Regulation of Secretion in Clara Cells

STUDIES USING THE ISOLATED PERFUSED RAT LUNG

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ABSTRACT Previous studies from our laboratory indicated that both beta-adrenergic and cholinergic agents stimulate in vivo secretion by rat bronchiolar Clara cells. Those studies also provided support for an in-series beta-adrenergic-cholinergic stimulation of secretion. To further explore the regulation of secretion in Clara cells, and to do it in the absence of systemic influences, we have used the isolated ventilated perfused rat lung. We have again used morphometry and electron microscopy to assess secretion by measuring the volume density (fraction of cell volume) of the secretory granules of bronchiolar Clara cells. We found that in the isolated perfused lung, as in the intact animal, isoproterenol stimulated secretion in Clara cells and that this effect was blocked by the beta-adrenergic antagonist propranolol. Pilocarpine, unlike its action in the intact animal, did not stimulate secretion in the isolated lung; rather it inhibited the secretory effect of isoproterenol. Increased tidal-volume ventilation stimulated secretion; propranolol did not block this effect. Analogs of cyclic (c)AMP and of cGMP also stimulated secretion by Clara cells. These findings indicate that there are at least two mechanisms by which Clara cells can be stimulated to secrete. One seems to be beta-adrenergic-cAMP mediated but the triggering event is unknown. The other is initiated by increased tidal volume and cGMP may be involved in the intracellular mediation of this stimulatory event. Finally, we found evidence of beta-adrenergic (stimulatory) -cholinergic (inhibitory) antagonism in the regulation of secretion in Clara cells.

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INTRODUCTION

Clara cells are epithelial cells which have ultrastructural characteristics of cells with a major secretory function (1–5). They are located mainly in small conducting airways (3–4), and are thought to secrete components of the extracellular lining of these airways (6). The extracellular lining of this region may be of particular importance because noxious agents inhaled into the lung commonly affect these airways (7), and because these airways are the site of obstruction early in the course of very common diseases such as chronic bronchitis and emphysema (8). In an effort to improve our understanding of this region of the lung, we used ultrastructural morphometric methods to study the in vivo regulation of secretion in Clara cells of rats (9). We found that both pilocarpine and isoproterenol stimulated secretion, as evidenced by a fall in the volume density (fraction of cell volume) of the secretory granules in these cells; atropine and propranolol, respectively, blocked secretion stimulated by these agonists. We found unexpectedly that propranolol, a beta-adrenergic antagonist, was as effective as atropine in blocking the stimulation of secretion produced by pilocarpine. This suggested that an in-series cholinergic-adrenergic mechanism might be involved in the regulation of Clara cell secretion.

The in vivo nature of our previous studies limited further delineation of this potential regulatory mechanism; we could not, for example, control the potential systemic effects of the pharmacological agents on Clara cell secretion or determine if the secretagogues were acting in series within the lung or if one arm of the in-series stimulatory mechanism was extrapulmonary. We have therefore undertaken the present study using

the isolated ventilated perfused lung (IVPL)[1] as a system with which we might explore the regulation of Clara cell secretion in the absence of potential systemic influences on secretion.

METHODS

Animals. We used male Long-Evans-descent hooded rats weighing between 200–250 g (Charles River Breeding Laboratories, Inc., Wilmington, Mass.). They were maintained on a 12-h light–12-h dark cycle and were allowed food (Wayne Lab-Blox, Allied Mills, Inc., Chicago, Ill.) and water ad lib.

Isolated ventilated perfused lung system. The animals were anesthetized by giving pentobarbital sodium (30 mg/kg) intraperitoneally, and were killed by exsanguination while the lung was excised and placed in the perfusion chamber. The method of perfusing and ventilating the isolated lung has been previously described in detail (10). In essence, the excised lungs were kept at 37°C, and ventilated at 40 breaths/min with warm humidified gas (95% O_2:5% CO_2) at a tidal volume based on the rat's body weight (11). We used Krebs-Ringer bicarbonate medium (12) containing fatty-acid-poor 5% bovine serum albumin, as the perfusion medium. The perfusate (40 ml) also contained glucose (5 mM) and normal rat plasma levels of 20 amino acids (13). The perfusate was delivered to the lung at a pressure of 20 cm of water and was recirculated.

