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REVIEW

Studies of Human Airways in vitro: A Review of the Methodology

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The pathophysiology of human airway narrowing is only partly understood. In order to gain more insight in the mechanisms of human lung diseases and potential beneficial therapeutic agents, adequate models are needed. Animal airway models are of limited value since lung diseases such as asthma and chronic obstructive pulmonary disease (COPD) are unique to humans and because the mechanisms of airway narrowing differ between species. Therefore, it is important to perform studies on human isolated airways. We describe the models that have been developed to study airway function in vitro, emphasizing human airway preparations. The easily prepared airway strip and ring preparations are described first. The potential damage during preparation and the interference with airway structure are important drawbacks in these preparations. Lung parenchymal strips, described next, were designed in order to study responsiveness of small airways. However, parenchymal strips are anatomically complex, and responsiveness is determined by the relative amounts of airway and vascular smooth muscle. The lack of reproducibility between species and even within one animal limits their usefulness. Airway tube preparations, in which luminal and serosal stimulation can be separated, enable us to study the modulatory role of the airways epithelium in vitro. Furthermore, airway compliance can be measured. In the isolated perfused lung preparation, relationships between the airways and the vascular system are preserved and the interaction between these two systems can be studied. Weight gain due to fluid extravasation is a problem in this model which has not been used yet to study human lungs in vitro. Next, methodological aspects such as tissue handling and storage, recording of responses, removal of the epithelium, and electrical field stimulation are discussed in some detail. Although animal airways tissue can be studied immediately after removal, human tissue is often obtained with some delay. However, this seems tenable since electron microscopy of lung tissue obtained at autopsy showed that recovery of the preparation occurs during incubation of carbogenated Krebs–Henseleit (K-H) buffer. Dissected airways can be stored overnight in cooled K-H buffer until up to 55 hr after resection without losing viability. Commonly used physiological salt solutions which bath the tissue contain osmotic molecules, ions important for contractility, glucose as a substrate, and a bicarbonate-carbon dioxide buffer. In studies of isolated perfused lungs, a colloid should be added in order to prevent edema. The responses of isolated airways strips and rings are recorded under isometric or isotonic conditions. Smooth muscle contraction in vivo, however, is auxotonic; the elastic load on the smooth muscle increases during airway narrowing. In perfused airway tubes responsiveness is measured under auxotonic conditions as a change in perfusion pressure or flow. Next, removal of epithelium from isolated airways is discussed. Although mechanical denudation is widely used, more physiological methods that mimic the epithelial damage found in asthma may well be preferable and these methods are described in some detail. Finally, the methodology of electric field stimulation (EFS) is described. EFS is delivered via electrodes suspended in the organ bath. According to the stimulus parameters chosen, autonomic nerves or smooth muscle cells are stimulated. An important side effect of EFS is the generation of oxygen radicals in carbogenated K-H buffer which may alter airway tone directly, or oxidize agonists added to the organ bath. It is concluded that although our knowledge of the pathophysiology of airway disease is rapidly increasing, the role of the bronchial circulation is poorly understood. Therefore, the development of a method to study the interaction between the ventilatory and the vascular systems in the isolated human lung is a major challenge.

Keywords: Isolated airways; Airways smooth muscle; Airway epithelium; Electrical field stimulation; Airway responsiveness; Bronchial circulation

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Introduction

The pathophysiology of human airway narrowing that characterizes asthma and chronic obstructive pulmonary disease (COPD) is only partly understood. Inflammation of the lung, especially of the airways, is found both in asthma (Djukanovic et al., 1990) and in COPD (Cosio et al., 1980) and may play a pivotal role in the development of bronchial hyperresponsiveness in asthma (Juniper et al., 1981; Cockcroft, 1988) and COPD (Yan et al., 1985; Bahous et al., 1985). In order to gain more insight into the underlying processes of human lung diseases, animal models have been developed. In most of these animal models, the immediate (IAR) and the late asthmatic reaction (LAR) can be induced by allergen challenge (Michoud et al., 1978; Wanner and Abraham, 1982; Hirshman, 1985; Snapper, 1986; Hayes et al., 1992). However, important differences between species exist (Snapper, 1986). Therefore, it is important to study pathophysiological and pharmacological aspects of human airway disease in human isolated airways. Most in vitro studies of human airways have been performed on tissue derived from smokers with or without COPD who were operated on because of bronchial malignancy. These studies have shown that the sensitivity of isolated airways is not related to the sensitivity to inhaled histamine or methacholine in nonasthmatic subjects (Vincenc et al., 1983; Taylor et al., 1985; Cerrina et al,. 1986; de Jongste et al., 1987a) indicating that airway hyperresponsiveness may not result from an intrinsic abnormality of airways smooth muscle. In the rarely available airways of asthmatic patients increased (Schellenberg and Foster, 1984; de Jongste et al., 1987b; Bai, 1990) as well as decreased (Goldie et al., 1986; Whicker et al., 1988) responses to contractile agonists have been reported.

These apparently contradictory results, that may well be due to methodological factors (de Jongste et al., 1989), and the lack of correlation between in vitro and in vivo measurements illustrate that in vitro data should be interpreted with caution.

Nevertheless, the study of pharmacology of human airways in vitro may provide insight into the pathogenesis of human airway disease. For instance, the role of the airways epithelium (Flavahan et al., 1985; Aizawa et al., 1988; Fedan and Frazer, 1992) and the nonadrenergic, noncholinergic (NANC) nervous system (Richardson and Béland, 1976; de Jongste et al., 1987c; Ellis and Undem, 1992) in airway responsiveness have been studied in detail in isolated airways. Furthermore, studies in vitro offer the opportunity to test the effects or side effects of novel pharmacological compounds potentially acting on lung tissue. The relaxant effects of the potassium channel opener cromokalim (Cortijo et al., 1992), originally developed for the treatment of hypertension, and of the phosphodiesterase inhibitors rolipram and SK&F 94120 (Belvisi et al., 1992a) on human isolated airways are examples of such in vitro studies.

A variety of models for animal and human isolated airways in vitro has been described since Williams' (1840) demonstration of the contractile mechanisms in isolated lungs. This review will briefly discuss these models, their historical backgrounds, their applications, and their restrictions. Furthermore, methodological aspects such as preparation and overnight preservation, epithelium removal, and electrical field stimulation are discussed, emphasizing human airways.

