigmine; i.e., the data are paired. Rat airway generations are
marked on the upper x-axis according to Yeh et al. (44). A one-phase
exponential-decay described the relationship reasonably well. $r^2$, goodness
of fit; data show the results from 29 PCLS from 5 rats.

Fig. 6. Effect of the thromboxane receptor antagonist
SQ29548 on bronchoconstriction induced by EFS or
U46619. A: first and second train: $4 \times$ EFS ($F = 50$ Hz,
$B = 1$ ms, $A = 200$ mA, TW $= 2.5$ s, TR $= 60$ s) in 3.3 min
in the presence of 10 μM neostigmine; third train: addition
of 10 μM of the thromboxane-prostanoid-receptor agonist
U46619; $n = 4$. The entire time course for a single exper-
iment with one slice is shown on the abscissa. B: same
experiments as in A with the addition of 10 μM SQ29548 15
min before the second train; $n = 5$. C: bar graph summary
of the minimal airway area obtained in the EFS trains in A
and B. D: bar graph summary of the airway area obtained 15
min after U46619 addition in A and B. ex. U46619, exog-
enous U46619; $^*P < 0.05$; data are shown as means $\pm$ SD
(A, B) or means $\pm$ SD (C, D).
when neostigmine was present. However, as the maximum contraction was always reproducible, we conclude that the lack of complete recovery in Figs. 3, 4, and 6 is not a major limitation. It is important though to note that the different stimulation trains were separated by at least 15 min to assure full recovery of the ASM to its physiological ground state before the next experimental setting was examined. In addition this period was used to equilibrate the slices with the pharmacological agents to be studied next.

Cholinergic neuronal response in PCLS. Acetylcholine release from parasympathetic nerve terminals regulates the airway tone in various species including humans (6, 10, 17). The enhanced airway contraction after addition of neostigmine, an acetylcholine esterase inhibitor (Fig. 3), indicates locally increased concentrations of acetylcholine at the neuroeffector junction (24, 27). Atropine, an unspecific inhibitor of muscarinic receptors, almost completely inhibited the neuronal cholinergic contraction in PCLS, even in the presence of neostigmine (Fig. 3). Atropine most probably prevents ASM contraction by blockade of the M3 (and M2) receptors on the postsynaptic membrane. Thereby the M3 receptors cannot initiate the contraction through the inositol trisphosphate pathway (6, 7, 28, 33). The small residual bronchoconstriction in the presence of 10 μM atropine may be explained by the blockade of presynaptic muscarinic receptors by atropine, preventing the autoinhibitory effect of acetylcholine release (6, 7), and/or the release of substance P that might potentiate the release of acetylcholine from cholinergic nerves (6) and trigger the release of bronchoconstrictive tachykinins from excitatory NANC (14).

Reactivity of different airway generations. PCLS have previously been used to study airway responses along the bronchial tree. Smaller airways are more responsive to methacholine, serotonin, the TP receptor agonist U46619, and allergens in lungs of different species (26, 42, 43). Endothelin-1 contracted airways independent of their size (26), indicating that smaller airways are not more sensitive in principle. Here we show that smaller airways are less sensitive to EFS (Fig. 5). This observation can be explained most likely by the decreasing innervation of the bronchial tree toward the peripheral regions (6). Thus parasympathetic bronchoconstriction in the airways appears to be balanced by inversely arranged innervation and acetylcholine responsiveness: rich innervations with low acetylcholine responsiveness in large airways opposed to more sparse innervations with high sensitivity in smaller airways.

Role of TP receptor in neuronal activation. Subthreshold doses of TP receptor agonists (e.g., U46619); i.e., doses that do not evoke airway contraction via the TP receptor themselves, have been shown to increase parasympathetic cholinergic bronchoconstriction in mice (as measured by R₅) (3), guinea pig (R₆) (39), and dogs (isometric tension) (2, 37). Interestingly, in the study of Takata and colleagues (37) the effect was only found for bronchial smooth muscle, whereas tracheal smooth muscle was unaffected suggesting regional differences in airway responsiveness, with smaller airways being more susceptible to thromboxane. Additionally, Saroea and colleagues (35) suggested U46619-induced bronchoconstriction via acetylcholine release in asthmatic subjects. Moreover, rat tracheal prostanoid synthesis can be stimulated by activation of muscarinic receptor-linked Ca²⁺ mobilization (20). These observations suggested a possible influence of endogenously released thromboxane on EFS in our model. However, the TP-receptor antagonist SQ29548 had no effect (Fig. 6), indicating that EFS-induced airway contractions in the rat are not influenced by thromboxane. This is confirmed by the observation that even a precontracting concentration of U46619 did not alter the response to EFS (Fig. 7). These observations are in line with other in vitro studies. For instance, Aizawa and Hirose (1) measured airway contraction of canine tracheal strips in response to acetylcholine or EFS either in presence or absence of PGF₂α or stable thromboxane A₂ and found no difference. They therefore suggested that the interaction of thromboxane and cholinergic pathways depends on stimulation of vagal sensory endings and activation of the reflex pathway in vivo. This conclusion is supported by studies from Underwood and colleagues (39), who discriminated strictly between in vitro and in vivo responses to PGD₂ and 9α,11β-PGF₂. In vivo, atropine antagonized prostaglandin increased airway resistance similar to the TP receptor antagonist SK&F 88046, whereas in vitro (tracheal strip) only TP receptor antagonists were effec-

