Chapter 20

Assessment of Airway Hyperresponsiveness in Murine Tracheal Rings

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Abstract

Isolated tracheal rings have often been used to directly measure the contractile output of airway smooth muscle (ASM). Here, we describe the method for excising murine tracheas, mounting tracheal rings in organ baths, and measuring the isometric forces generated by the ASM when stimulated by drug additions or electric field stimulation. The apparatus for the setup and the pathways responsible for stimulation are detailed. Examples of the responses and analyses of two types of ASM stimulation are illustrated: (1) the carbachol concentration–response curve and (2) the frequency–response curve elicited by electric field stimulation.

Key words Airway smooth muscle, Tracheal rings, Isometric force, Electric field stimulation, Concentration–response curves, Hyperresponsiveness

1 Introduction

Airway hyperresponsiveness (AHR) occurring in asthma is characterized by an excessive narrowing of the airways leading to an elevated resistance to airflow and, at times, complete obstruction of the airway lumen [1–5]. It is generally believed that the basis for AHR is complex and multifaceted, involving inflammation, alterations in airway smooth muscle (ASM) function, remodeling of the structures of the airway wall (including the ASM itself), and asthma-induced production of various spasmogens. The active element in airway narrowing is, of course, the contraction and shortening of the ASM. What is not so clear is the extent to which the contractile function per se of the ASM is altered in asthma. It may be that the basic contractile function of the ASM is normal as in the healthy lung, but that other factors, such as airway remodeling, inflammation, or presence of other contractile agents, result in excessive narrowing. On the other hand, asthma may involve an enhanced responsiveness of the ASM itself and excessive shortening of the muscle leads to airway obstruction.

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The development of in vitro methods has been helpful in dissecting a direct role for changes in ASM responsiveness per se in asthma from an indirect role involving other factors. A number of articles have been devoted to these methods [6–11]. In this chapter, the apparatus used for isometric contractile measurements as well as the solutions necessary to maintain the tissue in a viable state are described in detail. Descriptions of the isolation and in vitro mounting of the mouse tracheal ring for measurement of isometric force development are presented. In addition, the preparation and administration of commonly used contractile agents as well as the method to electrically induce contraction of the ASM are presented. The chapter offers examples of contractile responses to pharmacologic agents and electric field stimulation, as well as insight into the analysis of the data.

The methods described here are confined to the isometric contractile response of larger airway segments such as the trachea or primary bronchi. It is the most commonly used and easiest approach to measure the contractile output of ASM, although it is not without its own pitfalls in interpretation. Other methods, such as isotonic contractions and lung slices, have also been utilized and these have their own particular advantages for addressing issues of airway function. For example, airway narrowing is produced only when the ASM shortens. Too few studies have employed isotonic or auxotonic contractions, rather than isometric contractions, to characterize the effects of asthma on ASM contractile function and the contribution of afterload to the response of the muscle. Lung slices [12] offer another approach for examining changes in ASM properties, especially with visualization at the cellular and subcellular levels. The isometric method described in this chapter represents a good first approximation for examining the contribution of ASM to asthma-induced AHR.

2 Materials

2.1 Solutions (See Note 1)

1. Normal physiological salt solutions (PSS) (2 l): 119 mM (13.91 g) of NaCl, 4.7 mM (0.7 g) of KCl, 1.18 mM (0.32 g) of KH_2PO_4, 1.17 mM (0.58 g) of $MgSO_4 \cdot 7H_2O$, 18.0 mM (3.02 g) of $NaHCO_3$, 0.026 mM (0.1 ml of 0.5 M) of EDTA (*see* **Note 2**), 11.0 mM (3.96 g) of glucose, 12.5 mM (8.56 g) of sucrose (*see* **Note 3**), 2.0 mM (400 ml of 10.0 mM $CaCl_2$ stock solution) of $CaCl_2$ (*see* **Note 4**). The solution is bubbled with 95 % O_2–5 % CO_2 (pH 7.4 at 37 °C) (*see* **Note 5**).
2. High K^+ PSS: This is the same as the normal PSS except that the NaCl and KCl are adjusted: 56.7 mM (6.628 g) of NaCl and 67 mM (9.99 g) of KCl.
3. Stock carbachol (carbamylcholine) solution consists of 10 mM carbachol in normal PSS (*see* Table 1).

