Studies on the Regulation of Secretion in Clara Cells with Evidence for Chemical Nonautonomic Mediation of the Secretory Response to Increased Ventilation in Rat Lungs

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ABSTRACT Using electron microscopy and morphometric methods to assess secretion, we previously found that two times tidal volume ventilation of isolated perfused rat lung stimulates secretion by bronchiolar Clara cells; this effect is not prevented by β-adrenergic blockade (*J. Clin. Invest.* 1981. **67**: 345–351.). In this study we used the isolated perfused rat lung and the anesthetized mechanically ventilated rat, to further study the mechanism by which large tidal volumes stimulate secretion by Clara cells. With the perfused lung we found (*a*) α-adrenergic inhibition did not block the secretory effect of ventilation at two times normal tidal volume; (*b*) indomethacin completely blocked the secretory action of two times tidal volume ventilation; (*c*) medium previously used to perfuse lungs ventilated at two times tidal volume, but not medium previously used to ventilate lungs at normal tidal volume, stimulated secretion by Clara cells when used to perfuse fresh lungs ventilated at tidal volume; (*d*) addition of prostacyclin to the fresh perfusate increased secretion by Clara cells of lungs ventilated at normal tidal volume. In anesthetized mechanically ventilated rats, sighs stimulated secretion by Clara cells; this increased secretion was inhibited by indomethacin but not by cholinergic blockade (bilateral vagotomy). These studies indicate that increased volume ventilation stimulates secretion by Clara cells in vivo and in vitro; they provide evidence that chemical nonadrenergic, noncholinergic mechanisms are involved in this secretion, and that prostaglandins may be the chemical messenger coupling the mechanico-secretory events.

INTRODUCTION

Clara cells are epithelial cells located mainly in small conducting airways (1). They have morphologic characteristics of cells in which protein synthesis and secretion is a major function (2, 3). These anatomic features, and the ultrastructural similarity between Clara cell secretory granules and the extracellular layer lining terminal bronchioles (4, 5), has led to the notion that Clara cells are the major site of synthesis and secretion of components of the extracellular lining layer of small conducting airways. The presence of these secretory cells in small airways plus the evidence that one of the earliest abnormalities in chronic bronchitis and emphysema is obstruction of airflow, due in part to the presence of excessive secretions in the lumen of bronchioles (6), suggests Clara cells may play a role in the disordered lung function that occurs in these diseases. However, this extracellular material, although harmful in excess, when present in normal amounts is thought to protect underlying cells from noxious agents inhaled into the lung.

These considerations led to a series of experiments in which ultrastructural morphometric methods were used to examine the regulation of secretion in Clara cells (7, 8). The studies showed large tidal volume ventilation of isolated perfused rat lungs stimulates secretion by Clara cells, and, that this effect is not prevented by β-adrenergic blockade (7). The present work was undertaken to study the mechanism by which in-

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METHODS

Animals. We used male Long-Evans descent hooded rats (Charles River Breeding Laboratories, Inc., Wilmington, MA). They were allowed food (Rodent Laboratory Chow 5001, Ralston-Purina Co., St. Louis, MO) and water ad lib. Lighting was provided from 7 a.m. to 7 p.m. daily. The animals were anesthetized by giving pentobarbital sodium ($\sim$60 mg $\cdot$ kg^{-1}) intraperitoneally, and were killed by exsanguination.

Studies using the isolated ventilated perfused lung. The isolated perfused lung system was the same as previously described in detail (9). Briefly, the excised lungs were kept at 37°C, and ventilated at 40 breaths $\cdot$ min^{-1} with warm humidified gas (95% O_2:5% CO_2) at a tidal volume (or two times tidal volume) based on the rats body weight (10). Krebs-Ringer-bicarbonate medium (11) containing fatty acid-poor 5% bovine serum albumin was used as the perfusing medium. The perfusate (40 ml) also contained glucose (5 mM) and normal rat plasma levels of 20 L-amino acids (12); it was delivered to the lung at a pressure of 20 cm H_2O and was recirculated.