Isolated Airway Preparations

Airway Strip and Ring Preparations

In 1912 an isolated large airway preparation was described by Trendelenburg. Isotonic recordings were made in bovine tracheal rings with or without cartilage, and the bronchodilator effects of caffeine, adrenaline, and atropine were demonstrated (Trendelenburg, 1912). In order to measure the responses of tracheal muscle of small animals in vitro, Castillo and De Beer (1947) used a chain of 10–12 tracheal rings. By means of the additive responses of the rings in the chain preparation, they were able to demonstrate bronchoactive effects of various drugs. The same principle was applied to human central bronchi (Hawkins and Schild, 1951; Rosa and McDowell, 1951). However, the tracheal chain is a laborious preparation that requires a lot of airway tissue, and each connecting knot is a potential source of tissue damage and mechanical instability. Several years later, spirally cut airway strips from animals (Patterson, 1958; Constantine, 1965; Persson and Ekman, 1976) and humans (Persson and Ekman, 1976; Brink et al., 1980; Goldie et al., 1982) were described. These were easier to prepare, but the preparation also caused tissue damage. The development of sensitive transducers made it possible to record isotonic shortening or isometric force development in strips or rings of dissected animal (Persson and Ekman, 1976; Hooker et al., 1977; Advenier et al., 1985) and human (Persson and Ekman, 1976; Finney et al., 1985; de Jongste et al., 1985; Jongejan et al., 1988) small airways. In Figure 1, the different airway strip and ring preparations are shown schematically. Theoretically, airway ring preparations have advantages over airway strips: the contraction of a ring is directly related to airway narrowing and, furthermore, the configuration of the smooth muscle bundles is largely preserved. A practical advantage is that only a small piece of tissue is needed. A disadvantage of the airway strips and -ring preparations is the inability to

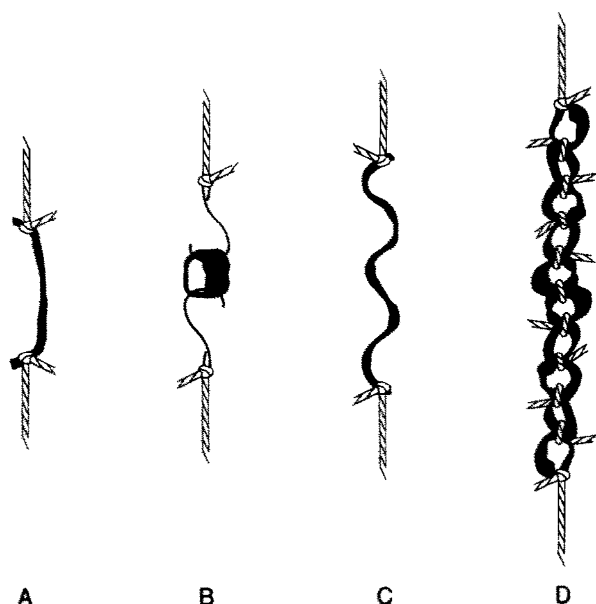

Figure 1. Schematic representation of the airway strip (A), airway ring (B), spiralized airway strip (C), and airway chain preparation (D).

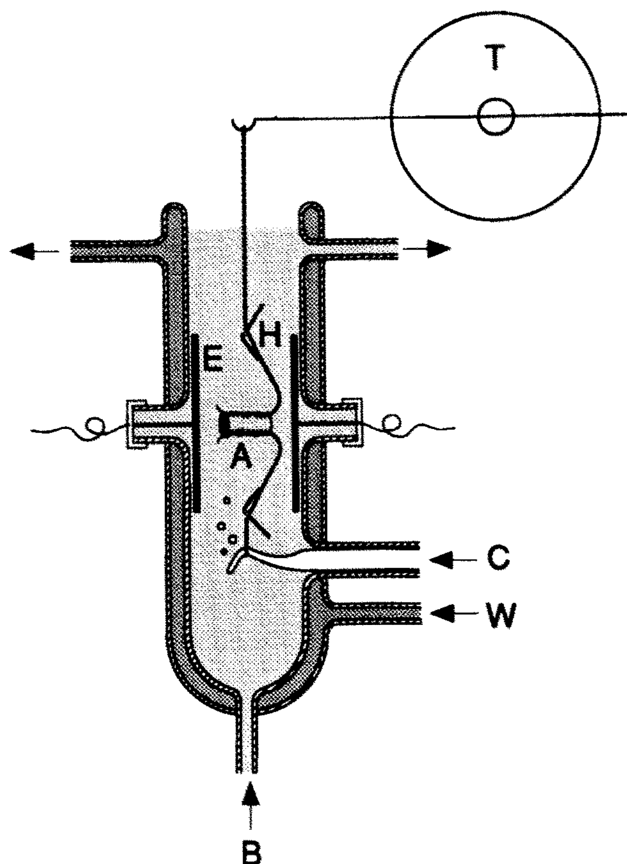

Figure 2. Schematic representation of an airway ring preparation mounted in a double-jacketed, glass organ bath. A, airway preparation; B, buffer supply; C, carbogen supply; E, platinum electrodes; H, stainless steel hookes; T, isotonic transducer; W, warm (37°C) water supply.

stimulate the mucosal or the serosal side selectively. Figure 2 displays an airway ring preparation in a conventional double-jacketed organ bath.

Lung Parenchymal Strips

The lung parenchymal strip was developed by Lulich et al. (1976) as an in vitro preparation to evaluate the actions of drugs on peripheral airways. Thin strips (approximate dimensions 20 × 3 × 3 mm) were dissected from a lung lobe and studied in a conventional organ bath. The preparation has been widely used in studies of both laboratory animal (Drazen et al., 1978; Chand et al., 1979; Kleinstiver and Eyre, 1980; Omini et al., 1990) and human (Ghelani et al., 1980; Finney et al., 1984; Saga et al., 1984) lungs. Marked differences in responsiveness to various agents of the smooth muscle of central airway strips and lung parenchymal strips were found, and it was assumed that the drug-induced effects in parenchymal strips reflected the responses of smooth muscle of small airways present in the bronchioles and alveolar ducts (Lulich et al., 1976; Drazen et al., 1978; Chand et al., 1979; Ghelani et al., 1980; Finney et al., 1984). However, since the responses of parenchymal strip preparations to sympathomimetic drugs were not consistent between species and even within a single animal, the involvement of nonairway components such as vascular smooth muscle (Mirbahar and Eyre, 1980; Goldie et al., 1980) and, probably, interstitial contractile cells (Kapanci et al., 1974) was suggested. Indeed, Bertram et al. (1983) showed that the type and size of responses of human parenchymal strips to the sympathicomimetic drugs serotonin and noradrenaline depended on the relative amounts of blood vessels and larger airways present in the airway preparation. Thus, noradrenaline will induce a contractile response in parenchymal strips containing more than twice as much vascular smooth muscle as airway smooth muscle, and noradrenaline will induce a relaxation when this ratio is lower than twofold (Figure 3). These relative amounts of the contractile components were determined with stereological analysis, a method that enables estimates of different parameters in a 3-dimensional body (Weibel, 1979), and a large variability in composition was shown between strips obtained from the same lung (Bertram et al., 1983). Similarly, the disparity of responses in different species can be explained by differences in composition; a slice of rat lung will comprise larger airways and blood vessels than would a similarly sized slice of human lung (Eyre and Mirbahar, 1981; Goldie et al.,

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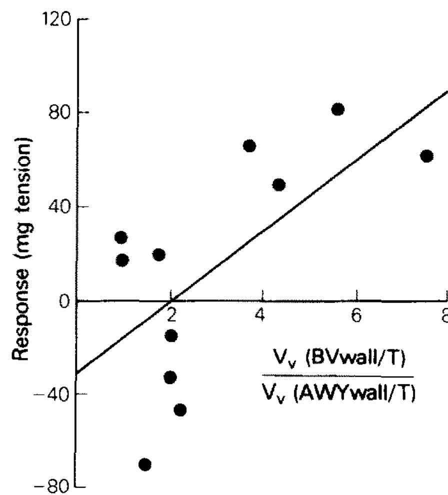


Figure 3. Relationship between the ratio of the volume densities of blood vessel wall to airway wall in tissue (V_v (BVwall/T)/V_v (AWYwall/T), and responses of human isolated lung parenchyma strips to noradrenaline (r = 0.65, $p < 0.05$). (Reproduced with permission of Bertram et al., 1983.)

