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Pharmacological techniques for the in vitro study of airways

Jeffrey S. Fedan[a,b,*], Michael R. Van Scott[c], Richard A. Johnston[a,b,d]

[a]Pathology and Physiology Research Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, 1095 Willowdale Road, Morgantown, WV 26505-2888, USA
[b]Department of Pharmacology and Toxicology, Robert C. Byrd Health Sciences Center of West Virginia University, Morgantown, WV 26506, USA
[c]Department of Physiology, The Brody School of Medicine at East Carolina University, Greenville, NC 27858-4354, USA
[d]Physiology Program, Department of Environmental Health, Harvard School of Public Health, 665 Huntington Avenue, Boston, MA 02115-6021, USA

1. Introduction

The purpose of this review is to describe some of the more commonly used isolated preparations of airways for in vitro experimentation and to discuss some practical considerations associated with their use. The types of experiments and protocols for which these preparations have been used are large and diverse. We will not attempt to review all of these applications, but will restrict the discussion to the use of preparations to examine airway smooth muscle and respiratory epithelium. Space will not allow inclusion of all the work that has been done using these preparations, and we apologize for not including some reports that would serve as equally good examples; however, there is much similarity in the manner in which each type of preparation is used, and any given paper can be a point of entry into that method. For the sake of expedience, we will highlight the preparations used in our laboratory. Some of these are novel and may be of interest to a wider community.

In vitro preparations of freshly isolated tissues allow investigation into effects and mechanisms under steady state experimental conditions with precise control of concentrations of substances and other experimental parameters. In the pulmonary research arena, investigators have utilized the information gained from isolated airway preparations to elucidate effects and processes that occur in vivo both in healthy mammals and in animal models of lung diseases. The preparations find usefulness both in defining fundamental cellular mechanisms as well as for screening the effects of agents of potential therapeutic benefit or that exert toxic effects. As such, the in vitro approaches for the study of airways have provided information that is critical to the interpretation of effects observed under in vivo conditions. On the other hand, it must be remembered that an isolated airway preparation, removed from its neuronal connections and contact with plasma-borne substances, both known and yet to be identified, may acquire new properties or lose some of the attributes it possessed in situ. Each of the preparations and techniques to be discussed has advantages and disadvantages that should be considered before adoption.

2. Tracheal strips

We will begin our discussion with the preparation that enjoys the widest use. Strips may be prepared from the trachea of every species used conventionally in pulmonary research. Likewise, strips can be prepared from the larger bronchi (extrapulmonary and intrapulmonary) of all but the smallest mammals. A major advantage of strip preparations is that large numbers of preparations may be obtained from each animal, allowing multiple conditions to be examined simultaneously in a paired experimental design. A disadvantage of strip preparations is the amount of surgical manipulation of the organ that occurs during preparation.

The trachea, mainstem bronchi, and lungs are exposed by an incision beginning at or below the level of the diaphragm. Staying on the midline, the rib cage is opened by cutting through the carina and continuing to the thyroid gland. After deflecting the ribs, the trachea, extrapulmonary bronchi, and lungs are readily apparent. As needed, the trachea and/or lungs are removed from the animal and placed in an oxygenated physiological salt solution (PSS; see below). It is best to remove the tissue of interest quickly, leaving fine cleaning to be performed after the organs are removed, when more control is possible. Adherent fat and connective tissue should be removed, as the possibility exists that mediators could be released from resident cells in these tissues.

Blood that coagulates on contact with the airways is very difficult to remove and is another potential source of

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* Corresponding author. Tel.: +1-304-285-5766; fax: +1-304-285-5938.

E-mail address: jsf2@cdc.gov (J.S. Fedan).

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mediators. The surgical field can be kept free of blood by exsanguinating the animal before removing the airways and lungs. In our laboratory this is accomplished by severing the abdominal aorta, hepatic vein, and heart.

Several options are available for the preparation of tracheal strips. In species containing large tracheas such as human, ferret, rabbit, dog, sheep, pig, and horse, it is possible to remove completely the smooth muscle band (the trachealis muscle) of the trachea from the cartilage ring and to prepare strips containing only the smooth muscle. The advantages of using preparations consisting only of smooth muscle need not be stated. The muscle extends beyond the border of the "C" ring and inserts into the cartilage. While immersed in oxygenated PSS (the preparation of all isolated airway preparations should be done with the tissue immersed in oxygenated PSS) ideally at body temperature, ring segments consisting of the width of one cartilage ring can be prepared using a scalpel to cut through the intercartilaginous spaces. The muscle can be cut into strips of desired width and length by cutting through the cartilage to open the ring, slightly stretching the opened ring and anchoring it to an appropriate surface (such as Sylgard), and bisecting the smooth muscle from the cartilage. Depending on the size of the trachea, many strips can be obtained from the muscle contained in one tracheal segment. Alternatively, a segment of trachea may first be opened by cutting longitudinally to give a "sheet" of muscle, from which strips may be cut. Generally speaking, it is far easier to attach ligatures to muscle strips that are anchored under a small amount of force, before the strip is severed from its attachments. The choice of ligatures is important. Braided silk is widely used, but its caliber must be appropriate for the force that the muscle will generate so that it will not become stretched by a contraction. When mechanical properties of the muscle are being studied (i.e., quick release, etc.), silk may be too compliant a material for ligatures and a nonelastic material, such as stainless steel wire, may need to be substituted. Trachealis smooth muscle is capable of generating great forces, on the order of ≥ 1 kg of force per g of muscle. Thus, the dimensions of the strip, which determines the ultimate force generated, should be appropriate for the size of the ligature and transducer.

It is difficult to prepare tracheal strips containing only the smooth muscle from small mammals such as mouse, guinea pig, and rat, although this has been accomplished using rabbit and guinea pig trachea (Raeburn et al., 1986). Preparation of strips of airways of small animals usually requires attaching ligatures to the cartilage (Justice et al., 2001). Using airways from small animals, we have found it essential to use a dissecting microscope during cleaning and strip preparation. The small amount of smooth muscle contained within only one cartilage segment may generate small contractions that require sensitive equipment to measure. As mentioned above, strips may be prepared from rings after cutting through the cartilage; the rings are often several segments wide to provide more muscle. After securing the strip to a suitable substrate, ligatures are tied to the cartilage at each end of the muscle band. Alternatively, the tracheal segment may first be opened longitudinally with microscissors. After anchoring, strips may be cut using a scalpel blade and pushing directly downward onto the support. We have found it easiest to use the latter method; it causes less distortion of the fragile preparations and can give rise to strips of nearly identical dimensions. In strips from small tracheas, the location of the ligature is important due to the natural curvature of the cartilage. When the ligature is placed as close as possible to the cartilage-muscle junction, measured force will reflect that imparted directly from the muscle onto the ligature. If the ligature is placed away from the muscle, a component of measured passive and developed force (see below) will be influenced artifactually by the force wasted between the muscle and the ligature, which reduces the curvature of the cartilage; a nonlinear transference of force to the transducer exists under these conditions.

Another technique for preparing strips from the trachea of small animals is to prepare a long, spiral strip or a chain of linked rings (Drazen & Schneider, 1978) to which a ligature is attached at each end. Aside from causing a misalignment of the smooth muscle from the longitudinal axis, with these preparations much force developed by the muscle is lost to the coil. In fact, the loss of measurable developed force is so large that little more force will be generated by one long tracheal strip or chain prepared from one trachea than is developed by the muscle in a linear tracheal strip two cartilage rings wide (unpublished observations). The number of experimental conditions that can be studied with spiral strips and chains is much reduced compared to that obtained with linear strips; fewer paired observations can be made and the number of animals that need to be used is increased.

3. Bronchial strips

The transition of the airways from trachea to bronchi is associated with the progressive loss of the obvious muscle band seen in trachea and extrapulmonary bronchi to bronchi in which the smooth muscle surrounds the airway as a band; cartilage is eventually lost in the bronchioles (Weibel, 1963). The airways of increasing generations of the tracheobronchial tree become shorter in length and, of course, smaller in the diameter. In order to prepare strips of bronchi it is necessary to surgically isolate the bronchi of interest from the surrounding parenchyma, while paying attention to the branching of the airways so as to be able to define the airway generation. The physical size of an airway of a given generation will depend on the size of the animal being used. In the lungs of large animals the intrapulmonary bronchi are still large enough to allow preparation of linear strips, along the lines discussed above for trachea. In smaller animals (mouse, rat, guinea pig, etc.) small bronchi may be studied by preparing rings. One example of this approach is found in the report by Szarek et al. 1995 in which rings from eighth generation bronchi from rats were prepared. When using airways this small, reasonably long segments are required in order to capture enough smooth muscle to generate measurable responses. Instead of inserting flexible suture into the lumen, which would result in distortion of the segment's geometry when knots are tied, two fine rigid metal wires are inserted into the lumen to anchor the segment and record force.