Studies using anesthetized artificially ventilated rats. Rats were anesthetized (pentobarbital sodium, $\sim$60 mg $\cdot$ kg^{-1}) and when anesthesia was achieved they were given tubocurarine chloride (15 mg in 0.5 ml of 0.15 M NaCl) intraperitoneally. The trachea was incised, a plastic endotracheal tube inserted and tied into place and ventilation was begun with humidified room air. The nomogram of Kleinman and Radford (10) was used to determine the tidal volume appropriate for the animal's body weight and ventilatory rate of 40 breaths $\cdot$ min^{-1}. Bilateral vagotomy was performed by identifying both cervical vagi, placing a ligature beneath each and then cutting the nerve. Sham-operated rats were treated in the same manner but the nerves were not cut.

Unanesthetized rats were given indomethacin (15 mg $\cdot$ kg^{-1} body wt subcutaneously) in ethanol (1.5 ml $\cdot$ kg^{-1} body wt). Control rats received an equal volume of vehicle alone. After 1 h the rats were anesthetized, given tubocurarine, an endotracheal tube was inserted and ventilation was begun as described above.

Electron microscopic and morphometric procedures. Lungs were fixed by infusing 2.5% cold glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, into the trachea at a transpulmonary pressure of 20 cm H_2O. The trachea was ligated, and fixation was continued by placing the lungs in fixative for 2 h at 0–4°C.

Preparation of tissue. A stratified sample of tissue was obtained by removing one piece from five different areas of lung: left upper, middle, and lower lung, and right upper and lower lung. These were placed in fresh 0.1 M cacodylate buffer. A dissecting microscope was used to identify areas containing bronchioles: blocks cut from these areas were washed three times in cacodylate buffer, kept at 4°C overnight, and then postfixed in 2% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.4. The tissue was then dehydrated and embedded (13). Tissue blocks were sectioned on a Porter-Blum ultramicrotome (DuPont Co., Instrument Products Div., Sorvall Biomedical Div., Newton, CT) with a diamond knife. Sections with a silver interference color were picked up on 200-mesh copper grids, stained with uranyl magnesium acetate and lead citrate (14), and examined with a Philips 300 electron microscope (Philips Electron Instruments, Inc., Mahwah, NJ).

Sampling procedures. The primary sample consisted of 50 tissue cubes (10 from each of 5 stratified areas). From the 5 sets of 10 numbered cubes, 1 cube was chosen, using a random number table, from each set to represent the secondary sample. Each of these blocks was sectioned at 1 μm for light microscopy and at 60–90 nm for electron microscopic examination. Nine electron micrographs of Clara cells were taken from each block. Thus, from each rat we used five blocks and made 45 micrographs. The micrographs of Clara cells were taken randomly and enlarged to a final magnification of 15,750.

Stereological procedures. Morphometric analysis was performed using the methods of Weibel et al. (15) with a double lattice test system. For calculation of volume density we counted enough test points in each animal to attain a relative error in each instance of 5% (15). The volume density and surface-to-volume ratio of the secretory granules were calculated by standard methods (15). We also calculated the fraction of cell volume as a function of the number of test points that fell on the Clara cell relative to the fixed number of points of the test system. Since the results from this calculation did not alter any of our findings we have not included this data. We interpreted a lack of change in the surface-to-volume ratio as evidence that secretory granule size was unchanged by the different experimental manipulations.

Drugs used. Drugs were added to the perfusate to achieve the following concentrations: cycloheximide, 0.1 mM; DL-isoproterenol, 0.5 mM; α-phenylephrine, 0.5 mM; phentolamine, 0.5 mM; indomethacin, 2 μg $\cdot$ ml^{-1} in 40 μl of ethanol. Prostacyclin was added to the 40 ml of medium at 0, 15, 30, and 45 min of perfusion to give 2.9 ng $\cdot$ ml^{-1} concentration of fresh prostacyclin at each addition. In the in vivo studies indomethacin (15 mg $\cdot$ kg^{-1}) was injected subcutaneously and control rats received the vehicle (100% ethanol) alone.

Statistical analysis. For each parameter measured or calculated from these measurements, the values for individual animals were averages per experimental group and the standard error of the group mean calculated (16). An analysis of variance for multiple group comparisons was used to test for significant differences between group means (17).