Experimental procedures. The drugs were added to the perfusate to achieve the following concentrations: pilocarpine (0.5 mM), atropine (0.5 mM), DL-isoproterenol (0.5 mM or 5 μM), propranolol (0.5 mM), N^6O^2-dibutyryl adenosine 3′:5′ cyclic monophosphoric acid (1.0 mM), N^6O^2-dibutyryl guanosine 3′:5′ cyclic monophosphoric acid (1.0 mM), 8-bromoadenosine 3′:5′ cyclic monophosphoric acid (1.0 mM), and 8-bromoguanosine 3′:5′ cyclic monophosphoric acid (1.0 mM). Control lungs were perfused with drug-free perfusate.

After 1 h, the perfusion was stopped and the lungs were removed from the chamber. We immediately infused 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 then ligated and fixation was continued as previously described (9).

Electron microscopic and morphometric procedures. The methods of preparing lung tissue for electron microscopic examination, the tissue sampling procedures and the criteria for identification of Clara cells were identical in the present study to those previously described in detail (9, 14, 15). Morphometric analysis was performed using the methods of Weibel (16). We used a double-lattice test system instead of the multipurpose test system used in our prior report (9); the morphometric procedures were otherwise the same as those used previously (9). The volume density of the secretory granules relative to the cell was calculated by dividing the number of test-system points which fell over the secretory granules by the number of test points which fell over the entire cell (including the secretory granules) (16), and multiplying this quotient by 100. It can be appreciated from this calculation that a change in the volume density could occur either because of an absolute change in the volume of secretory granules or from a change in cell volume without any change in the volume of secretory granules. To help exclude the second possibility, we have also expressed the volume density as a function of the test points that fell on the secretory granules relative to the fixed number of points on the test system. As before (9) the surface-to-volume ratio of the secretory granules was calculated by a standard method (16).

Lung weight. In some experiments the weight of the lung was measured at the end of the perfusion, after conducting airways had been removed at the hilum. The lung's dry weight was then obtained by heating pieces of lung at 60°C until two weights, 24 h apart, remained the same.

ATP measurements. At the end of the perfusion, the lungs were rapidly frozen by clamping them between blocks of aluminum cooled to the temperature of liquid nitrogen. Pieces of frozen lung (0.2–0.3 g) were quickly ground to a fine powder at the temperature of liquid N_2 and then immediately homogenized in 0.4 M perchloric acid at 0–4°C. The homogenized samples were kept on ice for 15 min and then centrifuged at 0°C at 15,000 *g* for 20 min. The acid-soluble supernatant material was collected and KOH added to it to bring its pH to between 5 and 7. These samples were kept on ice for 30 min to precipitate potassium chlorate. After another centrifugation at 0°C and 15,000 *g* for 20 min, the supernatant fluid was removed and immediately frozen for later determination of ATP using enzymatic methods: glucose-6-phosphate dehydrogenase and hexokinase (17). To determine the recovery of ATP in the assay procedure, we added a known amount of ATP to another piece of lung and subjected it to the same procedures.

Statistical analysis. For each parameter measured or calculated from these measurements, the values for individual animals were averaged per experimental group and the standard error of the group mean calculated (18). An analysis of variance was used for multiple group comparisons (19) and Duncan's multiple range test (20) or Kramer's extension of it (21) were used to test for significant differences between groups. An unpaired *t*-test analysis was used to compare means of two groups.