1984). It appears, therefore, that the anatomical complexity of the parenchymal strip restricts its value as a method to study small airway pharmacology.

Isolated Perfused Airways

The airway epithelium may modulate airway responsiveness through 1) the release of relaxing factors (Farmer and Hay, 1991), 2) the breakdown of agonists (Advenier et al., 1988; Devillier et al., 1988), and 3) acting as a physical barrier (Munakata and Mitzner, 1991). In human airway strips and rings, however, only a 1.5 to 2.5–fold increase in sensitivity to contracting agonists is found after mechanical removal of the epithelium (Raeburn et al., 1986a; Aizawa et al., 1988; Jongejan et al., 1991) probably because the stimulus reaches the smooth muscle not only via the mucosal side but also via the serosal side and the cut surface. Therefore, models have been developed that allow independent stimulation of rodent-intact tracheas and pig and human bronchial segments from the serosal and the mucosal side selectively. These airway "tube preparations" were perfused under conditions of constant flow (Munakata et al., 1988; Mitchell et al., 1989; Yang et al., 1991; Fedan and Frazer, 1992) or constant pressure (Mitchell et al., 1989; Sparrow and Mitchell, 1991; Omari et al., 1993), and responsiveness was measured as a change in perfusion pressure or flow, respectively. In these intact perfused airway preparations, the sensitivity to luminally applied contractile and relaxing agonists was much lower (over 30–fold) than that to serosally applied agonists. These differences were abolished after mechanical rubbing of the epithelium, indicating that the effect was caused by the presence of epithelium.

We developed a similar model to investigate the modulatory role of the epithelium in human peripheral airways (Hulsmann et al., 1992). Human isolated peripheral airway tubes were perfused with Krebs–Henseleit solution at a constant pressure of 6 cm H_2O (Figure 4), and responsiveness was measured as a change in flow. Accurate and reproducible measurements of sensitivity to metacholine were obtained. With this method we demonstrated a much greater modulatory role of the epithelium in human perfused peripheral airways than in peripheral airway strips (Hulsmann et al., 1993a). Apart from studying the modulatory role of the epithelium, airway tube preparations have been used to study other factors that determine airway narrowing such as preload and airway compliance. The effect of preload on airway narrowing has been studied in rabbit, pig, and human isolated airways. The transmural pressure in closed airway segments was varied between −10 and +30 cm H_2O, and the pressure change to field stimulation was recorded. It appeared that both the presence of cartilage and the transmural pressure determine the preload (and hence force) of the smooth muscle (Moreno and Paré, 1989; Sparrow et al., 1992; Figure 5).

Gunst and Stropp (1988) determined pressure–volume relationships in canine bronchi by measuring bronchial transmural pressure changes during inflation and deflation of the airway preparation with K-H solution. The compliance of contracted airways was lower than that of relaxed airways. Large airways contracted with acetylcholine (10^{-3} M) developed pressures >30 cm H_2O only near their maximal volumes, whereas small airways developed similar pressures at a much wider volume range (Figure 6). Furthermore, small airways were able to constrict to closure but large airways constricted only to 30% of maximal volume. These differences are probably due largely to differences in orientation of the smooth muscle tissue and in the amount of cartilage between large and small airways (Gunst and Stropp, 1988).

Isolated Perfused Lungs

The lung is supplied by both the pulmonary circulation and the tracheo-bronchial circulation. The tracheo-bronchial circulation may be important in the pathogenesis of asthma because of its involvement in the influx of inflammatory cells into the airways, in the development of airway wall edema, and in the clearance of bronchoactive mediators and inhaled drugs (Persson, 1986; Deffebach et al., 1987; Deffebach and Widdicombe, 1991; Widdicombe, 1992). In addition,

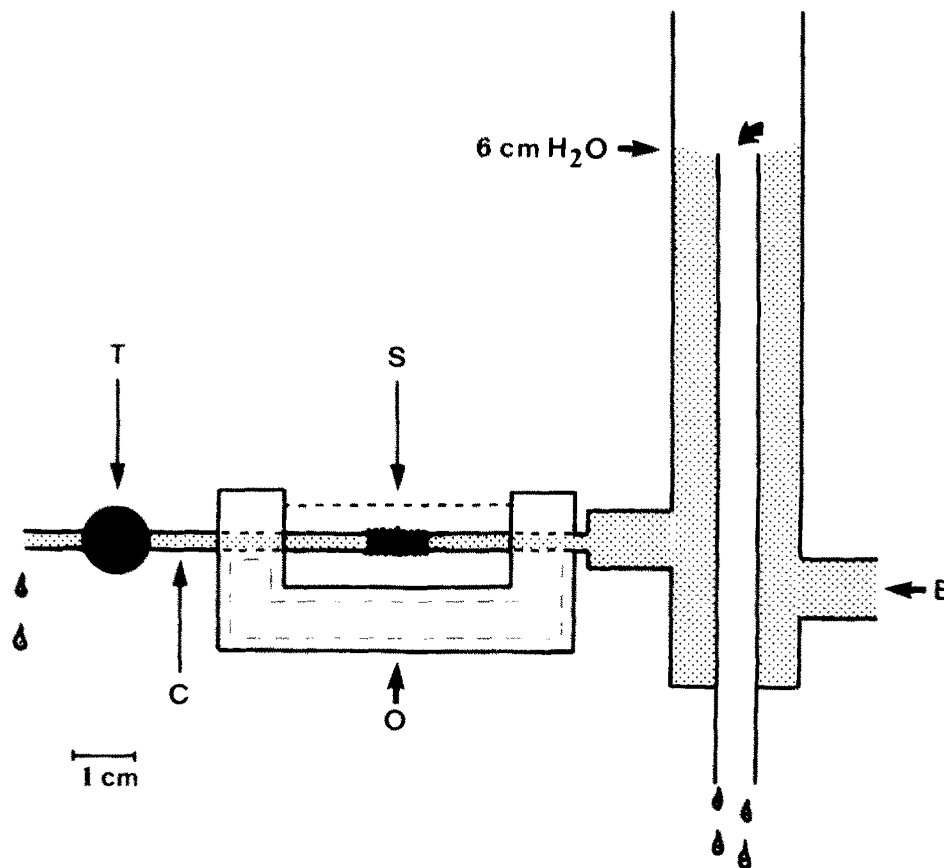
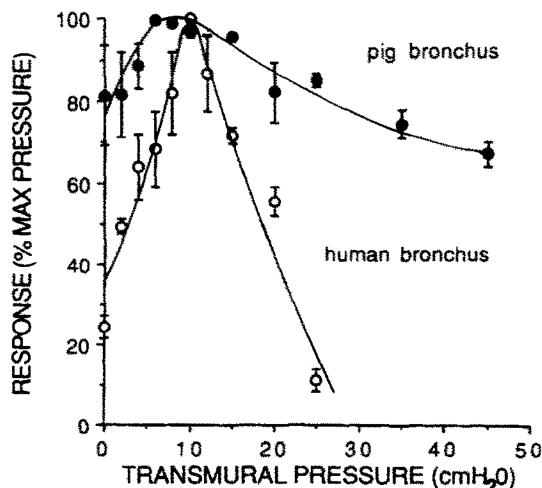


Figure 4. Schematic presentation of the human perfused airway preparation. The transmural pressure is maintained at 6 cm H_2O. B, buffer supply; C, stainless steel cannula; O, organ bath; S, airway segment; T, electromagnetic transducer. (Reproduced with permission of Hulsmann et al., 1992.)