Table 1
Drug solutions and additions for the cumulative concentration–response curve shown in Fig. 3

Stock volume (ml)	Stock concentration (mM)	Amount added (μmol)[a]	Final concentration (μM)[b]
0.01	0.01	0.0001	0.01
0.02	0.01	0.0002	0.03
0.007	0.1	0.0007	0.1
0.02	0.1	0.002	0.3
0.007	1.0	0.007	1
0.02	1.0	0.02	3
0.007	10	0.07	10
0.02	10	0.20	30

[a]Moles of drug added = (stock volume × stock concentration)

[b]Cumulative concentration = (amount added (μmol)/$10 \times 10^{+3}$ μl) + previous concentration. The bath volume is 10 ml

2.2 Trachea-Mounting Hardware (Fig. 1)

1. Ring mounting: The tracheal ring is mounted between two stainless steel rods (*see* **Note 6**). The upper rod is attached to a force transducer (*see* **Note 7**), while the bottom rod is attached to a micrometer used for setting length and adjusting the rest force. Both the micrometer and the force transducer are mounted on metal rods anchored either on a baseboard or on an upright board. The screw clamps allow adjustments to convenient heights.
2. Tissue bath: The tracheal ring and stainless steel mounts are immersed in the PSS contained in the inner compartment of a glass-jacketed tissue bath. Tissue baths can be obtained from several suppliers and are available in various sizes and configurations to accommodate the needs of the investigator. The bath has inlets and outlets for (1) heating fluid (jacket), (2) bathing fluid (PSS) changes (bath), and (3) gas bubbling. Most experiments are performed at 37 °C. Circulation of preheated water (*see* **Note 8**) through the tissue bath jacket assures that the PSS contained in the inner compartment that bathes the tissue remains at 37 °C. The input to the inner chamber is connected via Tygon tubing to a reservoir containing the PSS (also heated and gassed). The bathing PSS is refreshed by opening a clamp to the bath inlet of the inner chamber and allowing the heated PSS to be either pumped from the heated reservoir (not shown) or allowed to flow by gravity feed from the heated reservoir (not shown) located above the tissue bath (*see* **Note 9**). Gases are continuously bubbled through the input to the inner chamber via tubing connected to a pressure regulator controlling the flow of gas from the large stock gas tank.
3. Electric field stimulation (EFS) (Fig. 1b): The EFS of the tracheal ring is produced by two platinum plate electrodes

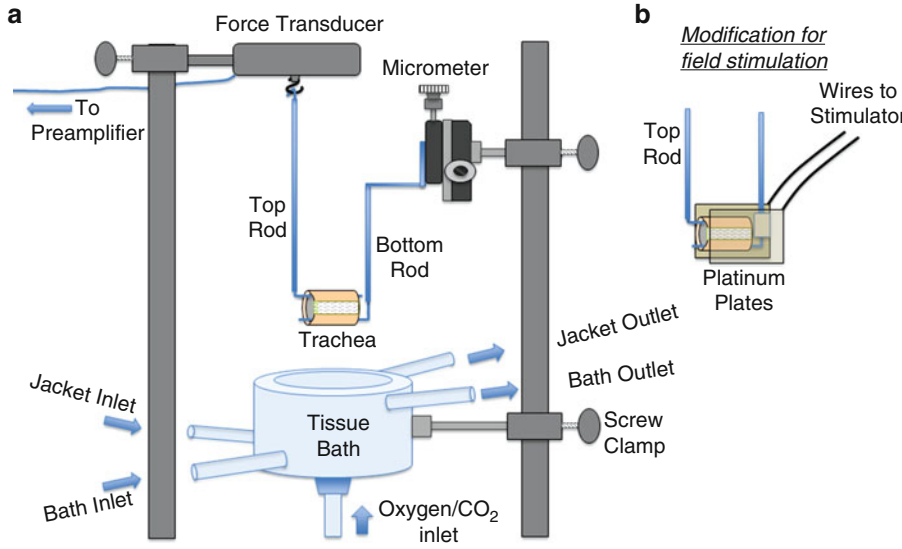

Fig. 1 Diagram of the apparatus used to measure the isometric contraction of isolated tracheal rings. (**a**) The jacketed tissue bath, force transducer, and micrometer are mounted on supporting rods. The use of screw clamps allows easy positioning of these elements. The trachea ring is mounted between the two stainless steel rods while the bath is in the lowered position. After the ring is mounted the bath (filled with heated, gassed PSS) is raised, thereby bathing the tissue. (**b**) Platinum electrodes connected to a stimulator are positioned side by side such that the tracheal ring lies between them. These electrodes should be mounted securely on the lower rod to insure that the distance between the electrodes does not change (reproduced from [10])

placed on either side of the mounted ring and connected to a Grass S88 stimulator (*see* **Note 10**).