RESULTS

Characteristics of the isolated ventilated perfused lung system. The lung dry weight, lung weight-to-body weight ratio and the ATP content of the lungs (Table I) were virtually the same as those reported by others using similar rat lung preparations (22–26). The fall in the flow rate of the perfusate of control lungs during the experiment was the same or less than that reported by others (26); the fall in the flow rate in lungs perfused with isoproterenol plus propranolol was greater than the decrease that occurred in control lungs. There was a 13% decrease in the percent dry weight in the lungs perfused with isoproterenol plus propranolol and in the lungs perfused with pilocarpine ($P < 0.05$ compared to control lungs). The ATP content of the lungs was the same under the different conditions in which it was measured.

In an attempt to obtain a more direct comparison of the effect on the Clara cell of in vitro ventilation and perfusion, we compared the volume density of secretory granules and of mitochondria in Clara cells after 1 h of in vitro perfusion and ventilation to values we had previously developed for Clara cells in lungs fixed immediately after killing (9). We found the following:

[1] *Abbreviation used in this paper:* IVPL, isolated ventilated perfused lung.

TABLE I

Effect of the Experimental Conditions on the Isolated Ventilated Perfused Lung

Parameter	Control	Isoproterenol	Isoproterenol + propranolol	Pilocarpine
Lung dry weight, %	18.0±0.4 (39)*	17.1±0.8 (3)	15.7±0.4 (3)	15.6±0.8 (4)
Lung weight/body weight, %	0.46±0.03 (39)	0.49±0.01 (3)	0.49±0.01 (3)	0.51±0.05 (4)
Perfusate flow, *ml/min*				
Initial	17.3±0.2 (39)	18.0 (2)	16.8±0.9 (3)	18.1±0.7 (4)
Final	15.3±0.4 (39)	17.5 (2)	12.0±1.0 (3)	16.9±1.0 (4)
ATP, *μmol/g dry lung*	11.3±0.3 (8)	11.7±1.2 (3)	10.6±0.9 (3)	10.9±0.6 (4)

The perfusion was for 1 h. The final concentrations of agonists and antagonists in the perfusate were: isoproterenol (0.5 mM), propranolol (0.5 mM), pilocarpine (0.5 mM), and atropine (0.5 mM). Mean±SEM are given.

* Figures in parentheses indicate the number of animals.

for lungs fixed immediately after killing, the volume density for secretory granules and for mitochondria of Clara cells from control rats was 4.2±0.3% (n = 3) and 8.0±0.3% (n = 3), respectively; the values for the same organelles in Clara cells from control IVPL were 3.7±0.5% (n = 6) and 7.8±0.5% (n = 3), respectively. The differences between the same organelles in these two groups were not statistically significant ($P > 0.05$). We wish to point out that the measurements in lungs fixed immediately after sacrifice were performed using a multipurpose test system (9), but morphometry performed on lungs fixed after 1 h of in vitro perfusion and ventilation was done using a lattice-test system. In comparative studies using both test systems on the same cells we found that the lattice-test system gave a volume density that was systematically lower (15%) than the multipurpose test system. Therefore, for the purposes of this comparison only, we have increased the volume density of the intracellular organelles measured with the lattice-test system by 15%. The volume density values given throughout the remainder of this paper were performed using the lattice-test system and have not been subjected to this adjustment.

Effect of pilocarpine and atropine. Pilocarpine given intraperitoneally caused a marked fall in the volume density of the secretory granules of Clara cells in the intact rat (9). In contrast to those results, pilocarpine did not cause a fall in the volume density of the secretory granules when used in the IVPL (Fig. 1). Although the differences were not significant, the slightly greater value for the volume density in the lungs exposed to pilocarpine and the virtually identical value of control compared to the lungs exposed to