RESULTS

Studies in the isolated ventilated perfused lung: alpha adrenergic mediation. The presence of α-phenylephrine lowered the volume density (Vv)[1] of Clara cell secretory granules and this effect was prevented by the α-adrenergic antagonist phentolamine (Fig. 1). Phentolamine, in a concentration that blocked the secretory action of α-phenylephrine, did not prevent two times constant normal tidal volume ventilation (2 $\times$ CTV) from lowering the secretory granule Vv (Fig.

[1] *Abbreviations used in this paper*: CTV, constant normal tidal volume ventilation; IVPL, isolated ventilated perfused lung; UM1 $\times$ CTV, used medium from lungs perfused for 60 min and ventilated constantly at normal tidal volume; UM2 $\times$ CTV, used medium from lungs perfused for 60 min and ventilated constantly at two times normal tidal volume; Vv, volume density.

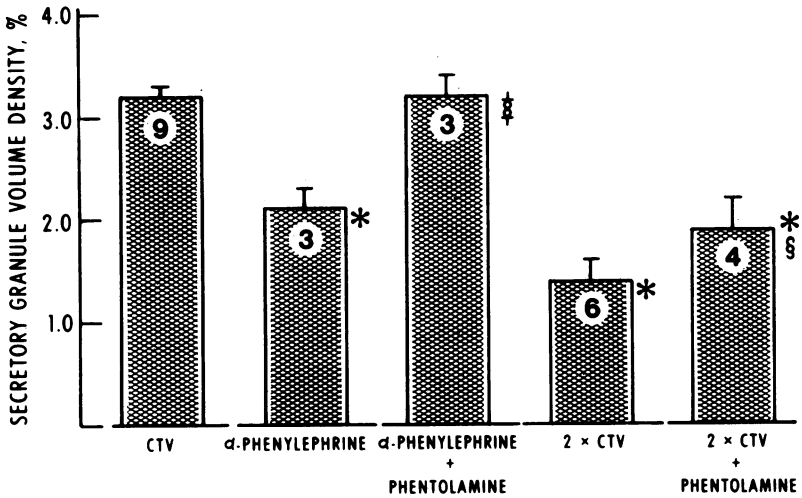

FIGURE 1 Effect of α-adrenergic agents and large volume ventilation on secretion. Excised rat lungs were ventilated and perfused for 1 h and then fixed for ultrastructural morphometric analysis. The final concentration of the pharmacological agents was 0.5 mM. Mean±SE are given. Figures within the bars indicate the number of experiments.

* $P < 0.001$ vs. CTV.

‡ $P < 0.001$ vs. α-phenylephrine.

§ $P > 0.05$ vs. 2 × CTV.

1). These changes in secretory granule Vv were not accompanied by changes in the surface-to-volume ratio ($\mu m^2/\mu m^3$) of these granules; the ratios were 10.1±0.2 (CTV), 10.8±0.2 (+ α-phenylephrine), 10.4±0.2 (+ α-phenylephrine + phentolamine), 9.9±0.1 (2 × CTV), 9.9±0.3 (2 × CTV + phentolamine); the differences between these groups were not statistically significant ($P > 0.05$).

Studies with "used" perfusate, indomethacin, and prostacyclin in the isolated ventilated perfused lung (IVPL). We have shown in the IVPL that β-adrenergic blockade (7), and α-adrenergic blockade (Fig. 1), do not inhibit the stimulatory effect of large tidal volumes on secretion by Clara cells. Furthermore, since all neural connections were severed in removing the lung from the thorax, this secretory response does not require vagally carried cholinergic mediation. These observations and considerations suggested that the increased secretion might be mediated by a chemical messenger present within the lung, i.e., that a paracrine mechanism might be involved (18). To test this possibility we did two series of experiments in which medium, previously used to perfuse one lung, was reused to perfuse a second "fresh" lung. In one series, we ventilated perfused lungs at 2 × CTV for 1 h, collected the perfusing medium (designated UM2 × CTV), and used it to perfuse fresh lungs ventilated at 1 × CTV. In a second series of experiments, lungs were ventilated at 1 × CTV and the medium (designated UM1 × CTV) was collected, and used to perfuse other fresh lungs ventilated at 1 × CTV. Used medium from lungs ventilated at 2 × CTV caused a significant fall in the Vv of secretory granules in Clara cells of fresh lungs ventilated at 1 × CTV (Fig. 2). In contrast, used medium from lungs ventilated at 1 × CTV, when used to perfuse lungs ventilated at 1 × CTV, did not lower the Vv of Clara cell secretory granules (Fig. 2).