4. Parenchymal strips

The question of the relevance of experiments using smooth muscle from large airways i.e., trachea to diseases involving smaller airways lurks in the background of such studies. While the role(s) of the smallest bronchi in pulmonary disease is of great interest, their small size makes it technically difficult to prepare them for in vitro examination using the surgical techniques described above. An alternative means of examining the properties of peripheral airways smooth muscle has been through the use of parenchymal strips (Drazen & Schneider, 1978; Gordon et al., 1984). The parenchyma is devoid of larger bronchi at the perimeter of the lung lobes. Strips (roughly 2 × 2 × 20 mm) are cut from the edge of lung lobes and attached in organ baths to transducers, as described below. Parenchymal strips respond both to airway smooth muscle contractile and relaxant agonists and share many of the pharmacological properties of preparations consisting only of airway smooth muscle. However, there are two concerns about parenchymal strips that are relevant to the design and interpretation of experiments. First, parenchymal strips are not homogeneous tissues, and they consist of vascular smooth muscle as well as other cells that are capable of developing force in the complex fabric of the lung. Consequently, a given response cannot be taken as reflecting solely the contribution of the smooth muscle in the small peripheral airways. Second, the interaction of respiratory smooth muscle with epithelium (see below) cannot be examined inasmuch as the epithelium cannot be removed from the airway wall.

5. Organ bath

Muscle strips are studied in an organ bath (chamber; Fig. 1) containing a gassed physiological salt solution (PSS). Several solutions are used, which differ in the composition and concentrations of the salts and buffer systems employed. A bicarbonate-containing solution will require gassing with CO_2 to set the pH. The solution we use in our laboratory [modified Krebs-Henseleit (MKH) solution] consists of (mM): NaCl (113.0), KCl (4.8), $CaCl_2$ (2.5), KH_2PO_4 (1.2), $MgSO_4$ (1.2), $NaHCO_3$ (25.0), and glucose (5.7), pH 7.4 (37°C); gassed with 95% O_2–5% CO_2. One observes small differences in the composition of the "Krebs" solutions used from laboratory to laboratory. Choosing the solution to use is an important commitment to make. It is inadvisable to make arbitrary changes in solute composition or to switch from one solution to another in the midst of a series of like experiments; the results will be affected by this decision. Solutions using artificial buffers such as Tris (which can interact with sodium channels) and HEPES are often gassed with 100% O_2, air, or air containing elevated O_2 concentration. It is our view that nonphysiological buffers should be avoided unless they are warranted by a specific experimental question (e.g., ion substitution). Discussions of the relative merits of these solutions have not been considered for some time, and the degree to which routine results are influenced by the solution chosen for experiments is an issue that has been largely dismissed. The temperature is usually kept at 37°C, but this may vary depending on the body temperature of the animal from which the tissue was derived or other experimental needs.

To measure responses of the smooth muscle preparation, one ligature or wire assembly is attached to a fixed holder at the bottom of the organ bath containing PSS, while the other is attached to a transducer to measure mechanical responses (i.e., contraction or relaxation) of the smooth muscle. There are two principal types of transducers that are used to measure mechanical responses of the smooth muscle: isometric and isotonic. A third type, auxotonic (Li & Stephens, 1994), provides valuable information but is not in widespread use and will not be discussed further here. Isometric transducers measure force generation while holding the preparation at a constant length established by the investigator. Isotonic transducers measure changes in muscle length under a constant imposed load. In most cases isometric transducers are more appropriate, especially when only a small amount of shortening will occur, as in the cases of tracheal strips containing a narrow band of smooth muscle and very small bronchial rings.

After mounting, the preparation is lengthened to place it at a length (L_o) at which it will develop maximal contractile force (see below). While monitoring the output of the transducer on the strip chart recorder or computer screen, the preparations are slowly lengthened to remove slack in the connections between the anchor and the transducer and to reach a predetermined optimum passive or baseline force (i.e., tension). Most smooth muscle preparations demonstrate stress relaxation (i.e., the initial passive force may "fade"), a normal property of smooth muscle. The stress relaxation will vary between types of preparations and species. The next steps to be taken depend on the preparation itself and the perceptions in the laboratory. In preparations that do not develop "spontaneous tone," such as those from the rat and dog, additional force may be applied after intervals to restore the desired force until the system stabilizes. Alternatively, a force may be applied initially that exceeds the desired passive force but that, after stress relaxation occurs, will decline to the endpoint passive force.

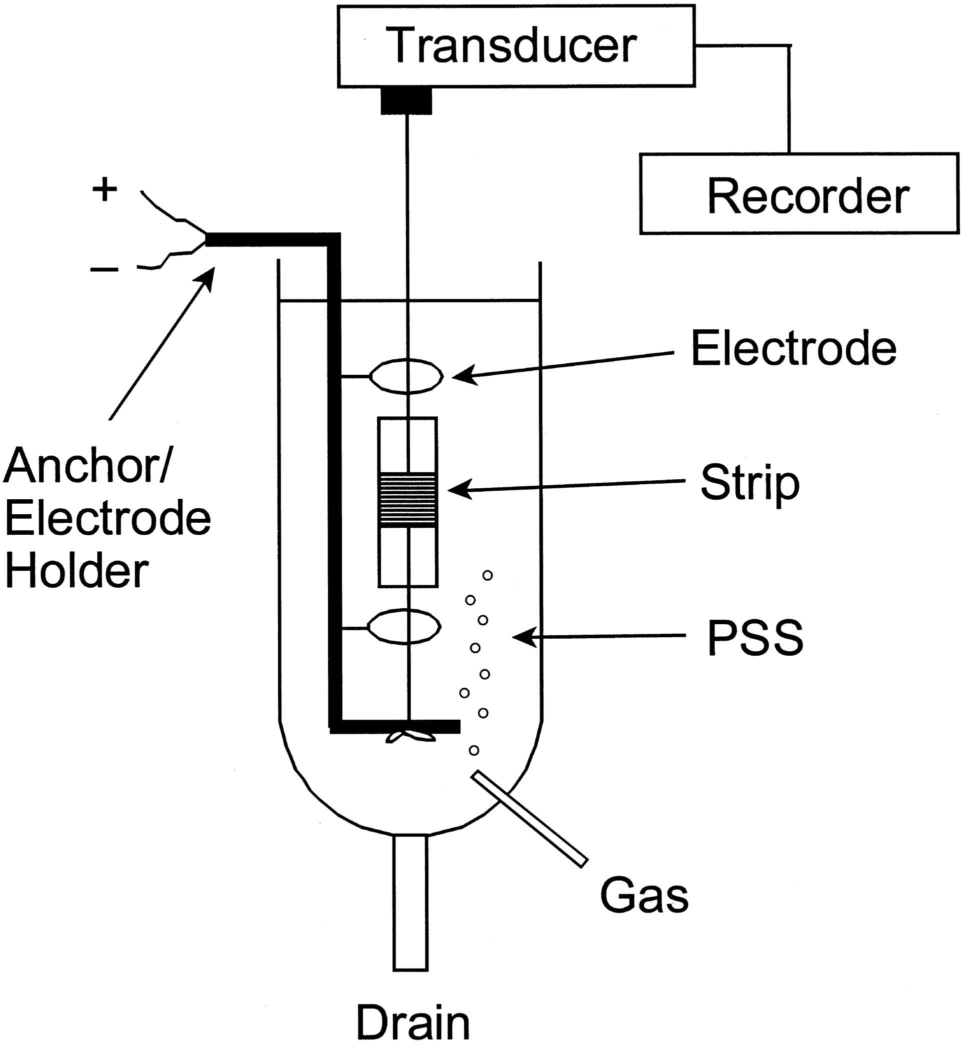

Fig. 1. Typical configuration of an organ bath for strip or ring preparation of airways. Shown here is an opened ring preparation of a larger airway containing a band of muscle (striped portion of strip). The strip has been attached at one end to the anchor and at the other end to the transducer. The bath contains PSS and is oxygenated with an appropriate gas mixture and maintained at a temperature relevant to the animal from which the tissue was taken. In this example the anchor is also equipped with electrodes, one at each end of the tissue, that would be connected to a stimulator (+ and –) for delivering electric field stimulation (see text).

The presence of spontaneous tone in the preparation may complicate the establishment of basal conditions. Spontaneous tone reflects an intrinsic activation of the smooth muscle, which the investigator has not caused. Guinea pig tracheal strips develop an appreciable spontaneous tone, which is due to the effects of released prostanoids (Hay et al., 1986). When force is applied to the strips to establish optimum length, the applied force opposes both the passive elastic properties of the muscle independent of spontaneous tone as well as the active force developed by the muscle, if any. Contraction of the muscle, at any given length, makes it resistant to stretch; at any given length, the force obtained in the presence of spontaneous tone will be greater. In our experience, the application of force to guinea pig tracheal strips to a level above the desired endpoint is followed by a rapid stress relaxation; this is followed by a gradual contraction due to spontaneous tone, which eventually stabilizes at or near the desired basal force. Only small adjustments are then necessary. Overadjusting the amount of applied force may eventually damage the smooth muscle. A narrow window of variability should be accepted.

Once basal force has stabilized at the beginning of the experiment, it is best not to make changes afterwards (i.e., after the muscle has been challenged with an agent), even if the baseline has changed. To do so is to alter the length of the muscle, which can affect subsequently the reactivity of the preparation to agents or procedures.

6. Equilibration/incubation period

Following the application of passive force, a mandatory period of incubation of at least 1 h should follow before any experimental intervention is begun. The PSS should be changed at regular intervals (15 min) to prevent buildup of metabolic products. This incubation also is necessary to allow the muscle to reestablish the normal ion content that goes awry during tissue preparation. For example, placing tissue specimens in ice-cold PSS while returning from the abattoir, or performing dissection at room temperature, results in inhibited production of ATP and inhibition of ATP-dependent ion transport systems. The cells lose potassium and gain sodium (i.e., become "sodium loaded") and experience alterations in resting membrane potential (Casteels, 1970). To perform experimental interventions under these conditions would lead to responses that are uncharacteristic of the tissues under their normal homeostatic conditions. Allowing an ample equilibration period at 37°C allows the muscle to generate ATP, reactivate ATP-dependent transport, and restore resting membrane potential.