4. Data acquisition: The signal from the force transducer is digitized using an A/D converter and amplifier and visualized on a personal computer (*see* **Note 11**). The system is calibrated each day prior to the commencement of the experiment (*see* **Note 12**).

3 Methods

3.1 Trachea Ring Isolation and Mounting

1. Prior to tissue isolation the force transducer is calibrated, the tissue bath is filled with normal PSS, and the airflow adjusted to obtain a light stream of O_2/CO_2.
2. Mice of approximately 2 months of age are deeply sedated with isoflurane and immediately sacrificed by cervical dislocation (*see* **Note 13**).
3. The skin (and fur) is removed from thorax to throat and the ribs are cut from the base of the sternum, laterally (on both sides) to the top of the heart. The sternum and ribs are then pulled forward to the throat to reveal the heart/lungs, thymus, trachea (ventral), and esophagus (attached to and dorsal to trachea).
4. The trachea is grasped via forceps above the pharynx and is excised by cutting below the bronchial bifurcation and above

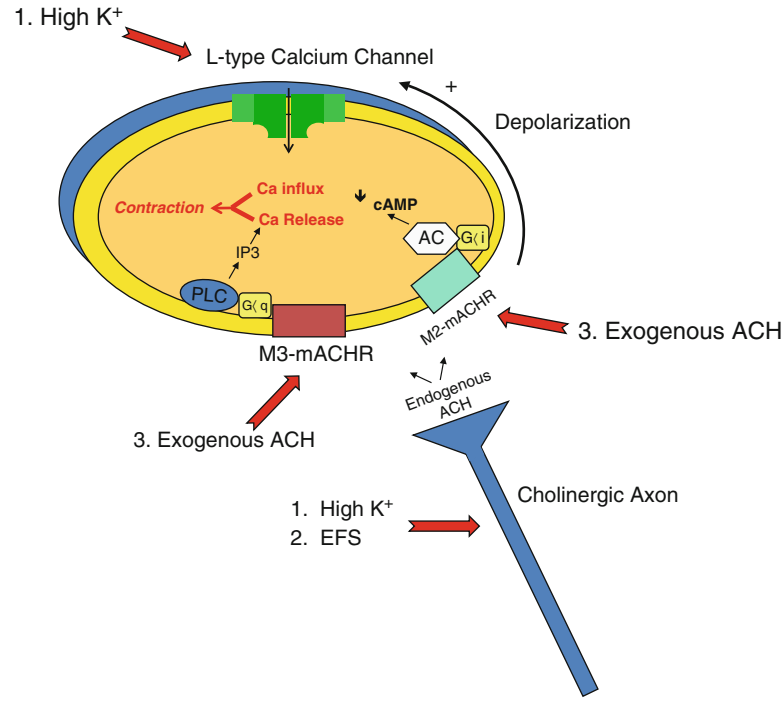

Fig. 2 Diagram of the major signaling pathways for an airway smooth muscle cell within the tracheal ring. Shown is L-type calcium channel activated by depolarization upon high K^+ stimulation (*1.*). M2 and M3 cholinergic receptors are activated by endogenous acetylcholine released from nerve endings (*1.* and *2.*) and exogenously applied acetylcholine (or cholinomimetic) (*3.*). The second messenger cascades leading to contraction are also shown. Not shown are other types of cells within the airway wall that may release other contractile and relaxing agents (reproduced from [10])

the forceps. The tracheal segment is placed in ice-cold oxygenated PSS where it is cleaned of loose connective tissue via fine scissors (*see* **Note 14**) and cut transversely into tracheal rings (*see* **Note 15**).

