Ultrastructural Localization of Phospholipases in the Clara Cell of the Rat Bronchiole

Kokichi Yoneda, MD

The Clara cell of the bronchiole is unique to the lung; the cell's function is not clear. The localization of the lipid-hydrolase enzymes phospholipase, lysophospholipase, and lipase was examined ultrastructurally in the Clara cell of the rat bronchiole. The secretory granules of the Clara cell showed a strong reaction of lysophospholipase and a weak reaction of lipase. Phospholipase activity was not detected intracellularly. These findings suggest that the Clara cell secretes lipase-phospholipase into the bronchiolar lumen, thus catabolizing the pulmonary surfactant phospholipids. (Am J Pathol 93:745–752, 1978)

THE NONCILIATED EPITHELIAL CELLS of the bronchiole, Clara cells, display a characteristic morphology and are one of the cells unique to the lung.[1] Their localization in the bronchiole suggests an important function in the peripheral airway. In spite of numerous morphologic and cytochemical studies, the function of the Clara cell is not clear.[2-7] The presence of abundant endoplasmic reticulum, membrane-bound granules, well-developed Golgi complex, and high oxidative enzyme activity suggests a secretory function. The formation and degranulation of the secretory granules under pilocarpine stimulation appear to confirm the secretory activity of these cells.[8]

Niden proposed that the Clara cell secretes pulmonary surfactant [9] and others support this hypothesis.[10,11] Recent studies, however, established the Type II pneumonocyte as the source of pulmonary surfactant.[12] Compared with the lamellar bodies of the Type II pneumonocyte, the secretory granules of the Clara cell show different morphologic and cytochemical features: crystalline configuration,[6] pepsin digestibility,[3] and amino acid uptake on autoradiography.[13] These findings indicate the presence of protein in the secretory granules of the Clara cell. Biochemical studies, on the other hand, suggest the existence of phospholipid catabolizing enzymes in the peripheral airway.[14] Based on the hypothesis that the Clara cell may participate in the catabolism of pulmonary surfactant phospholipids, we studied lipid-hydrolase activity of Clara cells cytochemically.

From the Laboratory Service, Veterans Administration Hospital, and the Department of Pathology, University of Kentucky College of Medicine, Lexington, Kentucky.

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Address reprint requests to K. Yoneda, MD, Laboratory Service (113CD), Veterans Administration Hospital, Lexington, KY 40507.

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The enzymes studied were phospholipase A, lysophospholipase, lipase, and acid phosphatase.

Materials and Methods

The experiments were repeated five times with reproducible results. In each experiment, 4 male Sprague-Dawley rats weighing 200 to 300 g were anesthesized by intraperitoneal injections of sodium pentobarbital. The chest was opened to collapse the lungs, the trachea was cannulated, and the lungs were inflated through the tracheal cannula with Karnovsky's paraformaldehyde–glutaraldehyde fixative [15] at 4 C in 0.2 M cacodylate buffer, pH 7.4, at a pressure of 25 cm of fixative. The pressure was maintained for 5 minutes, after which the trachea was ligated and the lungs were removed. The fixation was continued by immersion of the lungs in the cooled fixative for 1 hour. The lungs were cut by a Smith-Farquhar TC-2 tissue sectioner (DuPont Instrument Co., Newton, Conn) with a setting of 200 μ and rinsed in 0.2 M cacodylate buffer, pH 7.4, for 1 hour at 4 C prior to incubation.

Gomori's method for lipase cytochemistry modified by Nagata [16] was used for phospholipase A, lysophospholipase, and lipase. The incubation medium consisted of 1.0 ml of 5% substrate solution, 2.5 ml of 0.2 M tris-maleate buffer, 1.0 ml of 10% aqueous calcium chloride, and 20.5 ml of distilled water. The substrate solution employed was tween 80 (polyoxyethylene sorbitan mono-oleate) for lipase, palmitoyl-lysophosphatidylcholine for lysophospholipase, and dipalmitoyl phosphatidylcholine in 0.1% triton X-100 for phospholipase A. Phosphatidylcholine and lysophosphatidylcholine were tested for purity by thin-layer chromatography before use.[17] Two sets of controls were used in each of the experiments.