7. Resting optimum passive force

As alluded to above, passive force is applied to isolated airway smooth muscle preparations at the beginning of the experiment to set the length of the preparation at L_o, the length at which active force generation will be maximal. At L_o, the overlap of actin and myosin provides maximal cross-bridge formation and force development in response to contractile agents. It is essential that optimum resting force be established experimentally from the length–tension (force) relationship (the stress–strain relationship; Fig. 2) whenever an in vitro airway preparation is newly adopted. (These experiments can also be conducted with parenchymal strips, with the caveat that the underlying basis for maximal contractile activity may not lie in actin–myosin interactions in the airway smooth muscle contained in the strip.) The first step for establishing optimum basal force is to examine the literature for reports utilizing the preparation in order to gain a sense of the range of basal forces that have been employed. From this information the size of the passive force increments that will be added can be estimated. For example, if many investigators use a 1 g optimum passive force on a like preparation to establish baseline, it would not make sense to begin a length–tension study by applying 2 g of force at the outset. Likewise, it is intuitive that an airway smooth muscle with a large cross-sectional area (e.g., dog trachealis) will require more applied force to reach optimum basal force than a preparation containing a muscle with a small cross-sectional area (e.g., intrapulmonary bronchus). The underlying premise of the length–tension study is that the optimum resting force will be that which results in the greatest force developed by a stimulated tissue. Therefore, an agent or procedure that causes contraction of the muscle is needed. The choice can be eased by the pharmacological properties of the preparation as reported in the literature. Generally, histamine or acetylcholine (or analogs of acetylcholine such as methacholine or carbachol) are useful agonists for this purpose (histamine does not contract rat airways). A concentration must be chosen for use, and we recommend that it be one less than or equal to the EC50 value for the agonist; use of high concentrations of these agents (10^{-4} M) can result in desensitization of the muscle and spurious results. As an alternative, contractions may be elicited with electric field stimulation (EFS) to cause the release of neurotransmitters from efferent nerves (discussed below). The experimental protocol for length-tension experiments is simple and occurs as follows.

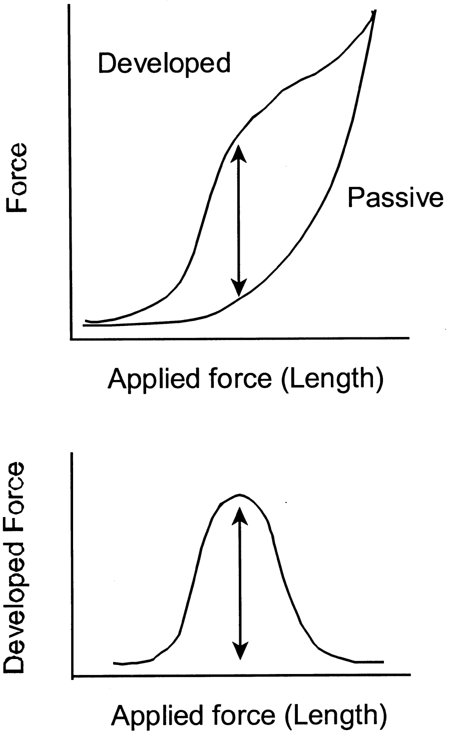

Fig. 2. Idealized results from an experiment performed to determine the length-tension (stress-strain) relationship of an in vitro smooth muscle preparation. (Top panel) The lower curve depicts the passive force resulting from the stretching of the preparation (i.e., tension increases when the preparation is lengthened). Notice that the passive force resulting from the stretch is not linearly related to length. The upper curve shows the force developed by the preparation in response to a stimulus that contracts the smooth muscle. The developed force is equal to the total force minus the applied force (arrow). (Bottom panel) The plot of developed force vs. applied force, derived from the data in the top panel, is bell-shaped. The peak of the curve reveals the passive force that, when applied to the tissue, gives rise to the greatest developed force. Thus, when this passive force is applied, the preparation will achieve a length (L_o), which is optimum for contraction.

The apparatus is configured to eliminate any "play" in the connections between the anchor in the bath and the isometric force transducer; the goal here is to apply the minimum force that makes the muscle and its connections taut. The resulting force is recorded and allowed to reach equilibrium. The tissue is then challenged with a contractile agent or EFS, and the response is allowed to reach its peak. Leaving the applied force unchanged, the preparation is washed repeatedly to allow it to relax fully to the prestimulus level. An additional increment of passive force is added again, and, after the force has equilibrated, the same stimulus is used to challenge the preparation. After the peak of the response the tissue is washed again until the prestimulus baseline is reached. The size of the second developed contraction will be larger than the first.

This process is repeated several times. At each stimulation period the value of the prestimulation basal force is subtracted from the total force to give developed force. Plots of passive applied, total, and developed force vs. applied force (length) will generally appear as shown in Fig. 2. Even though the abscissa of this figure expresses passive force applied to the preparation, what is actually being represented is the relationship between muscle length and its ability to generate force, independent of the passive elastic properties of the preparation. It is also possible to perform this experiment by measuring the actual length of the muscle and plotting length on the abscissa vs. forces on the ordinate. However, it is difficult to measure muscle length in the airways of small animals, and in the small bronchi of any animal, when the muscle cannot be surgically separated from surrounding tissues. The optimum passive force or basal tension is that one applied to the preparation at which the force developed by the muscle is maximal (i.e., the peak of the curve). Note that after this point is reached, the force generated by the muscle declines. The passive force will rise rapidly around this point, and the total force and the passive force curves will eventually converge at high passive force (unphysiological) conditions when the elasticity of the preparation becomes dominated by passive elasticity. Having determined with replicate experiments the optimum passive force for the preparation, that force may be applied thereafter in routine experiments; it is not necessary to perform a length-tension analysis for every preparation, with one proviso. If a treatment alters the passive elasticity of the preparation, application of the routine passive force will place the muscle at a length different than the control value (Fedan & Besse, 1980). Likewise, the manner in which the airway is prepared may result in a change in the length-tension relationship. For example, if the amount of muscle in the strip is changed or if the airway preparation is somehow reconfigured, the formerly used applied passive force will no longer apply, and a new length-tension relationship will have to be established.

8. Concentration-response curves

An important and convenient use of in vitro airway preparations is the establishment of concentration-response relationships for agents that cause contraction and relaxation. After the equilibration period under optimum basal force has ended, additions of contractile agonists to the bath will result in concentration-dependent responses. Airway smooth muscles usually maintain developed force in the presence of the agonist; at high concentrations of agonist desensitization may occur. A cumulative concentration-response determination is more expeditious than a noncumulative concentration-response determination. In the former, the response to a given concentration of agonist is allowed to reach its plateau, after which the next higher concentration is added to the bath without washout of the preceding concentration. Sufficient concentrations are added to allow the preparation to contract to its maximum response, beyond which no further responses can be obtained. In the second approach, agents are added one concentration at a time, with washout of the drug after the response to each addition has reached its peak. Time must be allowed for the muscle to relax to the baseline after washout. This time factor, the possibility that desensitization can occur when a large concentration precedes a smaller one, and large variability in the resulting data are disadvantages associated with the use of noncumulative concentration-response curves. In both cases the optimum passive force is subtracted from total force to obtain the developed force.

The goal of these studies is to determine the reactivity of the muscle to the contractile agent. The most important parameters used to describe reactivity are the EC50 and the maximum developed response (not the total force, which includes the passive force). The EC50 can be obtained using 4-parameter logit analysis, which is contained in many scientific graphics computer programs, or from linear regression of probit-transformed data. As no part of a concentration-response curve is a straight line, the use of a ruler or linear regression to estimate EC50 is not correct. When a great deal of control and uniformity is possible in preparing the strip preparations, such as those containing only smooth muscle, the maximum response can be expressed using units of force (e.g., *g* or Newton), which can then be normalized with respect to muscle mass or cross-sectional area. With small airway preparations, in which it is difficult to accomplish absolute uniformity from strip to strip and to determine muscle mass, maximum responses may show large variability from strip to strip. Therefore, maximum responses to an agent are normalized with respect to the contractile effects of an unrelated stimulant. For example, challenge of the preparations with 120 mM KCl, a depolarizing agent, will give a reference contraction against which responses to a receptor-acting agent can be normalized and compared. Likewise, responses to KCl can be normalized against a reference contraction to an unrelated agonist, such as methacholine or histamine. It must be remembered that these normalization procedures do not assist assessment of changes in absolute force development, as might occur after treatment of animals with agents, but only aid in normalizing the effects of agents on concentration-response curves obtained from the same preparation.

The existence of spontaneous tone can markedly affect the outcome of concentration-response curves for contractile agents. There is in all smooth muscles a ceiling of contractility beyond which force cannot be produced, regardless of the agent or concentration used to stimulate the tissue. If spontaneous tone is present and an agent or procedure is applied that decreases this tone, then contractions developed in response to a stimulating agent will appear larger (Fig. 3; Hay et al., 1986). As mentioned earlier, airway strips from different species vary in the degree to which spontaneous tone is present. Spontaneous tone is generally attributable to the release of prostanoids. The presence of spontaneous tone can easily be determined by examining whether a relaxation below basal force occurs in response to the cyclooxygenase inhibitor, indomethacin (5×10^{-6} M), or to smooth muscle relaxants such as sodium nitroprusside; or to a β-adrenoceptor agonist such as isoproterenol.