Figure 5. Relationship between transmural pressure and response to EFS of bronchial segments from pig (3 mm i.d.) and human (4 mm i.d.) lung. Closed segments were activated by electrical field stimulation (20 Hz, 0.5 ms, 60 V), and the increase in pressure was recorded via a transducer attached to a T-piece at one end of the preparation. The transmural pressure was changed by introducing different volumes of K-H solution into the segment. Means ± SEM, n = 3–4 animals. (Reproduced with permission of Sparrow et al., 1992.)



hyperemia and hyperpermeability of bronchial vessels may increase airflow resistance and airway responsiveness to bronchoconstricting agents (Persson, 1986; Lockhart et al., 1992). These aspects cannot be studied in isolated airways smooth muscle preparations. In isolated whole-lung preparations, however, relationships between airways and the vascular systems are preserved.

Models of dog, rat, rabbit, and guinea pig perfused and ventilated lungs have been described (Evans and Starling, 1934; West et al., 1964; Maloney et al., 1968; Levey and Gast, 1966; Hauge, 1972; Niemeier and Bingham, 1972; Ryrfeldt and Nilsson, 1978). After anesthesia, animals are tracheotomized, and a cannula is inserted into their trachea and connected to a ventilator. Then the thorax is opened, and heparin sodium is injected into either the right ventricle or intravenously. The pulmonary artery and pulmonary vein or the left atrium are cannulated, and the blood is flushed from the pulmonary circulation with K-H solution at 37°C. The lungs are either left in situ (Wang et al., 1992) or removed from the thorax and placed in a water vapor-saturated glass or perspex chamber warmed to 37°C. During the experiment the lungs are perfused via the pulmonary artery with oxygenated K-H buffer (37°C) containing 4%–5% bovine albumin or with whole blood

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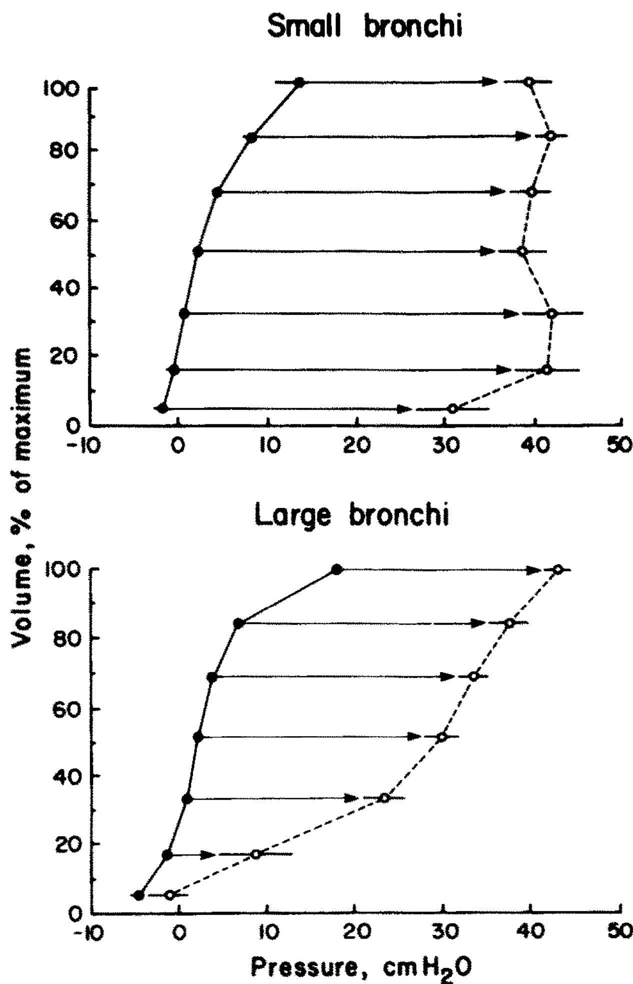

Figure 6. Maximal pressures developed by small ($n = 6$) and large ($n = 7$) bronchi. Closed circles represent passive pressure obtained 1 min after inflation. Open circles represent pressures obtained after contraction with acetylcholine (10^{-3} M). (Reproduced with permission of Gunst et al., 1988.)

under either constant pressure or constant flow conditions. In most studies the preparation is ventilated with preheated and humidified gas by creating a rythmically varying negative pressure (−3 to −12 cm H_2O) in the thorax chamber. Alternatively, positive pressure ventilation has been used (Levey and Gast, 1964; Hauge, 1967; McDonald and Heffner, 1991; Jensen et al., 1992). Weight gain during the experiment, indicating extravasation of the perfusate, is continuously monitored or measured before and after the experiment. In Figure 7 the experimental set-up of an isolated perfused and ventilated guinea pig lung is displayed.

The absence of perfusion of the bronchial circulation and lymphatic drainage are considered as major drawbacks in these preparations. However, after perfusion of the pulmonary artery with fluorescein isothiocyanate (FITC-D, MW 150,000) Kröll et al. (1987) found abundant presence of FITC-D in tracheobronchial tissue. This indicates functioning anastomoses between the pulmonary and the bronchial circulation, and this increases the validity of this model. In order to ensure oxygenation of medium-sized and large airways, Kröll et al. (1986) ventilated the isolated lung with supranormal O_2 tension.

The nonfunctioning lymphatic system, fluid extravasation, and the use of an artificial perfusion medium may lead to a weight gain during perfusion (Fisher et al., 1980; Kröll et al., 1986). Nevertheless, preparations can be used for several hours during which the lung function remains stable (Kröll et al., 1986). The model can be used for the measurement of lung resistance (R_L) and dynamic compliance (C_{Dyn}; Kröll et al., 1986) and for metabolic and pharmacological studies. Furthermore, Wang et al. (1992) showed that it is possible to measure capillary transit time in isolated rabbit lungs by fluorescence video microscopy. No studies in perfused ventilated human lungs have been described, probably because a fresh whole-lung preparation is rarely available.

It might be possible to develop a model for the perfusion and ventilation of human lung lobes or segments. However, the fact that the blood supply of a given ventilatory unit comes from several vascular units (Weibel, 1991) may provide a major problem in preparations of human lung segments.