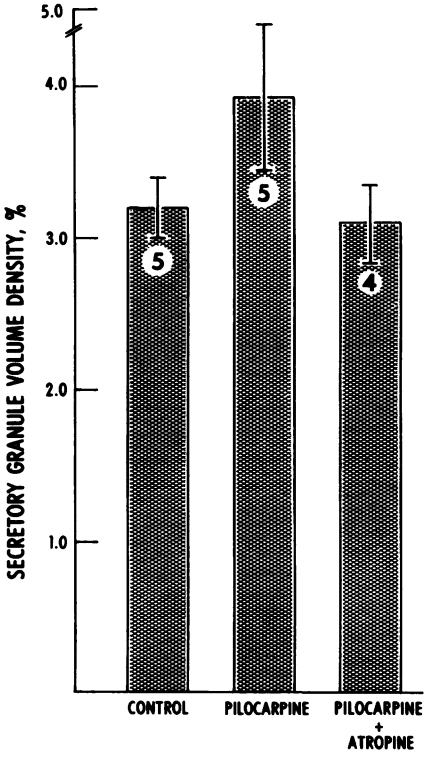

FIGURE 1 Effect of pilocarpine and atropine. Excised rat lungs were ventilated and perfused for 1 h and then fixed for ultra-structural morphometric analysis. The final concentration of pilocarpine and of atropine in the perfusate was 0.5 mM. In all the figures, the volume density of the secretory granules is relative to the number of test points that fell over the entire cell. Mean±SE are given. Figures within the bars indicate the number of experiments. $P > 0.05$ between group means.

pilocarpine and atropine suggested that pilocarpine might in fact be exerting an inhibitory effect on secretion (*vide infra*). Similar results were obtained when the volume density of the secretory granules was expressed relative to the fixed number of points of the test system: control (1.0±0.1% [n = 5], plus pilocarpine (1.2±0.1% [n = 5], and pilocarpine plus atropine (1.1±0.1% [n = 4]). The surface-to-volume ratio (square micrometer per cubic micrometer) of the secretory granules was the same in all groups: control (10.2±0.2 [n = 5]), plus pilocarpine (10.7±0.6 [n = 5]), and plus pilocarpine plus atropine (10.4±0.8 [n = 4]).

β-adrenergic effect and β-adrenergic-cholinergic interaction. Isoproterenol (0.5 mM) caused a marked fall in the volume density of the secretory granules and this fall was blocked by propranolol (Fig. 2); isoproterenol (5 μM) also caused a fall in the volume density to a value of 2.0 (not shown). Our results (Fig. 1) suggested that in the IVPL pilocarpine exerted an inhibitory effect on secretion. When we found that isoproterenol did stimulate Clara cell secretion we thought we could test more rigorously if pilocarpine inhibited secretion by observing its influence on isoproterenol-stimulated secretion. We found that pilocarpine inhibited the fall in secretory granule volume density produced by isoproterenol (Fig. 2). Similar results were obtained when the volume density of the secretory granules was expressed relative to the test system: control (1.0±0.1% [n = 5]), plus isoproterenol (0.47±0.09% [n = 5]), plus isoproterenol plus propranolol (0.87±0.07% [n = 3]), and plus isoproterenol plus pilocarpine (0.80±0.04% [n = 4]). These agents altered the volume density of the secretory granules without changing the surface-to-volume ratio (square micrometer per cubic micrometer) of these granules: control (10.2±0.2), plus isoproterenol (10.4±1.2), plus isoproterenol plus propranolol (9.5±0.4), and plus isoproterenol plus pilocarpine (10.2±0.2).