Fig. 7. U46619 does not prime the airway response to EFS. A subsequent EFS train was carried out on the same set of PCLS as depicted in Fig. 8. For both groups (± 10 μM SQ29548) no significant (n.s.) difference was found before (open symbols) and after (closed symbols) addition of U46619 (10 μM).
tive. Thus our findings suggest that a complete reflex arc is not present in PCLS.

An interaction of TP- and muscarinic receptor pathways may not be limited to prejunctional nerves but may also occur at the ASM itself. We therefore conducted cumulative dose-response curves with U46619 in presence or absence of atropine and found no difference (Fig. 8). This finding is in accordance with in vitro contraction of tracheal strips from guinea pigs, where comparable dose-response curves for U44069, PGD₂, and 9α,11β-PGF₂α in the presence or absence of atropine were obtained (39). In contrast, Allen and colleagues (3) observed a notable reduction in U46619 (50 – 500 nM)-evoked murine in vitro contraction of tracheal strips from guinea pigs, where comparable dose-response curves for U44069, PGD₂, and thromboxane A₂ in presence or absence of atropine and ASM itself. We therefore conducted cumulative dose-response curves with U46619 in presence or absence of atropine and thromboxane. However, their model presupposes the existence of an ASM M3 receptor to its cognate physiological ligand acetylcholine after stimulation of the ASM TP receptor by thromboxane. However, their findings by an increased sensitivity of the ASM M₃ receptor to acetylcholine in our system, since neostigmine had no effect in unstimulated PCLS (Fig. 3). Nonetheless, possible species differences can easily be studied with the help of PCLS (11, 25, 34, 42, 43).

Comparison to other in vitro models. Previously, EFS has been almost exclusively studied in the organ bath using trachea, bronchi, and parenchymal strips. In comparison, PCLS have both advantages and disadvantages.

First, in the organ bath, studies are limited to the trachea or upper bronchi or to the very distal part of the lungs as in parenchymal strips. PCLS from rat lungs allow to study nearly all generations along the bronchial tree. Though PCLS are very thin and therefore the percentage of neurons and especially ganglia will be lower than in tracheal and bronchial preparations. Second, airway contractions in the organ bath are either isotonic or isometric, whereas it is more autotonic (like in vivo) in PCLS. The airways remain embedded in the parenchyma that exhibits tending forces on the airways increasing with contraction. These tending forces are enhanced by using a Teflon ring that weighs on the outer parts of the slice, so that the tending forces increase during airway contractions. In the absence of the Teflon ring the whole slice contracted and no relaxation occurred, implying the lack of sufficient tending forces (Fig. 2A). So presumably, the Teflon ring acts like the attachment of the lung tissue to larger vessels and airways. Third, our PCLS setup requires only small buffer volumes of 1 ml, which can be further decreased to 0.4 ml. Fourth, PCLS allows one to analyze airway responses throughout the bronchial tree, which allows one to analyze airways down to a diameter of 50 μm (25, 42, 43). Thus the present method will help to further elucidate the role and the mechanisms of small airway innervation. In contrast to classic parenchymal preparations for the study of peripheral lungs, in which force generation from different sources (ASM, vasculature) cannot be distinguished, our model can specifically attribute contractions to airways or vessels along the entire tracheobronchial tree. And finally, the technique of PCLS is easily transferred to other species, including humans.

In conclusion, the present study shows that PCLS respond to EFS, indicating that terminal nerves remain intact in this preparation. In rats EFS-induced airway contractions are mediated by cholinergic nerve stimulation and airway innervations decrease with airway size. EFS of PCLS may serve as a model to study neurally mediated responses in large and small airways.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES
Innovative Methodology