In the first experiment, tissue was incubated in the same media as the experimental tissues, but without the substrate. The second control set of tissue was immersed in the respective buffer and heated for 1 hour at 60 C to inactivate the enzyme prior to incubation. The tissue was washed with three changes of tris-maleate buffer with a pH corresponding to that of the incubation medium. For the determination of the optimal pH of enzyme reaction, the pH of the incubation media used varied in increments of 0.5 and ranged from pH 4.5 to 9.0. All tissue was then incubated at 37 C for 18 hours. After incubation, the tissue was washed in 2% EDTA in 0.2 M cacodylate buffer, pH 7.2, for 5 minutes to remove calcium and was then immersed in 0.15% aqueous lead nitrate solution for 10 minutes at room temperature to substitute calcium. Thereafter the tissue was washed in 0.2 M cacodylate buffer for 5 minutes and postfixed in 2% osmium tetroxide in 0.2 M cacodylate buffer for 1 hour at 4 C.

Acid phosphatase was demonstrated according to Gomori's method.[18] The incubation medium consisted of 100 ml 0.05 M acetate buffer, pH 5.0, 1.0 ml 12% lead nitrate, and 10 ml 0.1 M sodium-beta-glycerophosphate. The incubation was carried out for 30 minutes and 1 hour at 37 C. After incubation, the tissue blocks were washed in 0.2 M cacodylate buffer and postfixed in 2% osmium tetroxide in cacodylate buffer for 1 hour at 4 C. After osmification, the tissue was dehydrated in graded ethanol solution, passed through propylene oxide, embedded in epon 812, and polymerized at 60 C for 48 hours. The thin sections were cut by a Porter-Blum MT2 ultramicrotome. All the sections were examined in a Philips 300 electron microscope.

Results

The results of the enzyme cytochemistry of the secretory granules of the Clara cell in comparison to the lysosomes of the alveolar macrophages are shown in Table 1.

Phospholipase A activity was not demonstrated intracellularly in the Clara cell at any pH. At the alkaline pH, linear lead deposits were seen

Table 1—Enzyme Cytochemistry of Clara Cells and Alveolar Macrophages

	Clara cell secretory granule	Alveolar macrophage lysosome
Phospholipase	–	–
Lysophospholipase	+ pH 7.5–8.0	+ pH 4.5–5.0
Lipase	+ pH 7.2	+ pH 7.2
Acid-pH-ase	± pH 5.5–6.0	+ pH 5.5–6.0

– = negative reaction; + = positive reaction; pH = optimal pH for reaction.

extracellularly in the bronchiolar lumen as well as attached to the cell surface (Figures 1 and 2).

Lysophospholipase activity was demonstrated most intensely at pH 7.5 to 8.0 in the Clara cells (Figure 3). The lead deposits were demonstrated at the periphery of the spherical as well as rod-shaped secretory granules. These deposits were also at the outer membrane of mitochondria and in the rough endoplasmic reticulum (Figure 4). Heavy deposits of the reaction product were seen in the discrete membrane-bound inclusions in basilar portion of the cell. Linear deposits were demonstrated extracellularly in the lumen and on the cell surface. Among the other cells, the lysosomes of the alveolar macrophages demonstrated a constant reaction with lysophosphatidylcholine at pH 4.5 to 5.0.

The ciliated epithelial cells show deposition of reaction product at the outer membrane of mitrochondria and in the endoplasmic reticulum. In the alveoli, the Type II pneumonocyte showed heavy deposits in lamellar inclusion bodies and multivesicular bodies. The Type I pneumonocyte did not show any reaction product. These reaction products were most intensely observed at an alkaline pH.

Lipase activity was demonstrated in similar locations as lysophospholipase activity; however, the deposition of reaction product was lighter.

Acid phosphatase activity was demonstrated in discrete-membrane-bound inclusions in the basilar portion of the Clara cell. Some of the apical secretory granules showed some deposition of reaction product; however, the majority of the secretory granules were free of reaction product. Alveolar macrophages showed heavy deposits of reaction product in lysosomes.

Discussion

In the present study, the author studied lipid hydrolase activity of the Clara cells. Any cytochemical findings should be augmented by a biochemical assay. In the lung, however, this is extremely difficult because of the heterogeneity of the component cell population. The presence of large amounts of phospholipids and phospholipases in the pulmonary tissue

hinders the correlation of biochemical data to cytochemistry. In that sense, this study is tentative concerning the function of Clara cells.