Concentration-response curves for the relaxant effects of agents are obtained from their effects on precontracted tissues. In preparations with spontaneous tone there usually is not enough tone to relax to produce meaningful data. The choice of agent to induce tone is usually not critical, unless specific drug interactions are to be avoided. Generally, a long-acting cholinomimetic like methacholine or carbachol will produce reliable, sustained contractions. Histamine and KCl also are used. The concentration of contractile agent is critically important. Ideally, a concentration of the contractile agent that is less than or equal to its EC50 is used to induce contraction. As the contractile agonist concentration is elevated further toward that giving the maximum response, it becomes more and more difficult to relax the airway smooth muscle; reactivity (EC50 and maximum response) to the relaxant is linked to the size of the initial contraction relative to the maximum response for the contractile agonist (Torphy et al., 1983). Quantification of the relaxant effects is often normalized as a percentage of the induced contraction. These values, or the raw data, may be utilized to quantify the EC50 and maximum response.

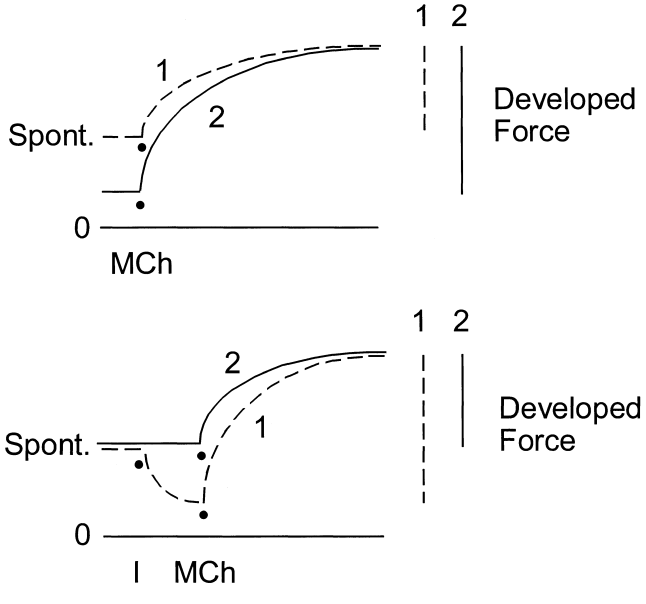

Fig. 3. Effect of spontaneous tone on contractile responses. (Top panel) Comparison of responses to methacholine (MCh) of two tissues. One (#1) develops spontaneous tone after the application of passive force (resting tension), while the other (#2) does not develop spontaneous tone (0 signifies no force). Preparation #1 *develops* less force in response to MCh, even though the maximum amount of obtainable force from each preparation is the same. (Bottom panel) The application of indomethacin (I) results in relaxation of preparation #1 to the level of passive applied force. Compared to preparation #2, MCh subsequently elicits a larger *developed* response in preparation #1. The maximum force that both tissues can develop is the same. Note: The use of a relaxant agonist, such as isoproterenol, instead of indomethacin would not yield the same result. In addition to relaxing the spontaneous tone to the level of passive applied force, isoproterenol would produce a functional antagonism of the response to MCh. As a result, the response to MCh would be inhibited.

9. Electric field stimulation

The neural control of airway smooth muscle is of great interest, especially since there are neural components in respiratory diseases such as asthma. The effectiveness of the antimuscarinic blocker, ipratropium bromide, in asthmatic patients and the neurogenic inflammation of the airways brought about by the release of neuropeotides from sensory nerves (Barnes, 2001) point to the central importance of efferent and sensory nerves in the airway wall. The study of the effects of neurotransmitters on smooth muscle in isolated airway preparations is quite facile and can be accomplished in virtually any airway preparation. Passage of an electric field across isolated airway preparations will cause activation of intrinsic nerves, the release of neurotransmitters, and responses of the smooth muscle to these neurotransmitters.

The apparatus for activating intrinsic nerves consists of platinum electrodes around the airway preparation, which deliver an electric field across the wall of the airway. The electrodes can be wire or flat stock, placed parallel to the preparation, or ring electrodes placed at each end of the preparation. Several manufacturers provide tissue-holding devices equipped with electrodes.

Delivery of EFS requires the use of a stimulator with which the frequency, duration, and voltage of square-wave electric pulses can be varied. Such stimulators are available from a number of manufacturers. The stimulation parameters used are very important; one desires a selective stimulation of the intrinsic nerves while avoiding direct stimulation of the smooth muscle. In order to verify that the observed responses are neurally and not directly mediated, it is necessary to examine the effect of tetrodotoxin (10^{-6} M), a sodium channel blocker that blocks action potential formation in nerves but that does not appreciably affect smooth muscle. If EFS-induced responses are blocked in the presence of tetrodotoxin, one has assurance that the responses occurred in response to nerve activation. The persistence of EFS-induced responses in the presence of tetrodotoxin indicates that the muscle has been stimulated directly. There is some diversity in the stimulus parameters used in different laboratories, and it is worthwhile searching the literature for parameters that have been used by others employing similar preparations. In recent studies on preparations of guinea pig and ferret trachea, we have utilized 10 s trains of square wave pulses of 130 V and 0.5 ms duration (Maize et al., 1998; Fedan, 2001). The voltage used should be "supramaximal," that is, of sufficient magnitude to excite all the nerves in the preparation (recall that nerves differ in their voltage thresholds for excitation). The configuration of the electrodes will determine the shape of the electric field, which will affect current density and, hence, the voltage required. There is a ceiling to the size of the voltage that should be used, because high voltages will stimulate the muscle directly. The stimulus duration parameter is also important in determining selectivity of the impulses for nerve vis-à-vis smooth muscle. The longer the pulse duration, the more likely that the muscle will be stimulated directly; a pulse duration of 0.1–0.5 ms usually will not affect the muscle. When a new preparation is set up, the investigator will want to vary the voltage and duration to obtain the largest responses while maintaining sensitivity to tetrodotoxin.

Airway smooth muscle is innervated by several types of efferent and sensory nerves. The innervation of the airways is a vast area of research and the details are beyond the scope of this review; we will highlight only the main points. The innervation of mammalian airways is not uniform across species (Richardson, 1979; Canning & Fischer, 2001). All mammalian airways appear to receive vagal parasympathetic efferents, which release acetylcholine as the neurotransmitter; acetylcholine contracts airway smooth muscle. The airways of some mammals are innervated by sympathetic nerves, which release norepinephrine to induce relaxation of the smooth muscle. Many species also contain innervation that is neither adrenergic or cholinergic in nature; this is referred to as "noradrenergic, noncholinergic" (NANC) innervation, and the term derives from experiments in which it was determined that blockers of cholinergic and adrenergic nerve activity did not inhibit completely neurally mediated responses of airway smooth muscle. The NANC system is comprised of an inhibitory component (the i-NANC system), which causes relaxation of the smooth muscle, and the excitatory component (the e-NANC system), which induces contraction of the muscle. It is currently believed that nitric oxide and vasoactive intestinal polypeptide (VIP) are the significant i-NANC neurotransmitters, whereas tachykinins such as substance P and neurokinin A are the main e-NANC neurotransmitters. Undoubtedly, more as yet undiscovered peptides are involved as i-NANC and e-NANC transmitters to airway smooth muscle.

Knowledge of these systems is vital for understanding responses of airway smooth muscle preparations to EFS. Depending on the species one is studying, many or all of these innervations may contribute to the observed responses, to give responses profiles that have several components (Fig. 4). Typically, under ideal conditions (see below), the response to EFS consists of an initial, transient contraction, which is cholinergically mediated and can be blocked with muscarinic antagonists such as atropine (10^{-7}–10^{-6} M). This is followed by a longer-lasting relaxation below the baseline, which is mediated by the concerted effects of adrenergic nerves, nitric oxide-releasing nerves, and VIP-containing nerves. This relaxation can be inhibited with a β-adrenoceptor antagonist such as propranolol (10^{-6} M) to block the effects of released catecholamine, with an adrenergic neuron blocker such as guanethidine (10^{-5} M) to prevent transmitter release from adrenergic nerves, and with Nω-nitro-L-arginine methyl ester (L-NAME; 10^{-4} M) to prevent the synthesis of nitric oxide in nitrergic nerves. Finally, a longer-lasting contraction may follow the relaxation, which is due to effects of e-NANC transmitters (i.e., tachykinins). The shape of the responses will depend on the frequency of stimulation (Fig. 4) and the population of nerves in a given airway.

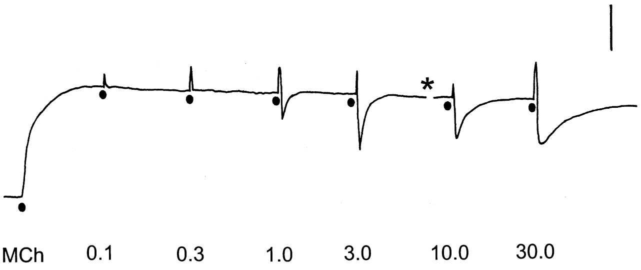

Fig. 4. Results of a frequency-response determination obtained from a guinea pig tracheal strip that was prepared from a segment two cartilage rings wide. The preparation is challenged with MCh (3×10^{-7} M; EC50) to induce tone. The dots indicate periods of EFS (120 V; 0.5 ms) delivered at 7 min intervals in the continued presence of MCh. The frequencies of stimulation are indicated below the dots. Note the frequency-dependence of the shape of the responses. Vertical bar: 0.5 g to the left of the asterisk, 1 g to the right of the asterisk.