Preparation and Overnight Storage

Animal airways tissue can be usually studied immediately after removal. Human airway tissue, however, is often obtained with some delay because pathological examinations have to be performed. In case of autopsy there may be even many hours of delay. In a study by Ferguson and Richardson (1978) lung tissue was obtained at autopsy within 5 hr of death. Electron microscopy of epithelium and smooth muscle cells showed swelling of mitochondria and endoplasmatic reticulum, condensation of cell nuclei and blebs in the cell membrane. In addition, in the smooth muscle cells disorganization and clumping of the contractile filaments was seen (Ferguson and Richardson, 1978). These changes were largely reversible after incubation of the tissue in organ baths containing carbogenated K-H buffer solution. Although this may indicate recovery of the preparation from the anoxic period, only brief functional studies were performed by the authors.

Bronchial tissue obtained at thoracotomies seems preferable because the anoxic damage and autolysis can be largely avoided when the tissue is submerged in cooled (0 to 4°C) K-H buffer solution immediately after surgical resection. de Jongste et al. (1985) de-

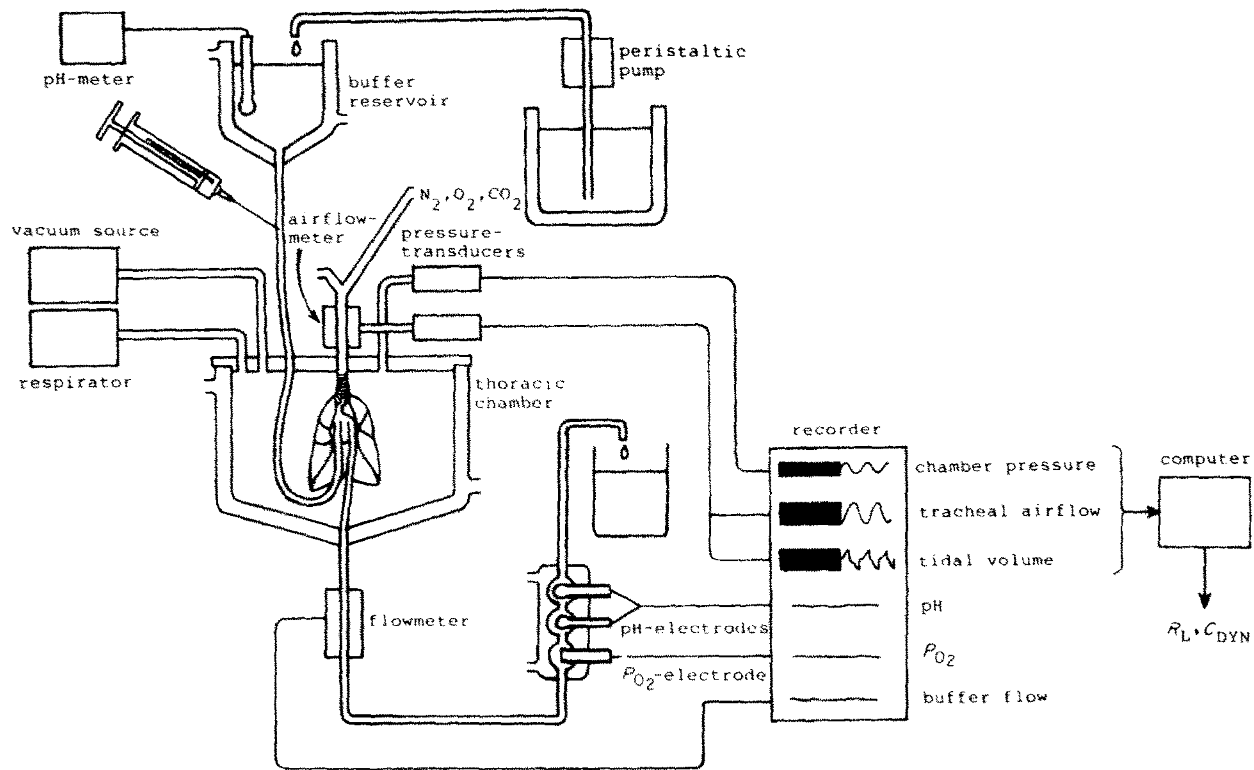

Figure 7. Experimental set-up for studies in isolated perfused lungs. (Reproduced with permission of Kröll et al., 1986.)

scribed a technique for preparation and storage of human lung tissue. After washing to remove blood, bronchi are identified on the cut surface of the excised lung tissue and cannulated with polyethylene tubes. Air is gently inflated to ascertain that an airway, and not a blood vessel, is cannulated. The airway is carefully separated from the surrounding tissue guided by the cannula. Thereafter, blood vessels, lymphatic tissue, and parenchyma are removed from the airway with iris scissors under a binocular preparation microscope (de Jongste et al., 1985). The airway tubes can be cut into helical or transversal strips or rings.

Tissue preparations can be stored overnight in cooled (4°C) Ringer's solution (Hawkins and Schild, 1951) or, preferably, cooled, and carbogenated K-H solution (Brink et al., 1980; de Jongste et al., 1985; Raeburn et al., 1986b). With K-H buffer, the response to methacholine remains unchanged until up to 55 hr after resection (de Jongste et al., 1985). To prevent bacterial overgrowth, we note antibiotics, for example, penicillin (3×10^{-5} g/L) and tobramycin (5×10^{-3} g/L) should be added.

Physiological Salt Solutions

In vitro studies are performed in glass or Plexiglass organ baths filled with a physiological salt solution. Plexiglass organ baths can be a problem because interaction of several drugs with synthetics has been described (Krieglstein et al., 1972).

A physiological salt solution is a solution of inorganic salts in which an isolated organ or tissue survives for some time and displays most of its normal functions. The critical ions in any salt solution are sodium, potassium, calcium, and bicarbonate. Sodium and chloride are the main osmotic ions; potassium, calcium, sodium, and magnesium are important for contractility. Bicarbonate is part of a bicarbonate–carbon dioxide buffer system. Ringer (1883) was the first to use a physiological salt solution in his studies on the frog heart. The first salt solution for mammalian tissues was devised for the heart by Locke (1901). He increased the salt concentration of Ringer's solution to increase the osmotic pressure and he added glucose (0.1%) to improve the survival time of the heart. Tyrode (1910) added phosphate to improve buffering and magnesium to maintain contractility of the smooth muscle preparation. The disadvantage of Tyrode's solution is its tendency to become alkaline and to precipitate calcium carbonate. In the solution of Krebs and Henseleit (1932), a higher concentration of bicarbonate is used, similar to that found in plasma. The solution should be gassed with carbogen (95% O_2, 5% CO_2) to achieve a pH of 7.4. For studies on tissue respiration,

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Krebs (1950) replaced part of the sodium chloride with sodium salts of fumaric-, pyruvic-, lactic-, or glutaric acid as additional substrates apart from glucose. The K-H solution is most widely used. In studies of perfused isolated organs, a colloid should be added to the physiological salt solution in order to prevent edema. Several colloids have been used including dextrans (Grönwall, 1957), polyvinylpyrrolidone (PVP; Ross, 1972) and bovine serum albumin (Schimassek, 1962). Most investigators use albumin, however, disadvantages of albumin are its tendency to lower the pH, to bind calcium ions, and to froth when aerated (Burton, 1975).