Since prostaglandins are released from lung cells by mechanical stimuli (19, 20) they could be the agents serving as chemical messengers in our experiments with used medium. We therefore performed two additional sets of experiments, one to determine if an inhibitor of prostaglandin synthesis would block the effect of 2 × CTV ventilation, and, if it did, to determine if a prostaglandin, which is known to be released from the lung during increased ventilation, would, when added to the perfusate of lungs ventilated at CTV, stimulate secretion by Clara cells. We found indomethacin, an inhibitor of prostaglandin synthesis (21), completely prevented the fall in secretory granule Vv that occurs during 2 × CTV ventilation (Fig. 2). Furthermore, prostacyclin, a prostaglandin released from lung cells during increased ventilation (20), stimulated secretion by Clara cells when it was added to the perfusing medium (Fig. 2).

These manipulations did not alter the surface-to-volume ratio of the secretory granules. These values, $\mu m^2/\mu m^3$, were 10.1±0.2 (CTV), 10.3±0.5 (UM2 × CTV), 10.2±0.2 (UM × 1CTV), 9.8±0.3 (2 × CTV + indomethacin), and 10.0±0.2 (+ prostacyclin).

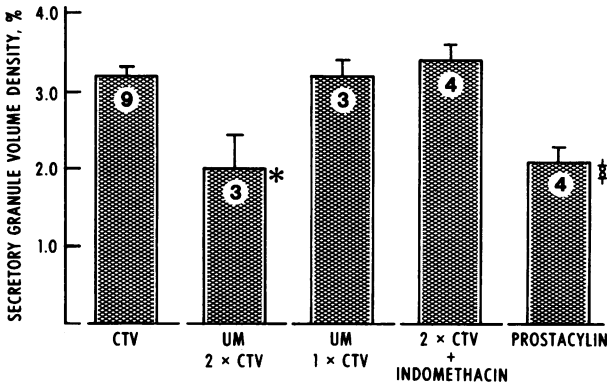

FIGURE 2 Effect of used medium, indomethacin, and prostacyclin on secretion. Lungs were perfused for 60 min and ventilated at normal tidal volume (CTV). Other lungs were ventilated at two times normal tidal volume (2 × CTV) with or without indomethacin (2 $\mu g \cdot ml^{-1}$). Some lungs were ventilated at tidal volume and perfused with medium previously used to perfuse lungs ventilated at two times tidal volume for 60 min (UM2 × CTV).Other lungs were ventilated at tidal volume and perfused with medium previously used to perfuse lungs ventilated at tidal volume (UM1 × CTV). Prostacyclin was added to 40 ml of medium at 0, 15, 30, and 45 min of perfusion to give 2.9 $ng \cdot ml^{-1}$ concentration of fresh prostacyclin at each addition. We chose this approach since the estimated half-life of this agent is about 3 min in blood (8). Mean±SE are given. Figures within the bars indicate the number of experiments.

* $P < 0.05$ vs. CTV or UM1 × CTV.

‡ $P < 0.05$ vs. CTV.

TABLE I

Effect of Experimental Conditions on Medium Flow in the Isolated Perfused Lung

Condition	Flow rate	
	Initial	Final
	$ml \cdot min^{-1}$	
CTV (9)	17.8±0.2	17.7±0.2
+ α-phenylephrine (3)	18.0±0.1	7.0±1.2*
+ α-phenylephrine + phentolamine (3)	17.8±0.2	17.7±0.2
2×CTV (6)	17.6±0.4	17.5±0.3
2×CTV + phentolamine (4)	18.2±0.2	17.5±0.3
UM2×CTV (3)	18.0±0.1	18.0±0.1
UM1×CTV (3)	17.8±0.2	18.0±0.1
2×CTV		
+ indomethacin (4)	17.6±0.3	17.1±0.3
+ prostacyclin (4)	18.5±0.2	18.5±0.2

The rate of medium flow was measured 1–2 min after the beginning of perfusion of the IVPL and again after 60 min. Values are mean±SE. Figures in parentheses indicate the number of animals. * $P < 0.001$ compared with initial flow rate.