Quite often the goal of EFS experiments is the generation of a frequency-response curve (Fig. 5), in which the size of responses as a function of stimulus frequency is plotted. In many preparations a single pulse of EFS will give rise to a response, but it is usually more instructive to utilize several frequencies to establish a frequency-response relationship. Frequencies spanning from 0.1 to 30 Hz give meaningful and physiologically relevant data. Some preparations will continue to respond to higher frequencies, such as 60 to 100 Hz, but these are nonphysiological rates and probably should be avoided even though the maximum response has not been obtained. In addition, higher frequencies of stimulation will tend to cause direct activation of the smooth muscle.

The relaxant effects of inhibitory transmitters will not be observed in preparations lacking tone, either spontaneous or induced pharmacologically. In such preparations only contraction will be seen. This is not because the inhibitory nerves have not been stimulated, but because tone must be present to allow visualization of the effects of the relaxant transmitters. Likewise, if the preparation is stimulated with a maximally effective concentration of a contractile agent, an EFS-induced contraction will be blunted and relaxant responses will be inhibited or completely abrogated, due to the inability of the relaxant transmitters to overcome the contractile stimulus. Thus, there is a pharmacological "win-

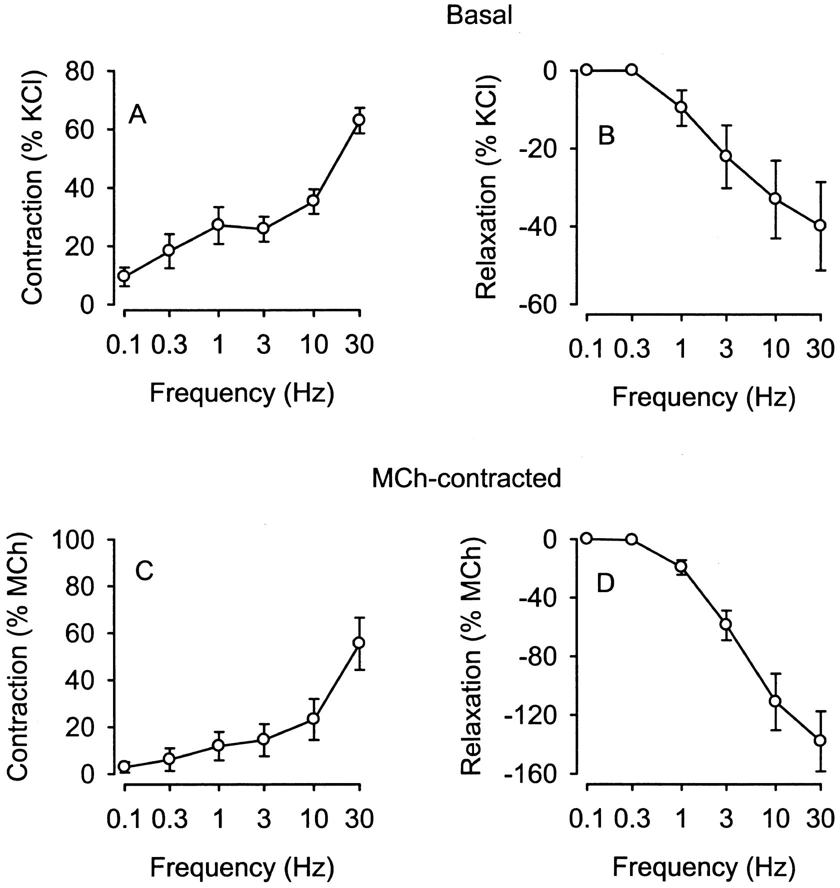

Fig. 5. Frequency-response curves obtained from guinea pig tracheal strips, derived from experiments such as that shown in Fig. 4. (A) Contractile and (B) relaxation responses obtained from strips under basal tone. The strips develop spontaneous tone, which allows relaxation responses to be developed. These results are normalized in terms of a reference response to 120 mM KCl, which was applied to the tissue at the beginning of the experiment. The KCl was not present at the time the frequency-response determination was conducted. (C) Contraction and (D) relaxation responses obtained from strips (from the same animal as A and B) that had been contracted with MCh (3×10^{-7} M; see Fig. 4). The responses are normalized with respect to the force induced by MCh. Note in panel D that the preparations relax beyond the level of MCh-induced force, due to the presence of spontaneous tone in the strips. Modified from Fedan (2001), with permission.

dow" that allows the effects of the excitatory and inhibitory transmitters to be expressed. In preparations such as guinea pig trachea, which develop spontaneous tone, the effects of the inhibitory transmitters can be visualized without inducing further tone; the results under these conditions are very similar to what is observed when tone is induced pharmacologically (Fig. 5). In preparations lacking basal tone, the application of a contractile agent such as methacholine will permit relaxant responses to be seen. In preparations at basal tone, relaxations to several higher frequencies of stimulation may relax the preparations to the level of passive force (i.e., the force that would be observed in the presence of indomethacin or a smooth muscle relaxant). An advantage of using a precontracted preparation is that the relaxation responses will seldom reach the level of passive force. One should remember when using an agent to precontract a preparation that the agent itself may affect neurotransmission. For example, added methacholine would be expected to inhibit prejunctionally the release of acetylcholine from cholinergic, parasympathetic nerves (Schultheis et al., 1994). The use of KCl to induce force could disrupt action potential propagation.

10. Modulation of airway smooth muscle reactivity by epithelium

Removal of the epithelium from airway preparations alters the reactivity of the smooth muscle to contractile and relaxant drugs (Flavahan et al., 1985; Hay et al., 1986). This phenomenon is widespread throughout mammalian species, including humans, and occurs in all generations of larger airways that have been examined. Several mechanisms are involved, and they have been reviewed (Fedan et al., 1988; Farmer & Hay, 1991; Goldie & Hay, 1997; Folkerts & Nijkamp, 1998). In strip or ring preparations of airways from which the epithelium has been removed, an increase in reactivity to contractile agonists occurs and contractile responses to EFS are heightened. An unidentified, nonprostanoid, non-nitric oxide substance known as epithelium-derived relaxing factor (EpDRF) is thought to mediate the increase in reactivity that is not otherwise explained by the loss of a diffusion barrier when the epithelium is removed. Thus, the integrity of the epithelium in an in vitro airway smooth muscle preparation can affect the responses to exogenous agents or EFS. Care is needed to preserve the integrity of the epithelium during preparation of the tissue to achieve the desired outcome. Alternatively, when removal of the epithelium is desirable, the effectiveness of the method used should be verified histologically. A variety of methods have been used to remove the epithelium, and they have been adapted to the size of the airway being studied. In large airways, rubbing the surface with a cotton-tipped applicator is effective, but other gentle abrasives are equally effective. Tiny brushes inserted into the lumen have been used to remove the epithelium from ring segments of small bronchi (Szarek, 1994). It is important that the epithelium be removed without damaging the smooth muscle. A pharmacological marker for testing the success of epithelium removal has been reported. In guinea pig tracheal strips arachidonic acid elicits relaxation in the presence of the epithelium and contraction in strips from which the epithelium had been removed (Farmer et al., 1987). The use of arachidonic acid as a marker for successful epithelium denudation has not been pursued further.

11. Isolated, perfused trachea preparations for measuring contraction and relaxation of airway smooth muscle

The use of tracheal and bronchial strips and rings affords the opportunity to obtain many tissue samples for study from each animal, thereby allowing for a paired experimental design and high data throughput. However, the preparations are no longer structurally representative of their state in situ. All surfaces of the preparation are exposed to agents applied to the organ bath, and effects that might depend on an “intact” airway or that otherwise are dependent upon the surface of exposure to an agent cannot be evaluated easily. The first attempt to study the properties of “intact” airways in vitro was reported by Farmer and Coleman (1970), who mounted guinea pig tracheas on an apparatus with which intraluminal pressure responses to EFS and the effects of blocking drugs could be measured. In their apparatus the lumen was filled with PSS, at constant volume under static conditions, and agents were added to the extraluminal (outer) bath. While it yielded very interesting data, this apparatus did not permit delivery of agents to the luminal fluid; hypoxia in the lumen and accumulation of substances from the epithelium could affect the results. More recently, the use of the isolated, perfused trachea has been reported (Munakata et al., 1988b; Munakata & Mitzner, 1991); its advantage over the preparation devised by Farmer and Coleman is the ability to deliver agents and oxygenated PSS to the lumen of the airway, as well as to the extraluminal bath, while measuring responses to the agents. When agents are added to the extraluminal bath, they have free access to the smooth muscle, which is located on the surface of the trachea, at least in small mammals. In order for agents to reach the smooth muscle when they are added to the luminal perfusate they must first cross the epithelium, a notable diffusion barrier. Thus, a comparison of responses after addition to the intraluminal bath vs. the extraluminal bath yields much information about epithelial integrity and about the ways in which the epithelium is involved in regulating reactivity of the underlying smooth muscle.