5. The tracheal ring is threaded over the two L-shaped stainless steel prongs (Fig. 1a) and the tissue bath is then raised so that the ring is immersed in PSS.
6. The micrometer is adjusted slowly to obtain a tension of ~1 g. Over the first 5–10 min, trachea passive tension (also called preload) tends to decline somewhat (stress-relaxation phenomenon) and the micrometer is used to adjust the passive tension at 1 g during equilibration (*see* **Note 16**). The trachea is allowed to equilibrate for at least 1 h before experimental challenges and the bathing PSS refreshed periodically.

3.2 ASM Stimulation (Fig. 2) (See Note 17)

1. High potassium contractions (Fig. 3a): The rings are challenged twice with the high K^+ PSS. The normal PSS is replaced by high K^+ PSS and the contraction is allowed to proceed until

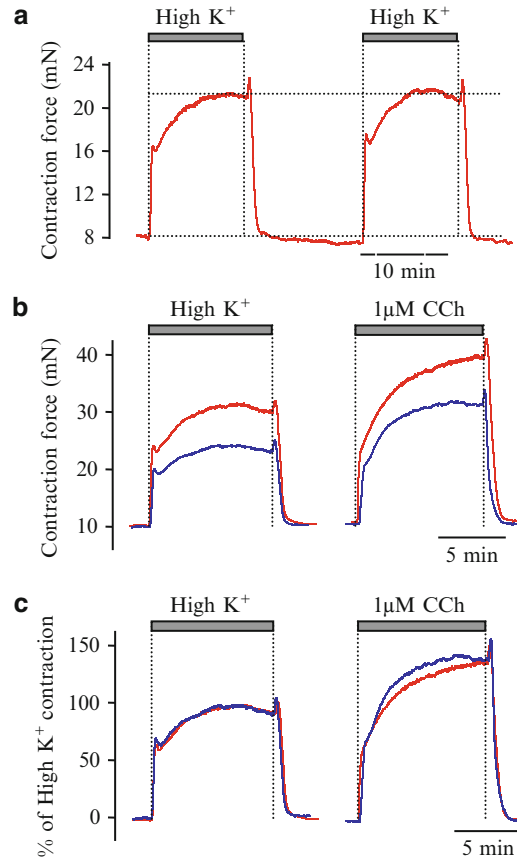

Fig. 3 Tracings of the contractile responses of tracheal rings to stimulation. (**a**) Duplicate contractile responses (mN) of a single ring as a function of time (min). Note the similarities of the sequential responses. (**b**) Tracings of the responses of two different rings to high K^+ (*left panel*) and 1.0 μM carbachol (CCh) (*right panel*). Note that one ring has a consistently larger response to both types of stimulation. (**c**) Normalization of the responses of the two rings to their respective maximum response to K^+ (100 %). Note the similarity between the two strips when the output is normalized to K^+ (reproduced from [10])

it plateaus out. The bath solution is exchanged with fresh PSS and the force allowed to return to baseline. The high K^+ challenge is then repeated a second time (*see* **Note 18**).

2. Pharmacologic stimulation (Fig. 4): Exogenous agents, such as ASM agonists and antagonists, can be added directly to the bath. In Fig. 4a, doses of carbachol stock were added to the tissue bath without rinsing between drug additions (cumulative addition), with each higher dose being added at the height of the contractile response from the previous addition. Figure 4b shows the composite log concentration (abscissa) vs. contractile response (ordinate) (*see* **Note 19**). Drugs can also be added to