Rate of perfusion flow. Only the presence of α-phenylephrine altered the rate of medium flow through the lung (Table I).

In vivo secretory studies. The IVPL has features that make it a useful model to use to study secretory activity of lung cells. However, it is an artificial system where, among other things, the nerves to and from the lung are cut. We therefore examined the effect of increased tidal volume breathing in vivo on secretion by Clara cells. We found a sigh (fourfold increase in tidal volume) every 5 min resulted in a substantial fall in the Vv of Clara cell secretory granules compared with those of Clara cells in CTV ventilated rats; this effect was not blocked by vagotomy (Fig. 3). However, as in the IVPL, the in vivo secretory response to sighs was blocked by indomethacin (Fig. 3). The surface-to-volume ratio ($\mu m^2/\mu m^3$) of the secretory granules was the same in all groups: CTV (9.4±0.1), CTV + sigh (10.0±0.2), CTV + sigh + vagotomy (9.9±0.2), CTV + sigh + sham vagotomy (10.1±0.3), indomethacin + CTV + sigh (9.5±0.3), and vehicle + CTV + sigh (10.3±0.4).

Inhibition of protein synthesis. Cycloheximide (0.1 mM) inhibited protein synthesis, as determined by the incorporation of [^{14}C]phenylalanine into trichloroacetic acid insoluble material, by >90% (data not shown). The Vv of Clara cell secretory granules after 60 min of perfusion and CTV in the presence of 0.1 mM cycloheximide was 2.9±0.3% ($n = 3$); in the presence of 0.1 mM cycloheximide plus 0.5 mM isoproterenol the Vv was 1.7±0.2% ($n = 3$, $P < 0.025$). The surface-to-volume ratio ($\mu m^2/\mu m^3$) of the secretory granules was 10.5±0.3 and 10.4±0.3 in Clara cells exposed to cycloheximide or to cycloheximide plus isoproterenol, respectively.

Intracellular destruction of secretory granules. In an attempt to determine if changes in the Vv of secretory granules were due to their intracellular destruction we examined all the photographs used in this study for evidence of lysosomal fusion with secretory granules. We failed to detect any evidence of lysosomal destruction of secretory granules.

DISCUSSION

Ultrastructural analysis of Clara cell secretion. Our studies on the regulation of secretion in Clara cells assume these cells are indeed secretory cells, and that a fall in Vv of their inclusions represents release of the contents of these granules beyond the cell. The first assumption seems relatively secure because of the ultrastructural similarity of Clara cells to many other cells that make and store proteins destined to be secreted (1–4). As previously considered (7, 8), a fall in Vv of the secretory granules, in response to a presumed secretagogue, could represent an increase in cell vol-

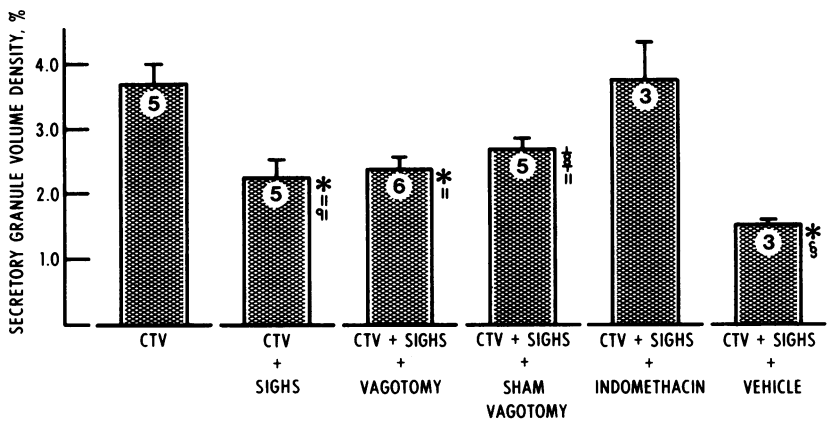

FIGURE 3 In vivo studies. Rats were anesthetized and ventilated at continuous tidal volume (CTV) at 40 breaths · min^{-1} for 60 min; other rats were ventilated at CTV with a 4 × CTV inflation (sigh) every 5 min for 60 min with or without having their vagus nerves cut. Indomethacin or the vehicle were given (Methods).

