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# ULTRASTRUCTURE OF THE EPITHELIUM OF TERMINAL BRONCHIOLES IN RABBITS AFTER THE ADMINISTRATION OF ACETYLCHOLINE

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**Abstract**

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In this experiment, the ultrastructure of the epithelium of terminal bronchioles was studied 5 and 20 minutes after intravenous administration of two different doses of acetylcholine. The administration of 0.5 mg acetylcholine resulted in pathological alteration of the cytoplasm of epithelial cells recorded 5 and 20 minutes post exposure. In the first phase, the secretion of Clara cells was inhibited. Twenty minutes after the acetylcholine administration, a mild stimulation of these cells to discharge the content of their secretory granules was observed. After the administration of 0.1 mg acetylcholine, the signs of pathological alteration of the epithelial cell cytoplasm were mild. Twenty minutes post exposure, the findings did almost not differ from those in the epithelium of healthy control rabbits. After an initial inhibition, the secretory activity of Clara cells was resumed and did not differ from that found in controls. No increased proliferation of epithelial cells was recorded in any experimental group.

*Airways, ciliated cells, Clara cells, cholinergic stimulation, electron microscopy*

Recently, the mechanisms controlling the functions of respiratory organs have become one of the main topics of authors dealing with the structure and function of the respiratory system. One of the most important mechanisms controlling the functions of airways, including the function of secretory elements and ciliary border, is the parasympathetic innervation with acetylcholine serving as a neurotransmitter (Fung et al. 1992; Kamijo et al. 1993; Ramnarine et al. 1996; Steel and Hanrahan 1997; Salathe et al. 1997; Wessler et al. 1998). The stimulating effect of the acetylcholine administration on the secretory cells of the lining epithelium of airways and digestive tube was repeatedly demonstrated in experiments *in vitro* or *in vivo* (Specian and Neutra 1980; Phillips and Wilson 1993; Tokuyama et al. 1990; Konrádová et al. 1996ab). The authors described the effect of the acetylcholine administration on the mucus-producing goblet cells. However, the reaction of the distal airways, where the secretory elements are represented by Clara cells, has not been studied in detail and therefore we investigated the effect of the intravenous administration of two different doses of acetylcholine on the ultrastructure of the epithelium of the terminal bronchioles in rabbits.

## Materials and Methods

Fifteen clinically healthy rabbits (males, body weight 1,520 g - 3,800 g) were used. Three untreated rabbits served as controls and 12 animals were divided in two experimental groups (n = 6). They were given 0.1 mg or 0.5 mg of acetylcholine chloride (Acetylcholinum ophthalmicum Dispersa, Ciba, Niederwangen, Belgium) i.v., respectively. The material was always collected from 3 rabbits 5 and 20 minutes after i.v. administration of each dose of the drug, respectively. In the animals, the thorax was opened under general anaesthesia (ketamine 35 mg/kg and xylazine 5 mg/kg i.m.), the lungs were removed and immediately perfused by 5% glutaraldehyde in 0.1 M cacodylate buffer

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(pH 7.2). From one pulmonary lobe, tiny pieces of the tissue were collected, fixed for 90 minutes in the same fixative and then for 60 minutes in 2% $OsO_4$ in 0.1 M cacodylate buffer (pH 7.4). The material was dehydrated in graded series of alcohol and embedded in a Durcupan-Epon mixture. Terminal bronchioles were localized in semithin sections stained with toluidine blue. Ultrathin sections were prepared on Ultrotome Nova (LKB, Broma, Sweden), contrasted with uranyl acetate and lead citrate and examined in JEM 100 C electron microscope (Jeol, Tokyo, Japan).

For quantitative evaluation, the total number of ciliated and Clara cells, and the functional state of Clara cells were recorded in the electron microscope using the method described in our previous paper (Uhlík 1996). The actual values are given in Table 1. To compare the results in all experimental groups, the $\chi^2$ test of homogeneity in frequency tables was used. To specify categories causing deflections from the hypothesis of homogeneity, adjusted standardized deviations were used.