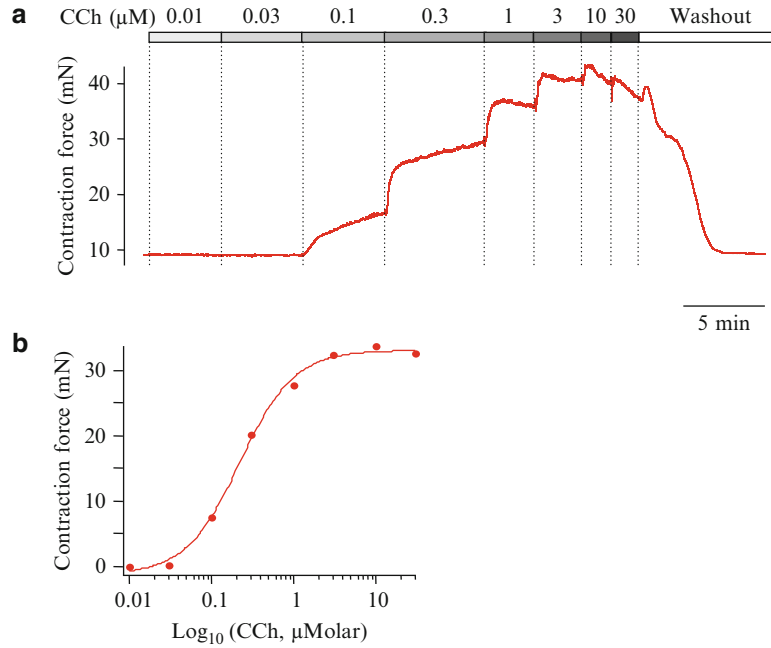

Fig. 4 Illustration of a cumulative concentration–response curve experiment. (**a**) Actual tracing of the contractile response to additions of carbachol. Numbers above the tracing indicate the concentration of carbachol in the bath after each succeeding addition. The protocol for making up the stock carbachol solutions and the appropriate volumes to be added are shown in Table 1. (**b**) Concentration–response curve for the data gained in (**a**). The maximum force developed for each carbachol concentration (mN) is graphed as a function of the log of the carbachol concentration (μM) (reproduced from [10])

the PSS prior to perfusion in the tissue bath. This can prove useful when a constant background of stimulation is required throughout the entire experiment. The normalization of the contractile output of a given tracheal ring presents some difficulty (*see* **Note 21**).

3. EFS: The stimulus parameters, such as voltage–response, frequency–response, etc., for a particular setup must be determined prior to beginning any series of experiments (*see* **Note 20**). The contractile response to increasing frequency (constant voltage, duration, and pulse width) during EFS is shown in Fig. 5a and the data are graphed in Fig. 5b as a frequency–response curve. The normalization of the contractile output of a given tracheal ring presents some difficulty (*see* **Note 21**).

3.3 Normalization and Data Analysis

1. Problems associated with setting the force or the length (between muscle clips) of the resting tracheal ring are discussed in **Note 16**. How to best to express the contractile output of a given ring also presents problems (*see* **Note 21**).

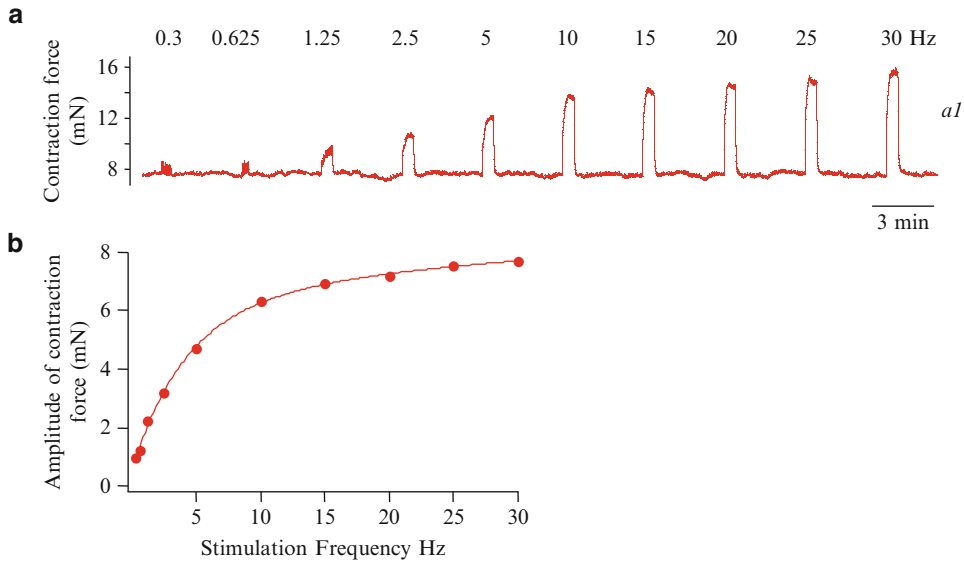

Fig. 5 Illustration of a force–frequency response curve experiment. (**a**) Actual tracings of the responses to different electrical frequencies (Hz) under conditions of 0.5 ms pulse and 40 V. The frequency, shown above the tracings, was varied from 0.3 to 30 Hz. (**b**) The contractile response to the EFS (mN) is graphed as a function of frequency (reproduced from [10])