A schematic of the principle used by Munakata et al. 1988a, 1988b, 1990), our laboratory (Fedan et al., 1990, 1999; Fedan & Frazer, 1992), and others (Hjoberg et al., 1999) is shown in Fig. 6. A measured length (e.g., 4.2 cm) of the trachea is removed from the animal, cleaned, and mounted on the holder. When the trachea is mounted on the holder, indwelling cannulas containing side holes are inserted into the lumen [see Munakata et al. (1988b) and Fedan & Frazer (1992) for the dimensions of the holder’s key components; Fig. 7)]. The purpose of these indwelling cannulas is to elevate fluid resistance; in their absence the fall in pressure along the length of the trachea would be very small and difficult to measure. Great care is used to make sure that the epithelium is not damaged while mounting on the holder. The holder contains rigid guides to allow the cannulas to slide into the lumen without damaging the epithelium. After tying the segment to the holder, the trachea is restored to its in situ length. The holder and mounted trachea are placed into a bath (the extraluminal bath) containing MKH solution, and the trachea is perfused

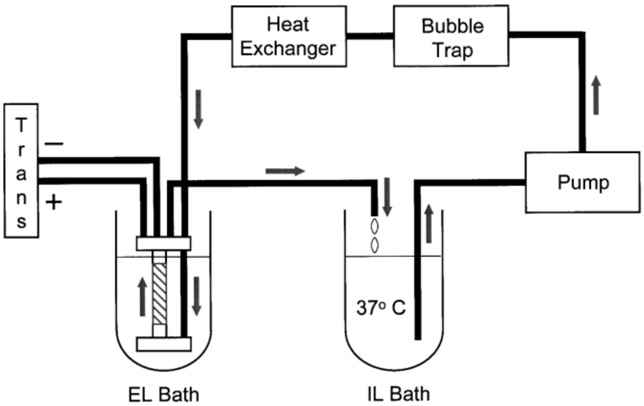

Fig. 6. Isolated, perfused trachea apparatus. The tracheal segment (hatched region) is attached to the perfusion holder and placed in the extraluminal (EL; serosal) bath containing MKH solution maintained at 37°C. The mucosal surface of the trachea is perfused with MKH solution, which is pumped to the holder from a separate intraluminal (IL) bath, also maintained at 37°C. The intraluminal perfusate recirculates back to the IL bath. The arrows indicate the direction of fluid flow. Indwelling pressure cannulas are connected to the positive and negative ports of a differential pressure transducer (Trans). A heat exchanger restores the temperature of perfusate to 37°C before it enters the holder. A trap is necessary to prevent entry of bubbles into the holder; at the low pressures of the system, bubbles create disruptive artifacts.

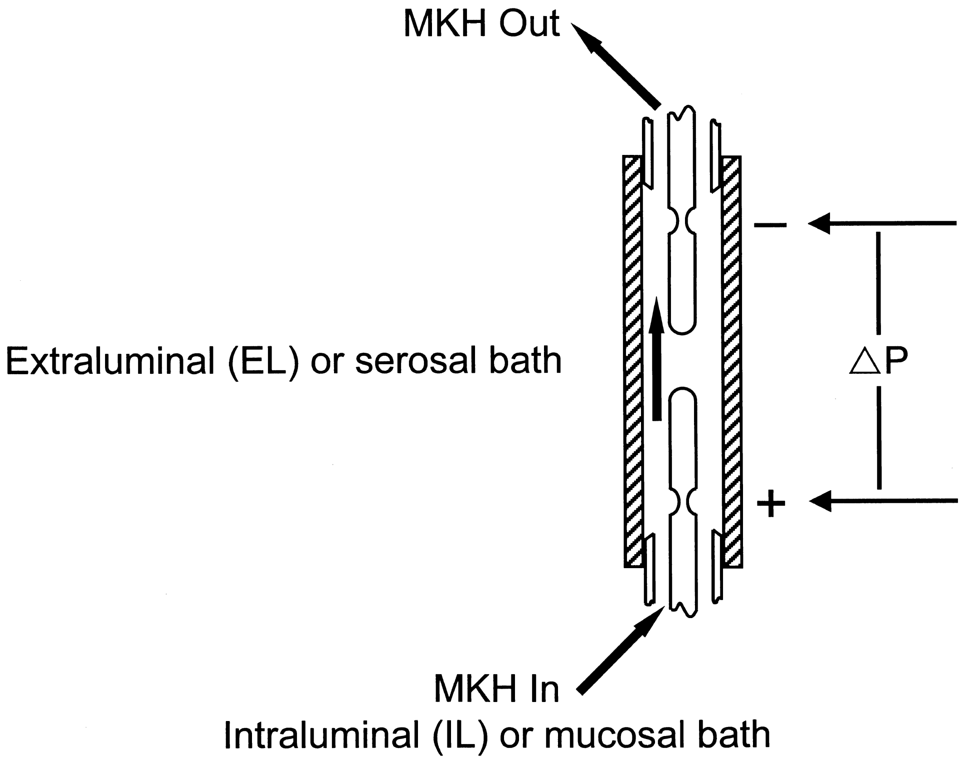

Fig. 7. Details of the holder used in the isolated, perfused trachea apparatus (Fig. 6). After the trachea (hatched) is tied to the holder, indwelling cannulas with side holes become inserted into the lumen. The cannula at the inlet (+) is connected to the positive side of a differential pressure transducer, while the cannula at the outlet (–) is connected to the negative side of the transducer, for the determination of inlet minus outlet pressure difference (△P). With constant flow of MKH through the tracheal lumen, △P varies with fluid resistance, which is a function of internal tracheal diameter. A decrease in tracheal diameter due to contraction of the smooth muscle will increase △P; relaxation decreases △P.

with MKH solution from a separate bath (the intraluminal bath). The side holes in the indwelling cannulas allow sampling of the pressure, and the cannulas at the inlet and outlet ends of the holder are attached to the positive and negative sides, respectively, of a differential pressure transducer to allow measurement of the inlet minus outlet pressure difference (△P). A pump is used to perfuse MKH solution through the tracheal lumen. When the smooth muscle contracts, decreasing diameter, fluid resistance and △P increase; likewise, fluid resistance and △P decrease when the smooth muscle relaxes. The hydrodynamics of the system have been modeled mathematically (Munakata et al., 1990). The apparatus is arranged so that during flow the transmural pressure difference is set to zero. Without this precaution the trachea could be distended by flow through the lumen, or it could collapse if the configuration of the system is such that the transmural pressure is negative, inside with respect to the outside, even with flow occurring. With transmural pressure set to zero, the smooth muscle will be placed at its in situ length under basal conditions. In the work of Munakata et al. the flow rate through the lumen was adjusted at the beginning of each experiment to achieve a desired endpoint value for △P under basal conditions. In our laboratory, the flow is kept the same in every experiment, and △P rises to whatever level is given by the diameter of the smooth muscle at rest, so as to ensure constant kinetics of agent delivery to the trachea.

The guinea pig trachea is long enough that, with indwelling cannulas inserted, a measurable fall in pressure occurs along its length. The technique is amenable for use in the rat trachea (Siegel et al., 2000). It has been possible to perfuse the mouse trachea without the need for indwelling cannulas; the diameter is small enough to allow absolute transmural pressure (not △P) responses to be measured (Fortner et al., 2001). We are not aware of any studies in which the airways of larger animals have been prepared for perfusion studies along the lines being described here. The fundamental problem is the difficulty of measuring changes in △P or absolute pressure in large diameter airways. Likewise, perfusion of short airway segments (e.g., extrapulmonary bronchi and higher generations of airways) while measuring pressure responses is not apt to be fruitful.

Another approach, devised by Pavlovic et al. (1989) for studies on rat trachea, has also been used for studying responses of perfused guinea pig trachea (Nijkamp et al., 1993). In this method small pins or hooks are inserted into the wall of the trachea, and isometric contractile and relaxant responses of the airway smooth muscle are recorded. An advantage of this technique over pressure-measuring approaches is that lower perfusion rates can be used, inasmuch as the measurement of a response is not dependent upon the flow of fluid through the lumen. This might be an important factor if shear stress during higher flow rates needed to measure pressure changes (△P or transmural) were to affect responses of the preparation. This possibility has been investigated and it has been found that the EC50 for reactivity to methacholine of the perfused guinea pig trachea was not affected by perfusion rate, whereas the maximum △P response was affected, as would be expected on hydrodynamic grounds (Fedan et al., 1995). Within bounds, the pharmacological properties of tracheas perfused for the measurement of △P are independent of flow rate. A potential disadvantage of measuring contractile force using the pin method is the possibility of leakage of agents from the intraluminal compartment into the extraluminal compartment. In volume terms this would seem to be an insignificant concern. However, larger concentrations of agents are required intraluminally than extraluminally to achieve the same effect (due to the epithelium; see below), and leakage when high intraluminal concentrations are used could give rise to enough of the agent in the extraluminal bath to exert a confounding effect.

Some representative results demonstrate the types of information that can be obtained using the perfused trachea, which are not possible using strip or ring preparations of airways. In guinea pig perfused trachea there are substantial differences in reactivity to contractile and relaxant agonists after they are applied to the extraluminal bath compared to the intraluminal bath. Fig. 8 shows results obtained for contractile responses to methacholine, in which it can be seen that the concentration-response curve for extraluminal application is located to the left of that for intraluminal methacholine. In addition, the maximum response to intraluminal methacholine is less than that obtained with extraluminal methacholine. Clearly, reactivity to methacholine in the extraluminal bath exceeds that in the intraluminal bath. In tracheas from which the epithelium has been removed by passing a small pipe cleaner brush through the lumen before mounting the trachea, both methacholine curves become nearly superimposable, with the intraluminal curve having shifted leftward and upward to the extraluminal curve location. This experiment illustrates the powerful effect that the epithelium has on reactivity, which is due to the diffusion barrier, the effects of EpDRF, and other mechanisms (Munakata et al., 1989; Fedan & Frazer, 1992).