Effect of cyclic nucleotides. We studied the effect of dibutyryl and 8-bromo-analogs of cAMP and cGMP on secretion. Because we did not find any differences between the analogs for each nucleotide on volume density of the secretory granules, we combined the results of these experiments. The analogs of both cAMP and cGMP decreased the volume density of the secretory granules (Fig. 3). These differences remained when the volume density of the granules was expressed relative to the test system: control (1.0±0.1% [n = 5]), plus cAMP analogs (0.81±0.07% [n = 6]), and plus cGMP analogs (0.46±0.06% [n = 3]). These changes occurred in the absence of any changes in the surface-to-volume ratio (square micrometer per cubic microm-

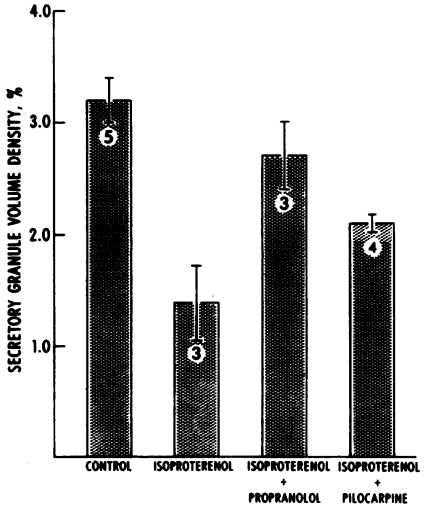

FIGURE 2 Effect of adrenergic and cholinergic agents on secretion. Excised rat lungs were ventilated and perfused for 1 h and then fixed for ultrastructural morphometric analysis. The final concentration of the pharmacological agents in the perfusate was 0.5 mM for each agent. Mean±SE are given. Figures within the bars indicate the number of experiments. $P < 0.001$ between Clara cells of control lungs and Clara cells of lungs perfused with isoproterenol alone. $P < 0.01$ between mean value of lungs perfused with isoproterenol alone and mean value of lungs perfused with isoproterenol plus propranolol or with isoproterenol plus pilocarpine. $P > 0.05$ between mean of control lungs and lungs perfused with isoproterenol plus propranolol: $P < 0.001$ between mean of control lungs and lungs perfused with isoproterenol plus pilocarpine.

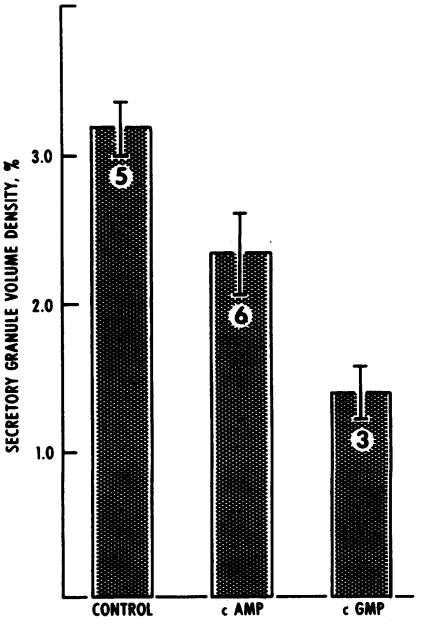

FIGURE 3 Effect of cyclic nucleotides on secretion. Excised rat lungs were ventilated and perfused as described in Fig. 1. The final concentration of the analogs of cAMP and of cGMP was 1.0 mM. Mean±SE are given. Figures within the bars indicate the number of experiments. $P < 0.001$ between control and cAMP and control and cGMP.

eter) of the secretory granules: control (10.2±0.2), cAMP (11.0±0.4), and cGMP (11.1±0.4).

Effect of ventilatory volume. A twofold increase in ventilatory volume produced a 50% fall in the volume density of secretory granules of the Clara cell; propranolol did not block the effect of ventilation (Fig. 4). The effects of these manipulations were similar when the volume density was expressed relative to the test system: control (1.0±0.1% [n = 5]), twice normal tidal volume (0.67±0.11% [n = 3]), and twice normal tidal volume plus propranolol (0.59±0.21% [n = 3]). The surface-to-volume ratio of the secretory granules was not changed by increased ventilation with or without propranolol: control (10.2±0.2), twice tidal volume (10.0±0.1), and twice tidal volume plus propranolol (10.9±0.4).