Table 1
Quantitative evaluation of the epithelium of terminal bronchioles in rabbits 5 and 20 minutes after i.v. administration of 0.1 and 0.5 mg acetylcholine (actual values)

| | Controls | | | | Ach 0.1 mg 5 min. | | | | Ach 0.1 mg 20 min. | | | | Ach 0.5 mg 5 min. | | | | Ach 0.5 mg 20 min. | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Rabbit | 1 | 2 | 3 | total | 4 | 5 | 6 | total | 7 | 8 | 9 | total | 10 | 11 | 12 | total | 13 | 14 | 15 | total |
| Ciliated cells | 127 | 77 | 80 | 284 | 52 | 62 | 24 | 138 | 32 | 84 | 136 | 252 | 51 | 43 | 102 | 196 | 17 | 49 | 33 | 99 |
| Clara cells | 123 | 96 | 98 | 317 | 65 | 78 | 32 | 175 | 76 | 122 | 173 | 371 | 88 | 79 | 98 | 265 | 31 | 50 | 20 | 101 |
| CC with granules | 79 | 73 | 81 | 233 | 55 | 70 | 22 | 147 | 63 | 108 | 119 | 290 | 77 | 48 | 90 | 215 | 11 | 37 | 16 | 64 |
| CC without granules | 44 | 23 | 17 | 84 | 10 | 8 | 10 | 28 | 13 | 14 | 54 | 81 | 11 | 31 | 8 | 50 | 20 | 13 | 4 | 37 |

CC = Clara cells, Ach = acetylcholine

## Results

### 1. The ultrastructure of the epithelium of terminal bronchioles in control rabbits

Terminal bronchioles of healthy control rabbits were lined by a simple epithelium where low columnar or cuboidal ciliated cells and high columnar Clara cells alternated almost regularly. In our previous paper, the arrangement and ultrastructure of both types of epithelial cells were described in detail (Uhlík 1996).

In the epithelium of the terminal bronchioles of healthy control rabbits, the Clara cells and the ciliated ones represented 52.7 ± 3.6% and 47.3 ± 3.6% of epithelial cells, respectively (Fig. 1). In the majority of Clara cells (73.5 ± 9.4%), secretory granules were discovered. The granules were not revealed only in 26.5 ± 9.4% of them (Fig. 2).

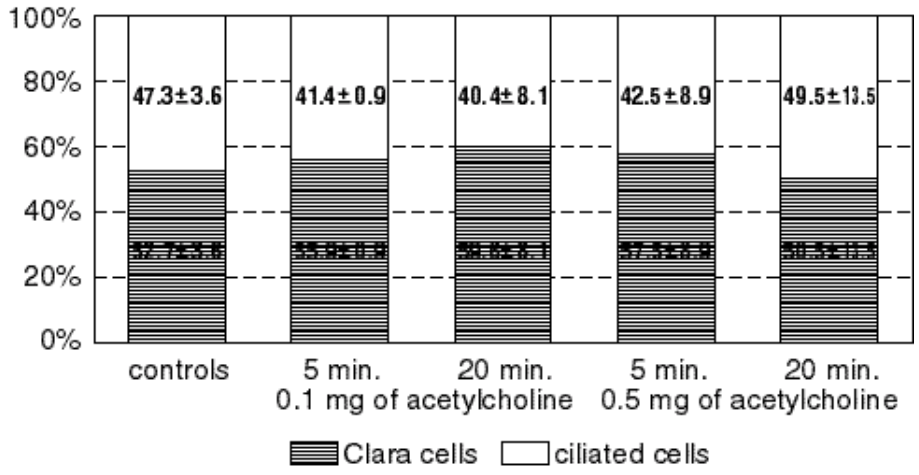


Fig. 1. Quantitative evaluation of the epithelium of terminal bronchioles in rabbits 5 and 20 minutes after the i. v. administration of 0.1 and 0.5 mg of acetylcholine.