In the lysophospholipase experiment, heavy deposits of reaction product were demonstrated in the secretory granules, at the outer membrane of the mitochondria, and in the endoplasmic reticulum. The phospholipase A experiment, on the other hand, failed to demonstrate any reaction product intracellularly. This negative finding, however, may not indicate the absence of phospholipase A activity in the Clara cell, since the substrate preparation used was in emulsion and the substrate may not have penetrated the cell. The lipase was demonstrated at the same locations as in the lysophospholipase experiment, although the deposits of reaction product were lighter. The comparison of activities of these two enzymes based on these experiments is not justified, since each experiment employs a different substrate preparation and the substrate penetration into the cell may be different. The existence of lipase in the Clara cell was documented by Caulet et al [5] on the light microscopic level. This study extended their findings to the electron microscopic level. These findings indicate the existence of lipase–phospholipase in the secretory granules of the Clara cell.

Lipase–phospholipase is a component of lysosomes.[19] The existence of these enzymes in the secretory granules of the Clara cell may indicate lysosomal enzyme activity, although acid phosphatase was not constantly demonstrated in the secretory granules in this study or in others.[7] The existence of lysosomal enzymes in secretory granules has been shown in several cells.[20-23] The function of lysosomal enzymes in secretory granules is not clear. The lipase–phospholipase, on the other hand, may be a secretory product of Clara cells. The intracellular localization is similar to that of lipase in the pancreatic acinar cell.[24] In this study, experimental groups showed constant heavy linear deposits of reaction product extracellularly in the bronchiolar lumen and on the cell surface. These were not demonstrated in control groups. In spite of their absence in the control groups, they may represent nonspecific lead deposits trapped in the hypophase of bronchiolar surfactant layer. It is also possible that they represent phospholipase activity of the hypophase of surfactant layer. The existence of phospholipase in the terminal airway was indicated by a biochemical study.[14] Another point of interest in this regard is the apparent optimal pH of the lysophospholipase in Clara cells in comparison to that of the lysosomes of alveolar macrophages. Sahu and Lynn demonstrated the existence of two kinds of phospholipase in the lung.[25] One is apparently derived from the lysosome of the alveolar macrophage and has an acidic optimal pH.[26] The other is an insoluble phospholipase and has

an alkaline optimal pH.[25] The latter is shown to increase in alveolar proteinosis. Our findings may indicate the Clara cell as a possible source of this enzyme. The study of Clara cells in alveolar proteinosis appears to be of interest. To clarify the function of the Clara cell, a different methodology, such as using a purified Clara cell population, was indicated.

The lysophospholipase was not limited to the macrophages and Clara cells. The ciliated epithelial cell and the Type II pneumonocyte showed the reaction. Presence of the reaction in ciliated cells is not surprising, since phospholipases appear to be microsomal enzymes and are shown to have wide distribution.[27] The reaction in the lamellar inclusion bodies of the Type II cell was expected, considering their active role in pulmonary surfactant synthesis, although the existence of phospholipid catabolizing enzyme in the lamellar bodies is debated.[28,29]

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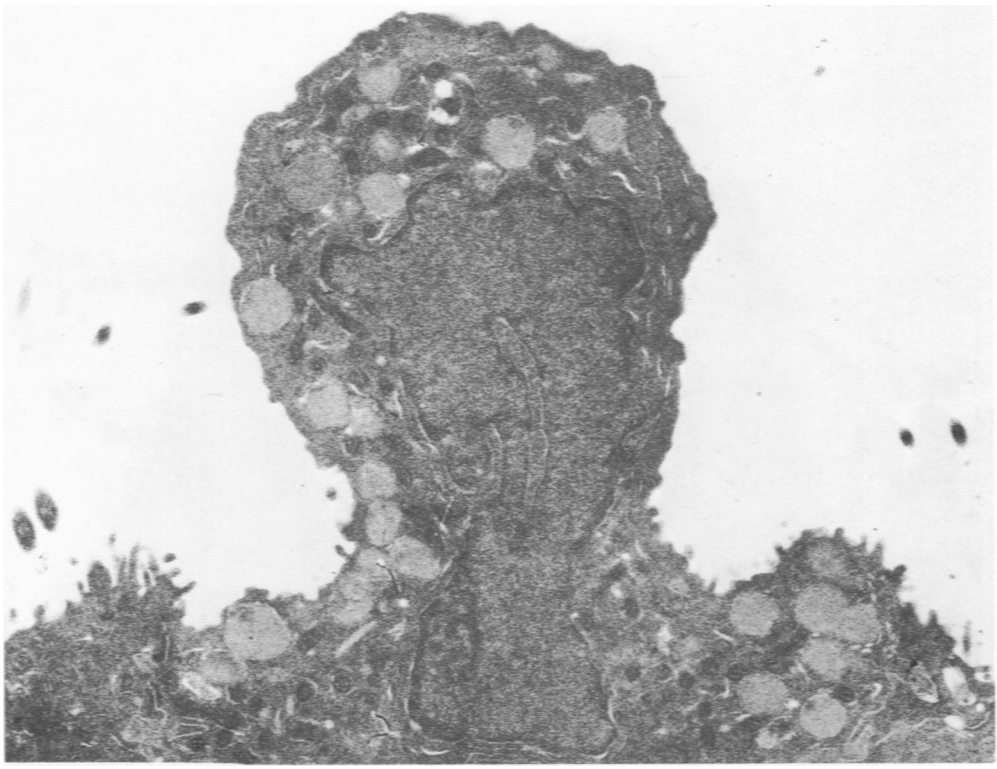