Although most physiological salt solutions are gassed with carbogen (95% O_2, 5% CO_2), perfused mammalian organs may need more oxygen than can be provided in this way. Oxygen transport can be increased by using erythrocytes or fluorocarbons (Sloviter et al., 1969; Goodman et al., 1973; Hartmann et al., 1984). Fluorocarbons are inert organic substances in which the hydrogen atoms are replaced by fluorine. They have the capacity to carry more oxygen than can be carried in human whole blood (Burton, 1975). In the perfused ventilated lung, however, addition of an oxygen carrier may not be necessary because during ventilation of the preparation with supranormal O_2 tension, tissue hypoxia does not seem to be a problem (Kröll et al., 1986).

Recording of Responses

Isometric, Isotonic and Auxotonic Recording

Mechanical muscle activity can be measured under isotonic, isometric, and auxotonic conditions. Under isotonic conditions, changes in length are recorded in a muscle to which a constant predetermined load is applied [Figure 8(a)]. Isometric recordings can be made by measuring changes in force of contraction in a muscle preparation which has a constant predetermined length [Figure 8(b)]. When changes in length and changes in force are measured simultaneously in a muscle preparation where the applied load increases while the muscle shortens, this is called *auxotonic* (auxanein (Gr.) = to increase) [Figure 8(c)]. In the early days of research of smooth muscle contractility in vitro, isotonic recording techniques were standard: The preparation was connected to the short arm of a lever, the long arm of which recorded changes in muscle length on a slowly moving kymograph. When isometric transducers became available length–tension relationships in canine tracheal smooth muscle were examined (Stephens and Van Niekerk, 1977; Figure 9). The preparations were stepwise stretched, and the resulting passive load and the total contractile force after EFS were

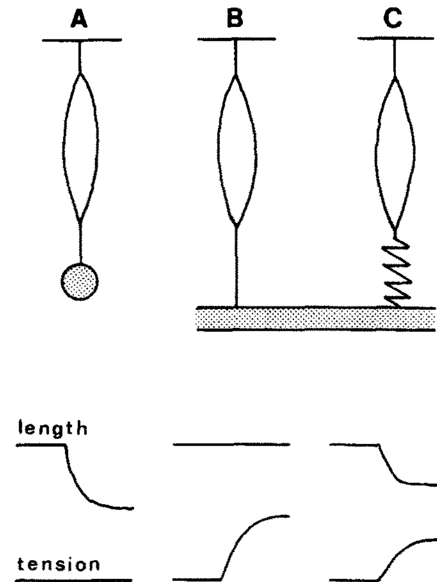
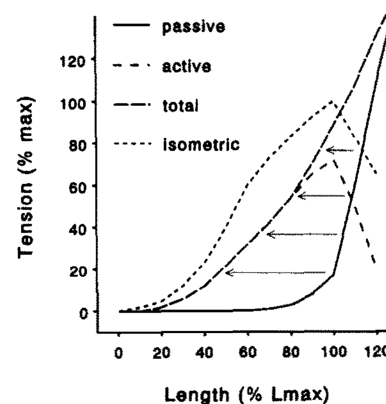


Figure 8. Recording of tension and length changes of smooth muscle obtained under isotonic (A), isometric (B), and auxotonic (C) conditions.

recorded. The active load causing isotonic shortening can be derived by subtraction of resting load from total force generated. The active force development in airway preparations increases with length until an optimal length (L_0 or L_{max}) is reached. The passive tension at L_{max} is only 5% to 10% of the active tension. The horizontal difference between the active and the total tension (arrows in Figure 9) represents the smooth muscle shortening under isotonic conditions. When the muscle is stretched beyond L_{max}, the maximum, active

Figure 9. Length–tension relationships of airway smooth muscle. The active tension curve is obtained by subtracting passive tension from total tension. The isometric curve is also shown. The arrows indicate isotonic smooth muscle shortening at different lengths. (Modified from Stephens and Van Niekerk, 1977.)



tension will decrease. At all muscle lengths, force development in isometric experiments is higher, indicating that in isotonic contraction the smooth muscle does not contract fully (Stephens and Van Niekerk, 1977). This incomplete contraction may be due to thickening of the muscle-hindering diffusion of the neurotransmitter and folding of contractile elements impairing optimal functioning (Michelson and Shelkovnikov, 1976; Stephens and Van Niekerk, 1977). At function residual capacity (FRC) in vivo, the tracheal smooth muscle may be stretched to around L_{max} (Moreno et al., 1986). The experiments of Stephens and Van Niekerk (1977) imply that, for optimal results, isolated airway preparations should be stretched to approximately L_{max} by either applying a weight (isotonic measurements) or increasing baseline length (isometric measurements).

In the rabbit bronchus, Armour et al. (1988) found a correlation between the maximal isometric force generation in response to carbachol and the amount of airway smooth muscle present in the airway preparation. In contrast, the maximal isotonic shortening was not related to the smooth muscle content of the preparation, and it was concluded that isometric measurements are preferable because they represent changes in smooth muscle contraction in response to an agonist more accurate (Armour et al., 1988).

In human small airways, however, only small differences were found between both methods, perhaps because the experiments were performed under near optimal tension and length conditions (de Jongste et al., 1987d). Although both recording techniques allow accurate and reproducible measurements of force generation, airway smooth muscle contraction in vivo is neither isometric nor isotonic, but auxotonic: during narrowing the load against which the smooth muscle shortens increases due to an elastic load provided by the surrounding structures (Moreno et al., 1986; Sparrow et al., 1992). This situation has been simulated in a study performed by Ishida et al. (1990) who measured the effect of elastic loads on smooth muscle shortening in pig isolated airways. It was shown that, at small loads, contractile responses were more or less isotonic, whereas, at large loads, minimal shortening was found indicating a isometric response (Ishida et al., 1990). Thus the size of the elastic load on the airway provided by the airway wall and the surrounding tissue determines the degree to which a contraction is isometric or isotonic.

Pressure or Flow Recording

The responses of perfused airway tube preparations are measured by recording changes in flow rate or perfusion pressure, depending on whether experiments are done under constant pressure or constant flow conditions, respectively. The configuration of the airway is left intact and the mode of contraction is more physiological than that in an airway strip preparation. However, in the constant flow model, the transmural pressure increases during contraction and this will influence the load on the muscle in an elastic (auxotonic) way. In the constant pressure model, airway closure may occur at higher doses of a contracting agonist (Mitchell et al., 1989), and this precludes accurate determination of the pharmacological sensitivity (EC_{50}) of the preparation.

In human peripheral airways perfused at a constant pressure, we were able to avoid airway closure by stretching the airway preparations to 140% of their initial length (Hulsmann et al., 1992).

Other Methods to Record Airway Responses

Other techniques to record airway responses have been developed and are briefly described below. These methods, however, are not commonly used, and their value remains to be established.

High-resolution ultrasonic imaging. In order to visualize airways smooth muscle contraction in vitro, Iizuka et al. (1992) introduced an ultrasonic catheter in porcine and human isolated bronchi. The ultrasound technique produced a three-layer image of the bronchial wall corresponding to the mucosa, cartilage, and adventitia. The muscle could not be distinguished from the mucosa. Dose-dependent responses to acetylcholine could be obtained, and it was found that human bronchus contracts elliptically, not circularly (Iizuka et al., 1992). Because the diameter of the transducer is 1.7 mm, the technique can be only used in airways of at least this size.