DISCUSSION

The results of previous experiments on intact rats led us to suggest that secretion by Clara cells might be mediated by cholinergic and adrenergic effectors arranged in series (9). The initial aim of the present study, to further explore this possibility, required that Clara cells be removed from systemic influences. The IVPL seemed to be an appropriate means to meet this requirement because: (*a*) using the IVPL avoids exposing cells to proteases, as would be required to isolate Clara cells; (*b*) in the IVPL Clara cells maintain nearly normal anatomical relations to other cellular as well as extracellular components of the lung; and (*c*) finally, Clara cells in the IVPL are studied under working conditions, i.e., while the lung is being ventilated.

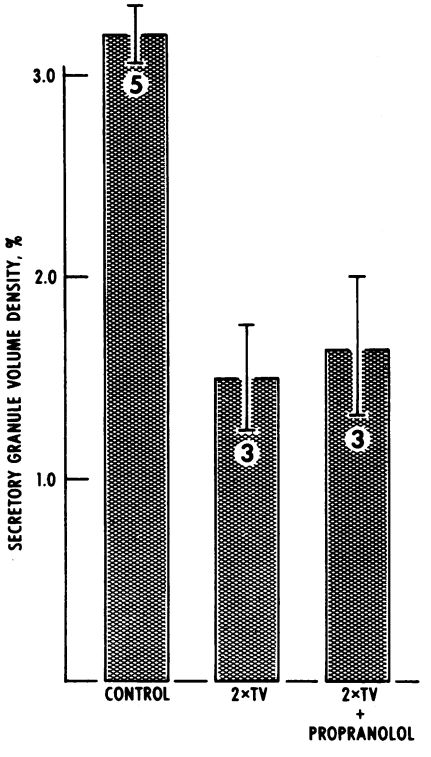

FIGURE 4 Effect of ventilatory volume on secretion. Excised lungs were ventilated and perfused for 1 h and then fixed for ultrastructural morphometric analysis. Control refers to lungs ventilated at a tidal volume based on the body weight of the rat (11); 2 × TV refers to lungs ventilated at twice this tidal volume. The final perfusate concentration of propranolol was 0.5 mM. Mean±SE are given. Figures in the bars indicate the number of experiments. $P < 0.001$ between control and 2 × TV and 2 × TV + propranolol. $P > 0.05$ between 2 × TV and 2 × TV +propranolol.

The IVPL, however, has certain potential disadvantages, including the ease with which it develops edema (22). We think that lungs in our control group did not have more than, at most, a minimal amount of edema. The reasons for this conclusion are (*a*) their percent dry weight was the same after 1 h of perfusion as we previously found at the onset of perfusion (10), (*b*) the ratio of lung weight to body weight was the same after 1 h of perfusion as in freshly excised lungs (23), and (*c*) the percent dry weight (18%) of control lungs was only slightly lower than predicted for bloodless lungs (24).

By these criteria the lungs in our control group and the lungs perfused with isoproterenol would be considered virtually free of edema. Lungs perfused with isoproterenol plus propranolol or with pilocarpine alone, did have edema as evidenced by the percent dry weight and by the fall in flow rate. We do not think the development of edema with propranolol plus isoproterenol limits the interpretation of our results, because propranolol, in spite of the edema, still acted as a beta-adrenergic antagonist. We cannot exclude the possibility that edema interfered with the reaction of pilocarpine in those lungs perfused with this drug, but we think this rather unlikely because the degree of edema is relatively small and because there was no change in the rate of flow of the perfusate.

Our second concern was with the degree to which the lungs' energy metabolism may have become impaired during perfusion. The ATP content after 60 min of perfusion (Table I) was the same as in freshly excised lungs.[2] This indicates that there was not an overall energy deficit in the perfused lung but provides little information on Clara cells. However, comparison of the secretory granules and the mitochondria of Clara cells of lungs fixed immediately after the animals were killed, with the same organelles in Clara cells of lungs fixed after 60 min of perfusion, failed to show any differences in volume density or size (as reflected in the surface-to-volume ratio) of these organelles. We thus believe that these conditions of perfusion and ventilation did not result in severe Clara cell damage which

[2] Chiang, M-J., and D. Massaro. Unpublished observations.

might be manifested by swelling of the mitochondria or secretory granules.