4 Notes

1. The PSS are prepared at the beginning of each week using ultrapure water and are stored at 4 °C in a refrigerator. Solutions are utilized within 5 days of preparation and any unused solution after that time is discarded. The composition of the tissue bathing solutions varies widely among investigators. Not only do the concentrations of the common salts vary, but also the buffer types and other additional compounds differ.
2. EDTA is included to bind and minimize the effects of copper, iron, and other trace metals that catalyze the oxidation of certain susceptible pharmacological agents (e.g., catecholamines).
3. Sucrose is included to correct the osmolarity of the solution. The cell membrane is virtually impermeable to sucrose.
4. The stock $CaCl_2$ should be added to the stirred solution last and somewhat slowly. If added too quickly in the absence of CO_2, especially when the solution pH is high, a white precipitate will form that usually can be reversed upon the addition of CO_2.
5. Bicarbonate buffer is the most commonly used buffer for these types of experiments, but organic buffers also have been used. Because the CO_2 content of different gas tanks may vary from the nominal value (depending upon the accuracy of the vendor)

it is a good idea to set the pH of each stock solution with HCl or NaOH at 37 °C.

6. The inclusion of any metals containing iron, copper, etc. in the solutions or in contact with the tissue should be avoided. These metals break down releasing contaminants that can adversely affect the tissue. Stainless steel is preferred for use in biological fluids. Small Parts Inc., Miami Lakes, FL, is a good source for stainless steel rods and other useful items for fabrication of the mounting hardware.
7. Many different force transducers are commercially available. The FT.O3C Force Displacement Transducer (Grass Technologies, Warwick, RI) is widely used. It is rugged and reliable and offers ample sensitivity for use with mouse tracheal rings.
8. Many heating circulator pumps are available on the market. We have tended to use those supplied by Haake Instruments or B. Braun.
9. Because of the tubing dead space between the reservoir PSS and the inner chamber of the tissue bath, the first fluid to flow into the chamber upon washing will not be at 37 °C, but at room temperature. This abrupt transient change in temperature can lead to a transient contractile response of the muscle (*see* Fig. 3).
10. The electrodes should be solidly mounted in the apparatus such that the distance between the electrodes does not change. The platinum electrodes can be attached to the bottom rod so that the distance between the plates is fixed (we use 4 mm). All wires and soldered connections are insulated from the bathing solution by coating with Sylgard (Silicone Elastomer; Dow Corning Corp., Midland, MI) to prevent leaching of deleterious metals into the tissue bath.
11. Two data-gathering systems are used in our laboratories: MacLab 8 A/D system, which is an older version of AD Instrument Powerlab hardware (ADInstruments, Inc., Colorado Springs, CO), and BIOPAC Systems (Goleta, CA). Macintosh computers are used to visualize the signals during acquisition and later during data analysis.
12. The complete system is calibrated prior to each experiment by hanging known weights (2–5 g) from the force transducer. Many studies report the force in grams or gram-weight, which is consistent with the calibration procedure. However, this expression is not formally correct. Force equals mass × acceleration and the correct quantity for force is the Newton (N). Since the acceleration due to gravity is 9.8 m/s, 1 g-weight is approximately 0.0098 N and is generally expressed as 9.8×10^{-3} N or 9.8 mN.