Sonomicrometry. Okazawa et al. (1990) measured length changes of canine trachealis muscles in vivo with sonomicrometry. This technique uses the transit time of ultrasound traveling between two piezoelectric crystals as a measure for the linear distance between these crystals. Small (1 mm) piezoelectric transducers were placed in the posterior tracheal wall in parallel with the muscle fibers. Length changes during mechanical ventilation and pressure–volume curves could be obtained. This method may be applicable in isolated large airways as well.

High-resolution computed tomography. With this technique, airways of 1–2 mm diameter can be visualized (Todo et al., 1986), and this method has been used to study carbachol-induced changes in airway dimensions in excised canine lung lobes (McNamara et al., 1992). The degree of airway narrowing could be accurately quantified, and it was shown that airway narrowing after carbachol is greatest in intermediate-sized airways (internal diameter: 2–6 mm).

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Photoelectric recording. Schabert et al. (1980) developed a photoelectric method to record changes in blood vessel diameter. A beam of parallel infrared light is directed at right angles to the blood vessel. The light passing the side of the vessel is a measure of the outer vessel diameter and is detected by a photocell. A decrease in vessel diameter causes an increase in photocell current. In isolated airways, photoelectric methods have been only used to study respiratory ciliary activity (Yager et al., 1980; Tamaoki et al., 1989).

Between-patient and Within-patient Variability

With the above-mentioned recording techniques, accurate and reproducible measurement of parameters such as tissue sensitivity (EC_{50}), maximal contraction, or relaxation and intrinsic (baseline) tone is possible in human airway strips, rings, and tubes (de Jongste et al., 1985; Jongejan et al., 1988; Hulsmann et al., 1992). However, for some purposes an in vitro model should be able to detect between-patient differences. de Jongste et al. (1985) showed that, despite large within-patient variability, significant between-patient differences in airway strips could be shown for EC_{50} and maximal response. In airway tubes, between-patient differences in EC_{50} and intrinsic tone accounted for more than 90% of the total variability (Hulsmann et al., 1992).

The finding of smaller within-patients variability in airway tubes compared to airway strips may indicate that within-patient variability in airway strips are largely due to disturbance of the airway structure during the cutting of the strips.

Intrinsic Tone

Isolated airway preparations may exhibit an intrinsic muscle tone. In guinea pig airways, this tone appears to be dependent on prostanoids and not on intrinsic innervation (Orehek et al., 1975). In human airways, however, the role of prostanoids is unclear since both enhancement (Ito et al., 1985) and reduction (Ito et al., 1989) of intrinsic tone have been described after inhibition of cyclooxygenase. In addition, peptidoleukotrienes may be involved because inhibition of 5–lipoxygenase decreased intrinsic tone (Ito et al., 1989). Mansour and Daniel (1986) expressed the responses of guinea pig tracheas on a scale between maximal relaxation and maximal tension in response to carbachol. It appeared that the responses to exogenous arachidonate were dependent on the intrinsic tone of the airway preparation. When this intrinsic tone was low, contraction was found; when it was high, relaxation was found. Their findings emphasize the importance of the expression of responses on a scale that displays the maximal active contractile range (MACR) in order to be able to compare responses of different airway preparations. Monitoring intrinsic tone is also relevant when EFS is used. With high intrinsic tone, EFS may predominantly give relaxations, whereas with low tone, contraction will result.

We routinely determine maximal contraction to exogenous cholinergic stimulation at the beginning of experiments, and maximal relaxation to β-adrenoceptor stimulation and calcium free buffer after completion of the experiments. Alternatively, a supramaximal dose of theophylline, sodium nitroprusside, or papaverine can be used to obtain maximal relaxation.

Removal of the Epithelium

Classically, the modulatory role of the airway epithelium is evaluated in paired observations of intact and epithelium-denuded isolated airway preparations. The epithelium is commonly removed by "gentle rubbing" with a wet gauze, and its effectiveness is verified histologically (Flavahan et al., 1985; Aizawa et al., 1988; Jongejan et al., 1991).

In guinea pig tracheas the effectiveness of epithelium removal can be also verified functionally by adding arachidonic acid which causes smooth muscle contraction in epithelium-denuded tracheas, whereas intact tracheas respond with relaxation (Nijkamp and Folkerts, 1987). This procedure has not been tested in human airways.

With mechanical rubbing, it is possible to remove over 95% of the epithelium leaving the basal membrane and the smooth muscle histologically intact. However, Franconi et al. (1990) showed that mechanical rubbing of the epithelium may lead to release of granules containing tryptase from mast cells present in the lamina propria.

In dog airways, tryptase causes hyperresponsiveness, probably due to an effect on Ca^{2+}-channels (Sekizawa et al., 1989). This mechanism may explain at least part of the hyperresponsiveness reported in epithelium-denuded airways. Furthermore, epithelial damage and denudation in asthma is not caused by mechanical rubbing but, probably, by the release of the basic proteins such major basic protein (MBP) and eosinophil peroxidase (EPO) and oxygen radicals released from inflammatory cells present in the inflamed airway (Frigas and Gleich, 1986; Barnes, 1989; Montefort et al., 1992). Deposits of EPO were found in areas of mucosal injury in asthmatics (Bousquet et al., 1992). Human MBP causes epithelial damage (Motojima et al., 1989) and hyperresponsiveness in vitro (Flavahan et al., 1988) and in vivo (Gundel et al., 1991).

Oxygen radicals increase the permeability of cultured epithelium (Welsh et al., 1985) as well as epithelium in the guinea pig trachea (Jeppsson et al., 1989), and the responsiveness of human airways is enhanced after damaging the epithelium with hydrogen peroxide (Hulsmann et al., 1993a). In a model of asthma it may be more appropriate to use these aggressive substances rather than mechanical rubbing to induce epithelial damage.

Apart from basic proteins and oxygen radicals, several other methods for damaging and removal of epithelium in vitro have been described.

Franconi et al. (1990) removed epithelium from animal and human airways by perfusing the preparations during 0.5–2 hr with pronase (protease type XIV; 1 mg/mL). Where the epithelium was effectively removed, the integrity of mast cells in the lamina propria and the smooth muscle was not affected.

Interferon-γ, produced by intraepithelial T-lymphocytes (Ebert, 1990) enhances tight junction permeability in a human intestinal epithelial cell line (Madara and Stafford, 1989) but has not been examined in airway epithelium.