The large decrease (about 50%) in the volume density of the secretory granules elicited by isoproterenol clearly indicates that Clara cells in isolated and perfused lungs can respond to a potential secretagogue. This response occurred at both a high (0.5 mM) and low concentration (5 μM) of isoproterenol. Because the IVPL was isolated from systemic influences and because ventilating gases remained constant, the secretory effect of isoproterenol was not due to an effect of the drug on these processes, as might have been the case in vivo. The failure of pilocarpine to stimulate secretion by Clara cells in the IVPL suggests that its in vivo stimulation of secretion (9) was mediated by an extrapulmonary mechanism. An alternative explanation for the in vivo effect of pilocarpine is that it altered ventilation or pulmonary blood flow and that these changes in some way stimulated Clara cell secretion.

We have so far found that three pharmacological agents (isoproterenol, cAMP, and cGMP) and one mechanical event (increased ventilatory volume) stimulate Clara cell secretion. The effect of isoproterenol and of cAMP are consistent with the findings in other tissues (25), in alveolar type 2 cells (26), and with the notion that cAMP is the intracellular mediator of the stimulatory effect on secretion of beta-adrenergic agonists; these results are also in accord with the observations that beta-adrenergic agonists increase the cAMP content of the lung (27, 28).

The mechanism by which pilocarpine inhibits the secretory effect of isoproterenol in the IVPL is unclear. Precedent for such an inhibition exists in the inhibition by muscarinic agonists of isoproterenol-stimulated cAMP accumulation in heart (29). However, to invoke this mechanism in the lung we must reconcile the cholinergic increase of the cGMP content of the lung (27) and our finding that analogs of cGMP stimulate Clara cell secretion. This apparent paradox could be explained by cellular or intracellular compartmentalization of the response of the cyclic nucleotides to adrenergic or cholinergic stimuli. Thus, although cholinergic agonists increase the cGMP content of the lung as a whole, they might not effect Clara cells the same way. Alternatively, pilocarpine might increase the cGMP content of Clara cells, but the cGMP produced could occupy receptor sites not related to Clara cell secretion; evidence for such a functional compartmentalization has been obtained from studies on isolated Leydig cells (30).

The stimulation of Clara cell secretion by increased ventilatory volume is interesting because increased tidal volume ventilation accelerates the rate of upward movement of the extracellular material lining pulmonary airspaces (31, 32). Greater rates of secretion during periods of increased tidal volume ventilation might be needed to meet the larger replacement requirement engendered by more rapid loss. The failure of propranolol to block the secretory effect of increased tidal volume breathing indicates this ventilatory event is not mediated by a beta-adrenergic mechanism.

The secretory effect of increased tidal volume and of cGMP are of particular interest when considered together and viewed in light of the report that large volume ventilation markedly increases the cGMP content of the lung (33). These observations suggest that cGMP may be involved in the intracellular mediation of the mechanico-secretion coupling. The triggering mechanism responsible for stimulating the increase in cGMP is unknown; possible initiating factors include mechanical deformation of the plasma membrane of the Clara cells, and the effect on Clara cells of bioactive materials released by mechanical deformation of the lung (34, 35).

In summary, our work suggests that there are at least two mechanisms by which Clara cells can be stimulated to secrete. One seems to be beta-adrenergic-cAMP mediated by the triggering event in unknown. The other is initiated by an increase in tidal volume; cGMP may participate in the intracellular mediation of this stimulus. Finally, we have found evidence for beta-adrenergic (stimulatory) -cholinergic (inhibitory) antagonism in the regulation of Clara cell secretion.

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