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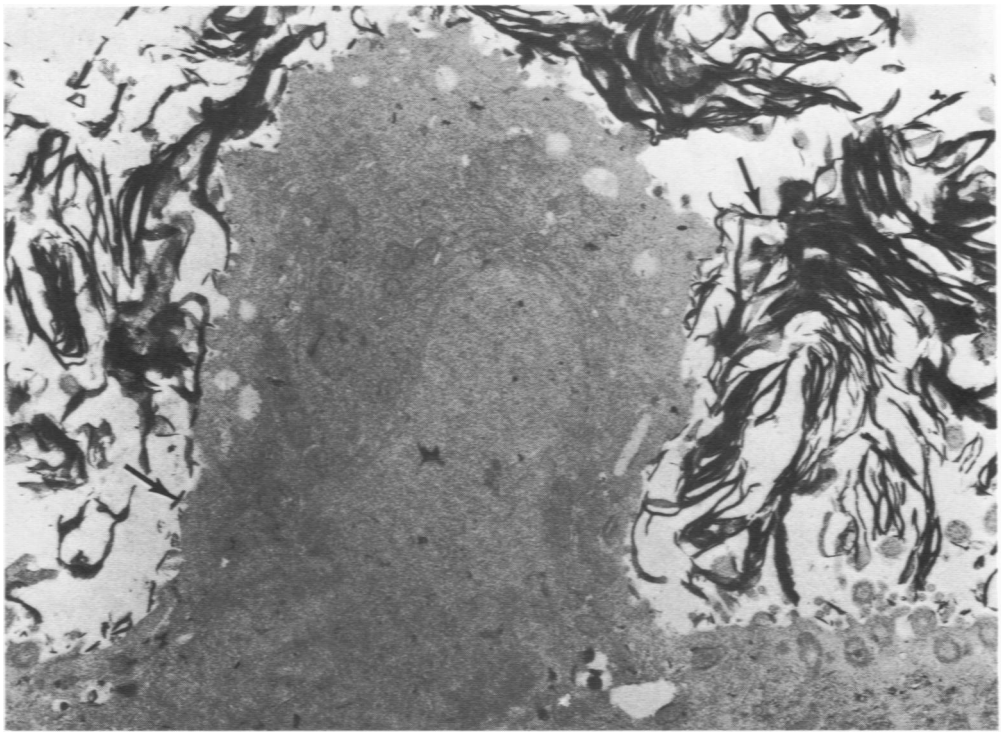