The perfused trachea preparation has been very helpful for examining the effects of altered osmolarity on the airways. These studies are relevant to the understanding of the mechanisms of exercise-induced asthma, in which the osmolarity of the airway surface becomes increased as a result of evaporative water loss, causing bronchoconstriction in asthmatic but nonasthmatic patients (Freed & Davis, 1999; Anderson & Daviskas, 2000). Fig. 9 illustrates the effect of elevated osmolarity on perfused guinea pig trachea, and these results confirmed the seminal observations by Munakata et al. (1988). After contracting the trachea with methacholine to induce tone, elevation of intraluminal osmolarity results in an osmolar concentration-dependent relaxation of the smooth muscle. The figure

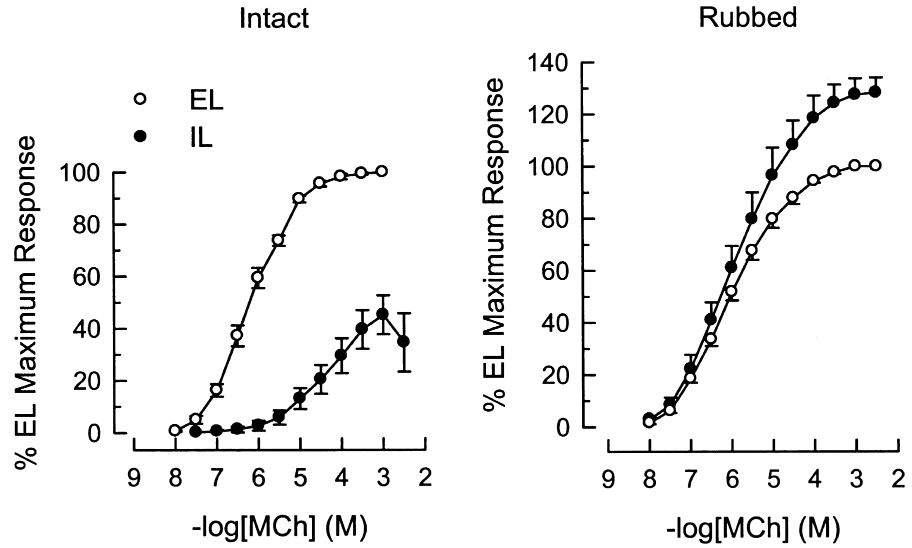

Fig. 8. Cumulative concentration-response curves for methacholine (MCh) from guinea pig isolated, perfused trachea after application to the extraluminal (EL) or intraluminal (IL) baths. The left panel shows the results from epithelium-intact tracheas (Intact); the right panel shows the results from tracheas from which the epithelium was removed by inserting a cotton-tipped applicator through the lumen before mounting the trachea to the holder (Rubbed). In intact tracheas, the IL concentration-response curve is displaced significantly to the right of the EL curve (IL EC50/EL EC50 = 818). The maximum response of the IL curve is significantly less than that for the EL curve. In rubbed trachea, the IL curve is shifted leftward from its original location and the maximum response is increased. In these figures the results have been normalized. The IL responses are quantified in relation to the EL responses obtained from the same trachea and are expressed as a percentage (% EL maximum response). Reproduced by permission, from Jeffrey S. Fedan, Long-Xing Yuan, Victoria C. Chang, Joseph O. Viola, Deborah Cutler and Loreen L. Pettit, "Osmotic Regulation of Airway Reactivity by Epithelium", *Journal of Pharmacology and Experimental Therapeutics*, vol. 289, no. 2, pp. 901–910, May 1999.

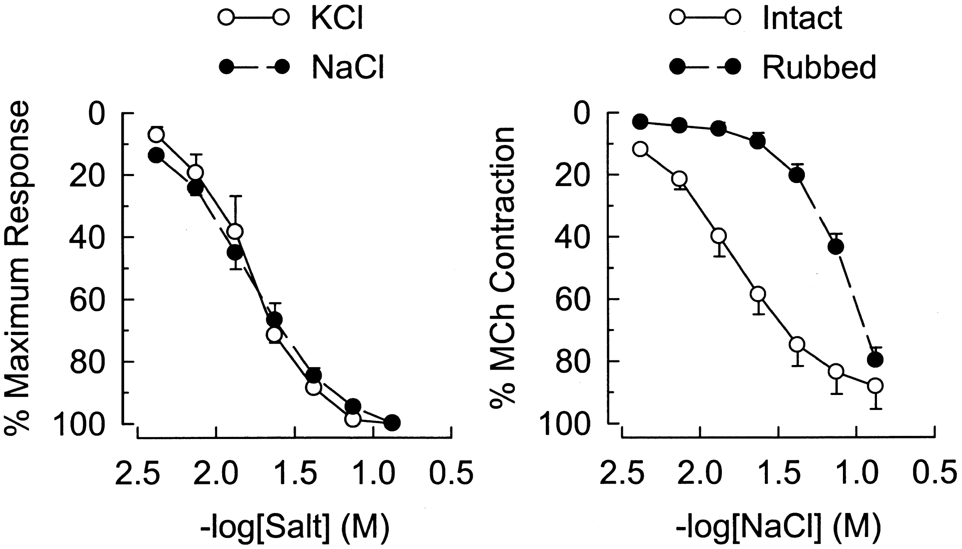

Fig. 9. Cumulative concentration-response curves showing the effects of intraluminally applied hyperosmolarity in guinea pig isolated, perfused trachea. The preparations were contracted with extraluminal methacholine (MCh; 3 × 10^{-7} M; EC50) to induce tone. (Left) In epithelium-intact tracheas, NaCl and KCl added to elevate osmolarity are equipotent in causing relaxation. (Right) The dependence of the relaxation (left panel) on the presence of the epithelium is shown. After removing the epithelium before mounting the trachea to the holder (Rubbed), the concentration-response curve for NaCl is displaced significantly to the right of the curve obtained from the epithelium-intact tracheas (Intact). These findings indicate that the relaxant response to elevated osmolarity is dependent upon the epithelium and involves the release of EpDRF. Reproduced by permission, from Jeffrey S. Fedan, Long-Xing Yuan, Victoria C. Chang, Joseph O. Viola, Deborah Cutler and Loreen L. Pettit, "Osmotic Regulation of Airway Reactivity by Epithelium", *Journal of Pharmacology and Experimental Therapeutics*, vol. 289, no. 2, pp. 901–910, May 1999.

shows results obtained using NaCl and KCl to elevate osmolarity; however, the effect shown is independent of the agent used to raise osmolarity. It may be surprising to the reader that KCl caused relaxation. Ordinarily KCl elicits contraction of smooth muscle following deplarization of the membrane potential. The application of KCl to the extraluminal bath, however, does cause contraction of the muscle. The role of the epithelium in the relaxation responses to intraluminal hyperosmolarity was made evident by performing experiments using epithelium-denuded tracheas. Fig. 9 shows that the relaxation response to NaCl was inhibited in epithelium-free tracheas. These and similar experiments using other osmolytes demonstrate that the relaxation response to elevated intraluminal osmolarity is epithelium-dependent and is mediated neither by a prostanoid nor nitric oxide, but by EpDRF (Munakata et al., 1990; Fedan et al., 1999). We have observed that epithelium-dependent relaxation responses are produced when challenging the trachea with extraluminal hyperosmolarity. In toto, the ability to evaluate phenomena such as these for polarity of effect reveals some distinct advantages of the perfused trachea preparation for studying the relationship between airway epithelium and smooth muscle.

Perfused airway preparations are amenable to studies using EFS to release endogenous transmitters (Fig.10). Platinum electrodes placed alongside the trachea in parallel to its long axis allow convenient delivery of electric impulses. In our hands there is very little, if any, spontaneous tone in perfused guinea pig trachea; indomethacin has virtually no effect on basal △P values, in contrast to the large reduction in force it produces in tracheal strips (this immediately suggests that prostanoid production is stimulated during preparation of the strips). The lack of effect of indomethacin in perfused trachea may reflect the gentler treatment of the airway as it is prepared for study. Therefore, examination of relaxant responses to EFS requires the use of a contractile agonist to induce tone. Neurogenic responses of the perfused trachea resemble in profile those obtained for strips, and a transition of response shape with increasing frequency also occurs (see above) (Fedan et al., 1999). Fig. 10 illustrates the frequency-dependence of contractile responses. The figure also shows that EpDRF, released by perfusing the trachea with hyperosmolar MKH containing elevated NaCl concentration, inhibited neurogenic responses.