13. We have found that tracheas from mice that are 2 months old or older are easily mounted. Younger mice may be used, but the smaller tracheas become more difficult to mount on the stainless steel rods. Some anesthetics should be avoided. For example, we have observed that Avertin (Tribromoethanol) has strong relaxant effects on ASM. The depth of anesthesia is appropriate when a toe pinch with forceps is unable to elicit a response.
14. The cleaning and ring preparation should be done as quickly as possible with minimal touching or damage to the tracheal segment. This stage can be facilitated if performed in a petri dish filled with silicone. By pinning the trachea below and above the bifurcation and pharynx, respectively, the danger of damage from too much handling with the forceps will be minimized.
15. Attempts should be made to keep the lengths (axial length) of the tracheal rings uniform. The output of any given ring is proportional to the number of muscle cells contracting in parallel. So, a ring that is twice as long as another ring should develop twice the force as the narrower ring. In addition, the transverse cut should also be uniform, i.e., 90° to the long axis of the trachea. A change in the orientation of the rings could lead to different force development, as shown for vascular smooth muscle strips [13]. It should be noted here that if in the course of remodeling due to asthma the ASM changes orientation, then the force development would be affected by the new orientation. Thus, the angle of the cut, as well as, possible reorientation of the ASM within the wall should be taken into account when analyzing the force output.
16. Our laboratory sets the rest force at 1.0 g, which is appropriate for the width of the rings utilized in our experiments. Preliminary experiments revealed that this rest force placed the rings at or near the length at which maximum force development occurs. The question of which rest force or rest length the ring should be set is problematic mainly because of the adaptation of the airway wall with regard to the force–length curve of the ASM [14]. The adaptation can occur quite rapidly and is observed as a shift of the curve along the length axis. At present, no universally accepted normalization point for the rest length or preload is available [14].
17. Figure 2 depicts the complex pathways leading to ASM stimulation. The depolarization due to addition of high K^+ PSS opens voltage-dependent calcium channels in the muscle allowing calcium influx and contraction. High K^+ PSS also opens voltage-dependent calcium channels in the nerve endings, causing release of acetylcholine and cholinergic-evoked contraction. Activation of the major cholinergic receptors (M_2

and M_3) by exogenous acetylcholine or a cholinergic mimetic causes contraction via second messengers. Electrical stimulation of the tracheal ring activates cholinergic nerves in the airway wall and release of acetylcholine, which in turn causes contraction of the ASM. Not shown are adrenergic receptors (B_2) on the ASM. Also not shown are several different other cell types (e.g., epithelia) that are found in the airway wall and that can produce and release other spasmogens or relaxing agents.

18. The high K^+ challenges serve three purposes. First, examination of this response can give an indication of whether the tissue was damaged during excision, preparation, and mounting. If the contractile response appears different from the usual response, it should be discarded. Second, we think of the high K^+ challenge as a "wake-up call" after the equilibration period and this is always included in our protocol for every experiment. Finally, the contraction to high K^+ is used to normalize the response to other contractile agents (*see* **Note 21**).
19. Most often the contractile response of the ASM to a particular agent is examined over the total range of effective concentrations. Such a concentration–response curve is usually obtained in a cumulative fashion, i.e., the concentration of the drug in the tissue bath is increased in an additive fashion without rinsing the bath between the additions of the drug. A representative experiment is shown in Fig. 4 where carbachol is added cumulatively to the tissue bath. Note that the range of concentrations extends over several log units and proceeds not at one-half log units, but at one-third log units. The use of one-third instead of one-half is mainly for appearance, i.e., data points graphed one-third between log units appear nearly equi-spaced between the log units. Table 1 demonstrates the drug preparation and addition procedure for the experiment shown in Fig. 4. The total volume of additions should not exceed 2 % of the initial bath volume. When additives are dissolved in organic solvents, the total volume of additions should be limited even further (<0.1 %) and preliminary test should be run to determine if the solvent itself causes any untoward effects. Moreover, the concentrations of drug stock solutions (stock) should be chosen such that convenient volumes can be added to the tissue bath. Table 1 lists the concentrations of the stock drug solutions, the volumes added to the tissue bath, and the final drug concentrations to which the trachea is exposed. Because dose–response curves are performed over several log units, the use of geometric means in data analysis should be employed [15].
20. For EFS each setup has its own peculiarities and therefore the stimulus parameters should be characterized empirically prior

to initiating any series of experiments. Contractile responses to stimulus durations, frequencies, voltages (strengths), and pulse durations are measured and the optimal settings chosen. In our setup where the plate electrodes are separated by about 4 mm, the optimal stimulation strength, frequency, and pulse width were 44 V, 30 Hz, and 0.5 ms, respectively.