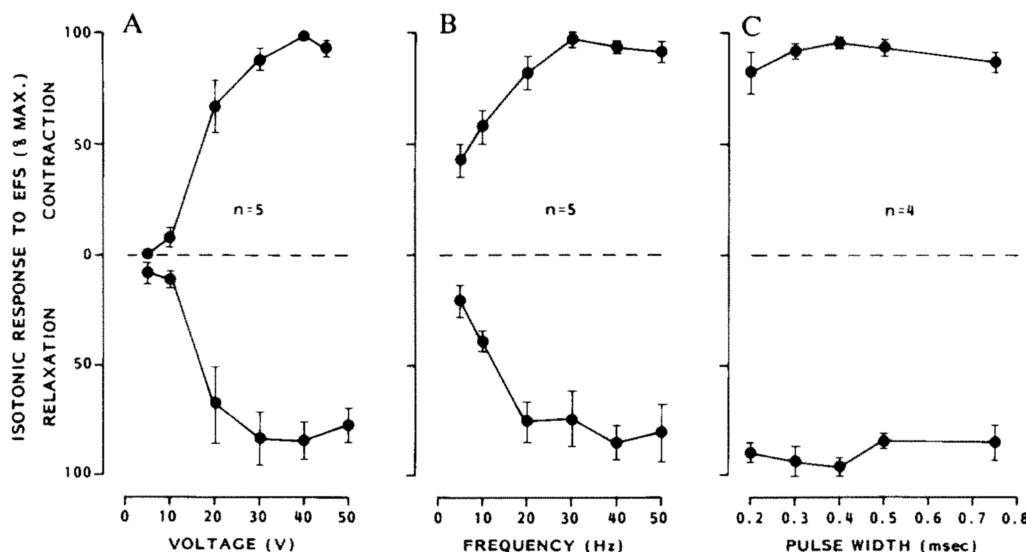
Perfusion of rat arteries with a hypotonic Tyrode solution (Pelissier et al., 1992) or the nonionic, nondenaturing detergent CHAPS (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate; Tesfamariam et al., 1985; Yang et al., 1989) resulted in a disruption of endothelial cells, and this method may be applicable for endothelial cell removal in airways as well. It appears, therefore, that there are alternatives for the mechanical removal of airway endothelium. These alternatives have the advantage that they may mimic the damage that is found in asthma (basic proteins and oxygen radicals) or that they produce less artifacts (pronase).

Electrical Stimulation

Basically, there are two methods of electrical stimulation of isolated organ preparations: contact stimulation using electrodes that are attached to the tissue and field stimulation via electrodes that are not in direct contact with the tissue. Also, one electrode may be in contact with the tissue, while the other, often a ring, is not (hybrid stimulation). Electric field stimulation is most commonly used to study neural responses in smooth muscle preparations including isolated airways. The technique is relatively simple platinum- or silver-silver chloride sheet electrodes are suspended close to, but not in contact with, the tissue, in an organ bath containing K-H solution (Figure 2). A stimulator generates rectangular pulses of short duration (0.1–1 msec) at a constant current. Voltage- and frequency-response curves can be obtained and the interval between stimuli and pulse width can be varied (Figure 10).

EFS was introduced by Paton in 1955 who demonstrated that single electrical pulses (1–25 V, 0.5 msec) elicited brief twitches (1 sec) in the guinea pig isolated ileum (Paton, 1955). Since the twitch was abolished by

Figure 10. Effect of change in pulse-voltage, -frequency, and -width on the contraction and relaxation responses of human bronchial strips. (A) Effect of voltage (0–50 V) with constant frequency (30 Hz) and pulse width (0.3 msec). (B) Effect of frequency (0–50 Hz) with constant voltage (30 V) and pulse width (0.3 msec). (C) Effect of pulse width (0.2–0.75 msec) with constant voltage (30 V) and frequency (30 Hz). Pulse trains of 30 sec. Contractions and relaxations are expressed as a percentage of the maximal response in a given strip. Mean values ± SEM of four to five experiments are shown. (Reproduced with permission of de Jongste et al., 1987e.)



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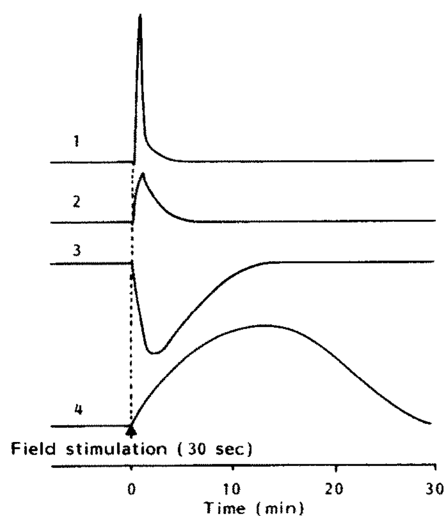
atropine, prolonged by eserine, and was insensitive to hexamethonium, he concluded that postganglionic nerve fibers were excited by EFS. In later studies it was confirmed that with appropriate stimulation parameters, nerve fibers can be activated selectively without stimulating the smooth muscle directly (Paterson, 1965; Duckles and Silverman, 1980). In human airways, EFS causes a fast, nerve-mediated cholinergic contraction followed by a slow nonadrenergic, noncholinergic inhibitory nerve-mediated (i–NANC) relaxation (Richardson and Béland, 1976; Davis et al., 1982; Taylor et al., 1984; de Jongste et al., 1987c). In addition, de Jongste et al. (1987c) found a rapid nonneural contraction and a sustained nonneural contraction resulting from synthesis of cyclo-oxygenase metabolites and leukotriene-like substances by fresh human airway tissue, respectively (Figure 11). These nonneural contractions may interfere with neural responses and should be taken into account as a confounding factor. In guinea pig airways vasoactive intestinal peptide (VIP) and nitric oxide (NO) may be the neurotransmitters of the nonadrenergic inhibitory nerves (Li and Rand, 1991; Lei et al., 1993), whereas in human bronchi the response may be due mainly to NO (Belvisi et al., 1992b; Ellis and Undem, 1992).

Although EFS is a useful tool to evoke neural responses in isolated airway tissues, the technique has an important side effect. During EFS with commonly used stimulation parameters activated oxygen molecules may be generated in carbogenated K-H buffer. These activated oxygen molecules have been shown to relax smooth muscle preparations directly (Greenberg et al., 1986) and may oxidize contractile drugs (Wyse, 1977; Hulsmann et al., 1993b). The inactivation of histamine by EFS may even occur at a frequency of 2 Hz (50 V, pulse duration 0.3 ms; Hulsmann et al., 1993b).

Figure 11. Schematic representation of time course, peak latency, and amplitudes of the four phases that constitute the response of fresh human bronchus to electrical field stimulation (EFS) in vitro. Phases are numbered according to peak latency after EFS: 1, cholinergic nerve-mediated, rapid contraction; 3, nonadrenergic inhibitory nerve-mediated relaxation; 2 and 4, nonneural contraction due to the release of cyclooxygenase and lipoxygenase metabolites, respectively. (Reproduced with permission of de Jongste et al., 1987c.)



Conclusions and Directions for Future Research

In the present overview, we discussed models that have been developed over the years to study the effects of drugs, inflammatory mediators, autonomic nerves, and epithelial cells on the responsiveness of airway smooth muscle in vitro. These models range from the simple bronchial strip preparation to the complex ventilated and perfused lung preparation. Although these models have substantially contributed to the progress in our understanding of the pathophysiology of asthma and COPD, the precise relationships between airway inflammation, bronchial hyperresponsiveness, and airway narrowing is still not clear. The relative contributions of inflammatory cells and their products, of autonomic nerves and of the tracheo-bronchial circulation to airway disease should be further investigated. With the currently available in vitro models, however, the role of the bronchial circulation in airway disease cannot be elucidated. Efforts should now be made to develop models in which the normal relationships between the ventilatory and the circulatory unit is preserved in vitro. The study of isolated perfused and ventilated lung tissue may be an important step in this direction, and it might be possible to develop such a model for the human lung.

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