12. Epithelial bioelectric measurements in perfused trachea

A primary physiological function of the respiratory epithelium is ion transport. Fluid absorption from the apical surface of the epithelium and maintenance of the airway surface liquid are accomplished through the concerted actions of ion channels and transporters located on the apical and basolateral epithelial cell membranes (Boucher et al., 1982; Clarke & Boucher, 1993). In most mammalian respiratory epithelium Na^+ and Cl^- channels are located on the apical membrane, whereas K^+ channels, the Na^+ -K^+-pump, and the Na^+ -K^+ -2Cl^- cotransporter are localized on the basolateral membrane (several ion channel subtypes

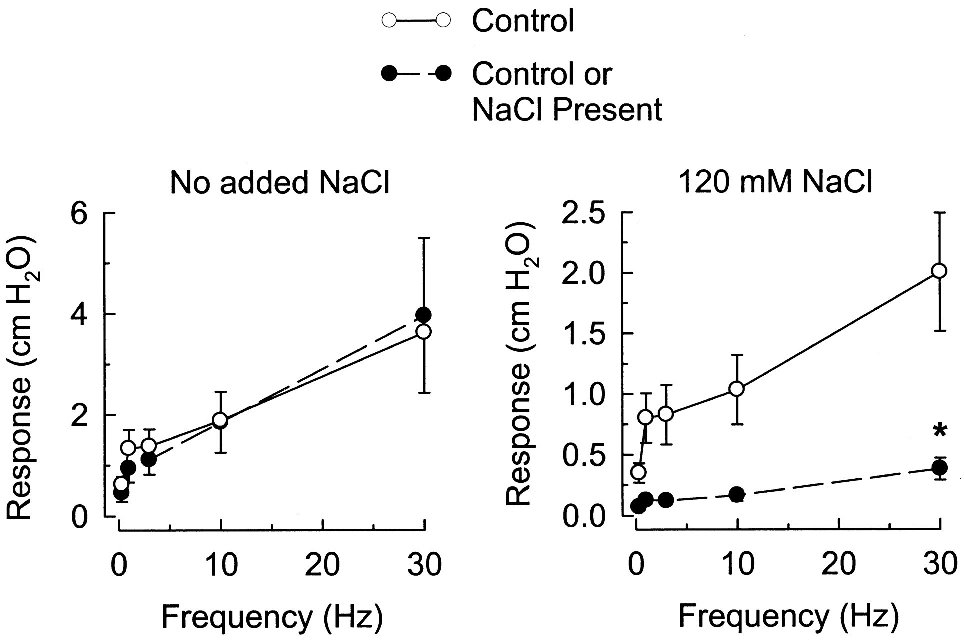

Fig. 10. Frequency-response curves obtained from guinea pig isolated, perfused trachea containing intact epithelium. This figure depicts typical frequency-response curves for the initial contractions (see Fig. 4), as well as the effect of raising the osmolarity of the intraluminal perfusate on the responses. Osmolarity was increased to cause the release of EpDRF. (Left) Two consecutive frequency-response curves in control tracheas showing the reproducibility of the responses in the absence of any treatment. (Right) In order to examine the effects of EpDRF on neurogenic contractions, the tracheal lumen is perfused with 120 mM NaCl during the second of two consecutive frequency-response determinations. This results in an inhibition of the contractile responses. The inhibition by KCl is abrogated in epithelium-denuded tracheas.

exist, but will not be discussed further). Concurrent activity in these ion transport pathways results in a transepithelial potential difference (V_T), with the apical bath negative with respect to the basolateral bath, and changes in the activity of one or more of the channels or transporters give rise to measurable alterations in V_T. While probing the potential relationship between hyperosmolarity-induced relaxation responses of the perfused trachea and epithelial ion transport in perfused guinea pig trachea, we observed that amiloride, a Na^+ channel blocker, and 4,4′-diisothiocyano-2,2′-stilbene disulfonate (DIDS), a Cl^- channel blocker, inhibited EpDRF-mediated relaxation responses (Fedan et al., 1999). Therefore, it was decided to examine the relationship between epithelial bioelectric events and the EpDRF-mediated relaxation response to hyperosmolar challenge using a novel preparation.

This preparation utilizes a tracheal perfusion holder as described above, except it is fashioned from plastic rather than stainless steel to prevent current flow except at desired sites needed to make measurements (Dortch-Carnes et al., 1999). In places where stainless steel must be used, the part is painted with nail polish to provide electrical insulation. The side holes of each indwelling cannula serve as point sources for the measurement of pressure, in order to measure $\triangle P$ as before (see above), and they also serve as point sources for the measurement of V_T at the proximal and distal ends of the trachea. Ag/AgCl calomel half cells, one designated as serosal and placed outside the trachea in the extraluminal bath and others (mucosal) connected in the lines between the inlet and outlet cannulas to the differential pressure transducer, are used to measure the V_T. Each calomel half cell is in contact with the fluid bathing either the extraluminal or intraluminal surfaces of the trachea by means of voltage electrode cartridges filled with 3 M KCl in melted 4% bacteriological agar. The V_T is equal to the sum of the extraluminal, intraluminal, and offset potentials. Electrodes that are matched to less than 2 mV are used, and the remaining offset is compensated for electronically. This preparation allows the simultaneous measurement of changes in $\triangle P$ and V_T in response to challenge of the trachea with agents applied to the intraluminal or extraluminal bath.

The application of intraluminal amiloride causes a depolarization of V_T, which is indicative of functional Na^+ channel activity in the preparation (Dortch-Carnes et al., 1999). Fig. 11 shows results typical of the kind that can be obtained with this preparation. In association with the contraction to extraluminally applied methacholine (to induce tone in the trachea), the epithelium hyperpolarized. Upon stabilization of the responses, the intraluminal perfusing solution was made hyperosmolar with the addition of NaCl or sucrose to the MKH solution. A relaxation response to the osmolytes was accompanied by a depolarization of V_T. The transient contraction to sucrose was accompanied by a transient hyperpolarization of V_T. In further studies not shown here, it was determined that relaxation also occurred in response to hyperosmolar extraluminal solution, and the bioelectric responses preceded the mechanical responses, regardless of the bath to

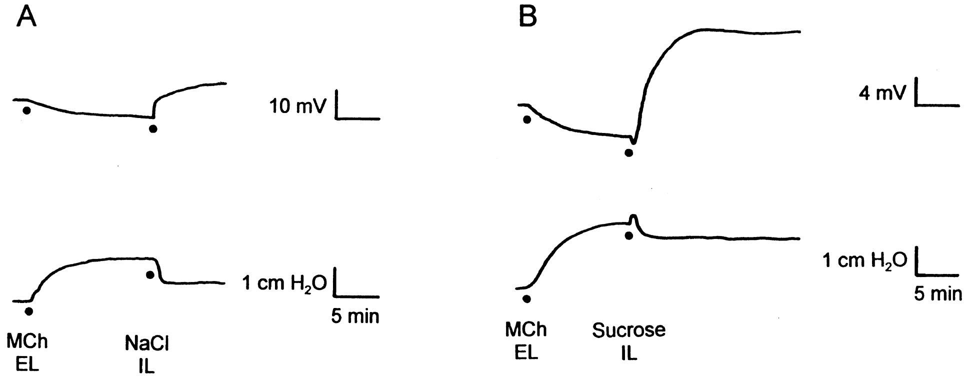

Fig. 11. Simultaneous measurement of bioelectric ($\triangle V_T$; upper traces) and mechanical ($\triangle P$; lower traces) responses of guinea pig isolated, perfused trachea, as described in the text. (A) Extraluminally (EL)-applied methacholine (MCh; 3×10^{-7} M) causes hyperpolarization of V_T in concert with a contraction. Raising the intraluminal (IL) osmolarity with added 120 mM NaCl causes depolarization of V_T and relaxation of the tracheal smooth muscle. A similar experiment using 240 mM sucrose added to the IL bath is shown in B. Note that the transient hyperpolarization of V_T upon addition of sucrose is accompanied by a transient contraction of the muscle. Reproduced by permission, from Juanita Dortch-Carnes, Michael R. van Scott and Jeffrey S. Fedan, "Changes in Smooth Muscle Tone During Osmotic Challenge in Relation to Epithelial Bioelectric Events in Guinea Pig Isolated Trachea", *Journal of Pharmacology and Experimental Therapeutics*, vol. 289, no. 2, pp. 911–917, May 1999.

which the osmolyte was added. Thus, the value of this preparation is that it provides insight into the temporal relationship between epithelial and smooth muscle events as well into the nature of the response of the epithelium to drugs in an "intact" airway. This information will aid in understanding the mechanisms involved in the communication between airway epithelium and smooth muscle.

Two other preparations have been developed to measure the effects of agents on epithelial bioelectric events and smooth muscle responses. In a very novel approach, Croxton 1993) used cable analysis to measure V_T, short circuit current (I_{sc}), and internal diameter of guinea pig tracheal segments while perfusing with PSS. He was able to calculate, from the electrical measurements made, the effect of contractile and relaxant agents on tracheal diameter. We have modified our plastic tracheal holders to accomplish similar ends (i.e., the measurement of I_{sc} and transepithelial resistance) and have been able to assess changes in tracheal diameter more directly from the simultaneous measurement of pressure changes (Johnston, Van Scott, and Fedan, unpublished findings). With the advent of these two techniques, we should look forward to the eventual ability to examine in greater detail the relationship between epithelial bioelectric events, the involvement of ion channels and transporters, and airway smooth muscle behavior.

13. Concluding comments

Although much progress has been made on many fronts, in this postgenomic era we are far from possessing any drugs that cure even a single, noninfectious pulmonary disease. Drugs continue to be developed that target airway smooth muscle in an attempt to alleviate patient symptoms. Therapies not necessarily directed at smooth muscle may still affect its characteristics and behavior, and this possibility must be known. There now exists several methods for in vitro examination of drug effects on airway smooth muscle separately and in relation to respiratory epithelium that, alongside molecular and in vivo approaches, will provide ongoing understanding of airway smooth muscle long into the future.

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