21. The contractile response of a tracheal ring is most accurately expressed as the force developed divided by the cross-sectional area of the smooth muscle within the ring, accounting for any change in ASM orientation. Because of differences between ring sizes attempts are often made to normalize force output to the ring size. However, in practical terms, laboratories have adopted a number of ways to express the contractile response and no uniformly agreed-upon normalization procedure is available. The following are the most commonly used approaches:
    (a) No normalization: This method presents the actual force development in either grams or in mN without any attempt toward normalization. The assumptions are that (1) the tracheal rings are cut at the same length and the same orientation and (2) the ASM mass and orientation have not varied from ring to ring.
    (b) Normalization to ring weight: To correct for differences in the length of the rings utilized, the rings are removed from the apparatus after the experiments and weighed, generally after light blotting. The force is then normalized to the wet weight. In our laboratory this approach was unsatisfactory because the variability using tracheas from smaller mice was too large. If the length of the ring between the mounting rods is known, the average cross-sectional area can be calculated using the density of the ring (e.g., 1.05 g/cm^3). Other laboratories normalize force output to dry weight or to protein content.
    (c) Normalization to a given contraction: Our laboratory normalizes force to the contraction obtained with high K^+ PSS (Fig. 3b, c). The tracings in Fig. 3b depict the contractile responses in mN of two different tracheal rings to high K^+ and carbachol stimulation, respectively. The outputs in mN between the two rings are different. However, as shown in Fig. 3c, the contractile responses are equivalent when the contractions are normalized to the respective maximum high K^+ contractions. Some laboratories normalize to the maximum response to cholinergic stimulation.

Acknowledgments

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References

1. Seow CY, Fredberg JJ (2001) Historical perspective on airway smooth muscle: the saga of a frustrated cell. J Appl Physiol 91:938–952
2. An SS, Bai TR, Bates JH et al (2007) Airway smooth muscle dynamics: a common pathway of airway obstruction in asthma. Eur Respir J 29:834–860
3. Fredberg JJ (2004) Bronchospasm and its biophysical basis in airway smooth muscle. Respir Res 5:2. doi:10.1186/1465-9921-5-2
4. Oliver MN, Fabry B, Marinkovic A, Mijailovich SM, Butler JP, Fredberg JJ (2007) Airway hyperresponsiveness, remodeling, and smooth muscle mass: right answer, wrong reason? Am J Respir Cell Mol Biol 37:264–272
5. Meurs H, Gosens R, Zaagsma J (2008) Airway hyperresponsiveness in asthma: lessons from in vitro model systems and animal models. Eur Respir J 32:487–502
6. Garssen J, Van Loveren H, Van Der Vliet H, Nikamp FP (1990) An isometric method to study respiratory smooth muscle responses in mice. J Pharmacol Methods 24:209–217
7. Hulsmann AR, de Jongste JC (1993) Studies of human airways in vitro: a review of the methodology. J Pharmacol Toxicol Methods 30:117–132
8. Fedan JS, Van Scott MR, Johnston RA (2001) Pharmacological techniques for the in vitro study of airways. J Pharmacol Toxicol Methods 45:159–174
9. Cooper PR, McParland BE, Mitchell HW, Noble PB, Politi AZ, Ressmeyer AR, West AR (2009) Airway mechanics and methods used to visualize smooth muscle dynamics in vitro. Pulm Pharmacol Ther 22:398–406
10. Semenov I, Herlihy JT, Brenner R (2012) In vitro measurements of tracheal constriction using mice. J Vis Exp (64):pii:3703, doi:10.3791/3703
11. Wright D, Sharma P, Ryu M-H, Risse P-A, Ngo M, Maarsingh H, Koziol-White C, Jha A, Halayko AJ, West AR (2012) Models to study airway smooth muscle contraction *in vivo*, *ex vivo* and *in vitro*: implications in understanding asthma. Pulm Pharmacol Ther 26:24–36. doi:10.1016/j.pupt.2012.08.006
12. Sanderson MJ (2011) Exploring lung physiology in health and disease with lung slices. Pulm Pharmacol Ther 24:452–465
13. Herlihy JT (1980) Helically cut vascular strip preparation: geometrical considerations. Am J Physiol 238:H107–H109
14. Bai TR, Bates JH, Brusasco V et al (2004) On the terminology for describing the length-force relationship and its changes in airway smooth muscle. J Appl Physiol 97: 2029–2034
15. Hancock AA, Bush EN, Stanisic D, Kyncl JJ, Lin CT (1988) Data normalization before statistical analysis: keeping the horse before the cart. Trends Pharmacol Sci 9:29–32