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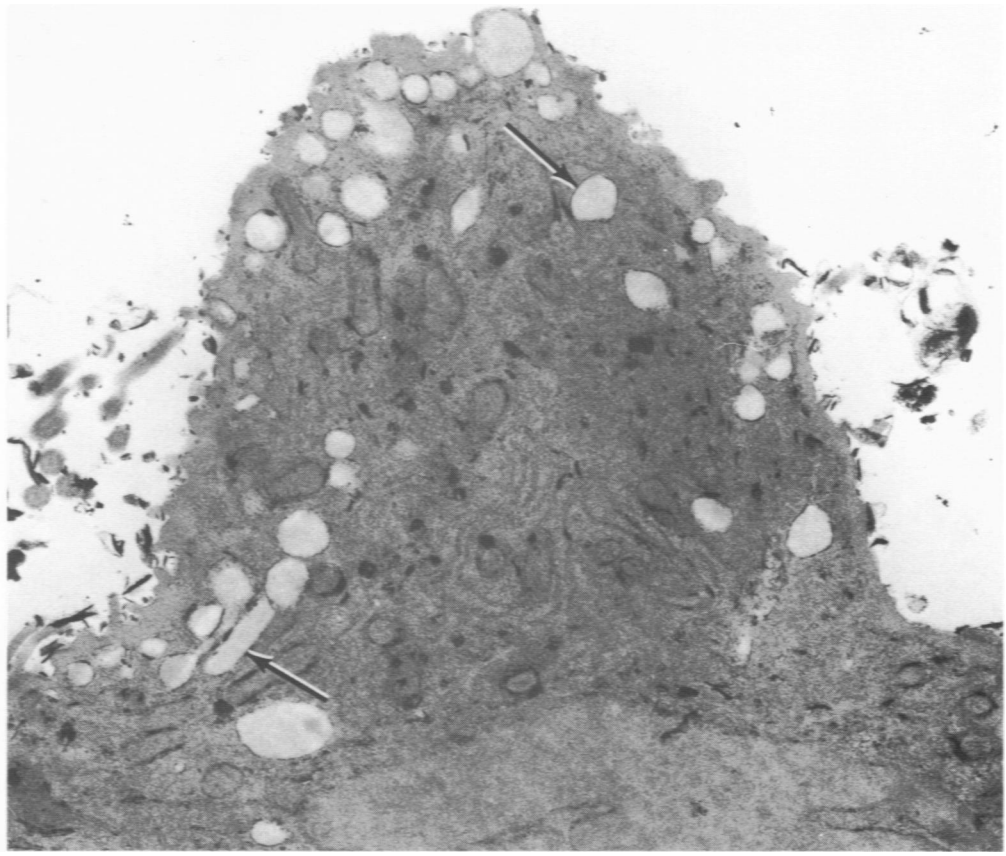
Figure 1—Control Clara cell. No reaction product is present. (×11,800) **Figure 2**—Clara cell in phospholipase A experiment. Linear lead deposits are seen on the cell surface and in the lumen. (×13,000)



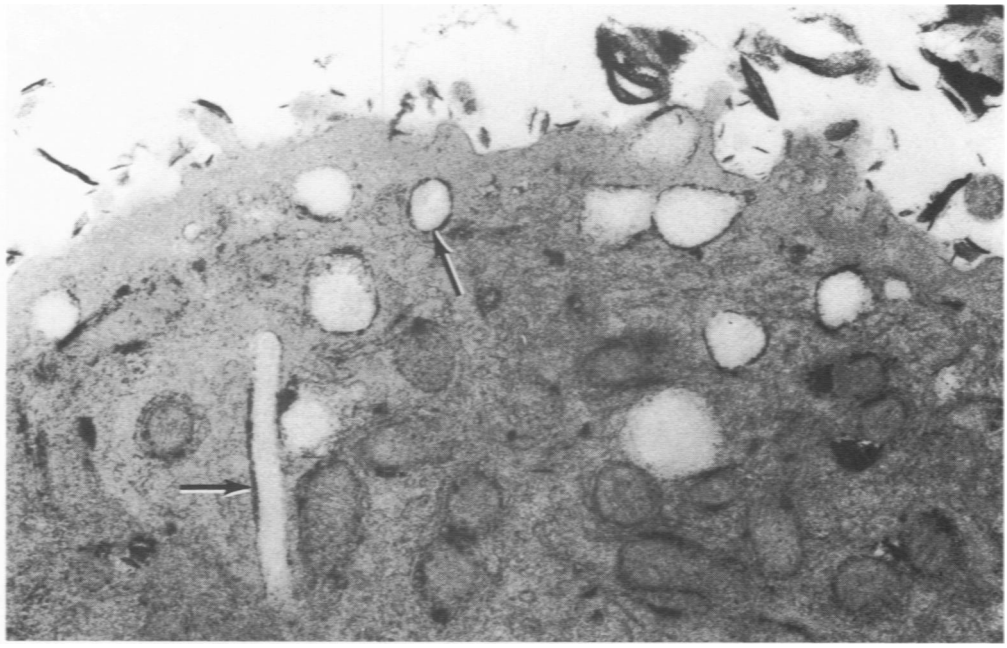


Figure 3—Clara cell in lysophospholipase experiment. Lead deposits are evident in secretory granules, mitchondria, and endoplasmic reticulum. Extracellular deposits are on the cell surface and in the lumen. (×16,500) **Figure 4**—Higher magnification of Clara cell in lysophospholipase experiment. Heavy lead deposits are seen at the periphery of spherical as well as rod-shaped secretory granules. (×35,000)