* $P < 0.01$ vs. CTV.

‡ $P < 0.05$ vs. CTV.

§ $P < 0.01$ vs. CTV and vs. + sighs + indomethacin.

‖ $P > 0.05$ between groups.

¶ $P > 0.05$ vs. CTV + sighs + vehicle.

ume rather than a change in the volume of the secretory granules. We think our studies relating the number of points of the test system that fell on the Clara cell to the fixed number of points on the test system excluded this possibility.

However, in addition to representing secretion, a fall in the Vv of secretory granules could reflect a decrease in the synthesis of proteins that form the contents of the granule or the intracellular destruction of the granule. We do not think these mechanisms explain the fall in Vv for two reasons. First, when protein synthesis was inhibited by >90%, isoproterenol caused a decrease in Vv (42%) similar in magnitude to that produced by isoproterenol in the absence of an inhibition of protein synthesis (45%) (7). Secondly, we failed to detect ultrastructural evidence of destruction of secretory granules within lysosomes, this mechanism being the means by which such formed elements are destroyed (22).

Evidence against autonomic mediation of secretion triggered by increased volume breathing. The following observations argue against autonomic mediation of secretion stimulated by large breaths: (*a*) propranolol, in a concentration that completely blocks isoproterenol-stimulated secretion, does not diminish secretion stimulated by large breaths (7); (*b*) similarly, phentolamine, in a concentration that blocks secretion produced by α-phenylephrine, does not impair secretion stimulated by large breaths; (*c*) bilateral vagotomy does not impair secretion produced by sighs in the mechanically ventilated intact rat. Furthermore, stimulation of secretion in the isolated perfused lung by 2 × CTV ventilation indicates the afferent and efferent limbs of the stimulus-effector mechanism reside within the lung.

The afferent-efferent circuit coupling the mechanico-secretory events could reside within the Clara cells themselves. However, the results of the experiments with used medium suggest the presence of an intrapulmonary chemical intermediate bridging, or forming part of the bridge, between the mechanical event (sighs) and the response (secretion). This chemical(s) could arise within Clara cells; however, the secretion stimulating ability of medium from 2 × CTV lungs raises the possibility that it originates in other cells and travels through the interstitium to the Clara cell, i.e., that a paracrine event is involved in the stimulus-secretion coupling (18).

Our study provides two pieces of evidence that this chemical agent is a prostaglandin. The first is the inhibition by indomethacin, both in vitro and in vivo, of the fall in Vv produced by large tidal volume ventilation. This drug influences several enzymes and has many other actions; however, in the concentration used in vitro (~5 μM) its major effect is the inhibition of prostaglandin synthetase (21). The second, a more direct evidence that prostaglandins affect secretion by Clara cells, is the substantial fall in secretory granule Vv that occurs when prostacyclin is added to the perfusing medium. However, in view of the multicellular make-up of the lung, we cannot conclude that prostacyclin acts directly on Clara cells. Furthermore, prostacyclin, a powerful vasodilator (23), could theoretically stimulate secretion by increasing the delivery

of substrates to the Clara cell. However, prostacyclin did not alter the flow rate of medium in the IVPL.

Possible coordinated secretion by Clara cells and type 2 alveolar cells. The demonstration that sighs in the anesthetized rat stimulate secretion by Clara cells suggests this event plays a role regulating secretion in these cells in spontaneously breathing rats. It is of interest that a large inflation in the excised lung (24) and sighs in the anesthetized rat (25) also stimulate secretion of surfactant by type 2 alveolar cells. These observations suggest that secretion in terminal lung units (bronchioles and alveoli) may be coordinated and that sighs, which are normal ventilatory events (26), may be the trigger for secretion under resting conditions. The interstitial space of alveoli and bronchioles communicate, and the flow of interstitial fluid is from alveoli to bronchioles, hence, this coordination could be effected by an agent released by alveolar interstitial cells. Furthermore, since increased volume ventilation accelerates the rate at which the lining material in these terminal units moves cephalad (27, 28), augmented secretion, triggered by increased volume ventilation, would serve to meet the increased replacement requirements.

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