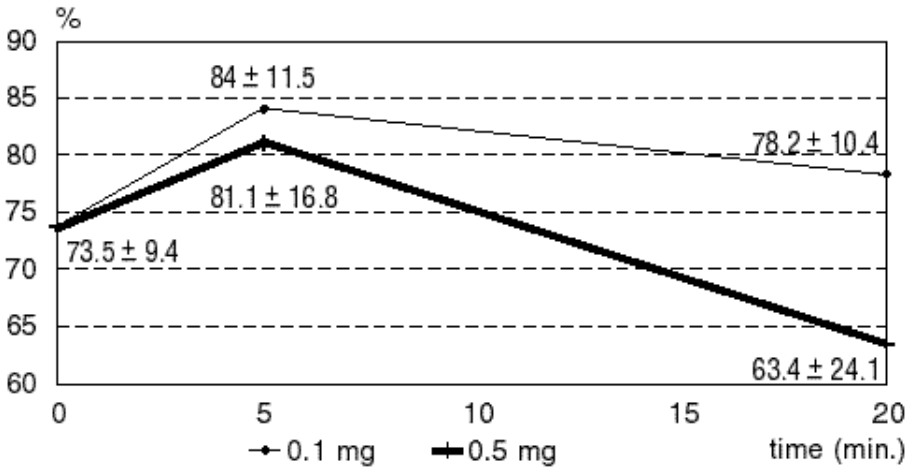


Fig. 2. Clara cells containing secretory granules in the epithelium of terminal bronchioles in rabbits 5 and 20 minutes after the i. v. administration of 0.1 and 0.5 mg of acetylcholine.

## 2. The ultrastructure of the epithelium of terminal bronchioles in rabbits 5 minutes after i.v. administration of 0.5 mg acetylcholine

Five minutes after administration of 0.5 mg acetylcholine, the terminal bronchioles were lined by an altered simple epithelium consisting of ciliated and Clara cells. Intercellular spaces were narrow, apical junctional complexes remained intact (Plate V., Fig 3).

The cytoplasm of ciliated cells contained voluminous lysosomes and many smaller vacuoles. Mitochondria revealed an electron-lucent matrix and altered cristae. Cisternae of the Golgi complex were dilated. On the apical surfaces of the ciliated cells, the formation of cytoplasmic protrusions sometimes containing axonemes of degenerating cilia was observed (Fig. 4). Less altered, pathological cilia were often encountered, too.

In the cytoplasm of Clara cells, a distinct dilatation of tubules of the smooth endoplasmic reticulum filled with an electron-lucent content was revealed. The alteration of the smooth endoplasmic reticulum was unequally pronounced in individual cells. In several of them, signs of the vacuolar degeneration (Fig. 5) and impairment of the apical plasma membrane were revealed. Remnants of their degenerated cytoplasm were then found above the epithelium in the lumen of terminal bronchioles (Fig. 6). In the less altered Clara cells, mitochondria with an electron-lucent matrix and dilated spaces of the rough endoplasmic reticulum were encountered. Secretory granules were usually present in the cytoplasm of Clara cells, but the exocytosis of these granules was noticed only exceptionally (Plate VI., Fig. 7).

Ciliated and Clara cells represented 42.5 ± 8.9% and 57.5 ± 8.9%, respectively (Fig. 1). 81.1 ± 16.8% of the Clara cells contained secretory granules in their cytoplasm (Fig. 2). This value differed significantly ($\alpha = 0.01$) from that revealed 20 minutes after administration of the same dose of acetylcholine.

## 3. The ultrastructure of the epithelium of terminal bronchioles in rabbits 20 minutes after i.v. administration of 0.5 mg acetylcholine

Twenty minutes after administration of 0.5 mg acetylcholine, the epithelium of terminal bronchioles was still altered. Intercellular spaces remained narrow and apical junctional complexes were intact (Fig. 8).

Ciliated cells contained slightly altered mitochondria, dilated Golgi complex, tiny vacuoles, numerous lysosomes and exceptionally isolated ciliated vacuoles in their cytoplasm. In the small apical cytoplasmic blebs, disintegrating axonemes of cilia were often contained. Differentiating ciliated cells were observed more frequently than in the previous experimental group.

In the cytoplasm of Clara cells, tubules of smooth endoplasmic reticulum were dilated, but the degree of their swelling was less pronounced compared with the previous experimental group (Fig. 8). Mitochondria revealed mild signs of pathological alteration and tiny lysosomes were occasionally observed. Apical cytoplasmic protrusions containing secretory granules were often found on the Clara cells.

The epithelium was composed of 49.5 ± 13.5% and 50.5 ± 13.5% of ciliated and Clara cells, respectively (Fig. 1). Secretory granules were found in only 63.4 ± 24.1% of Clara cells (Fig. 2). This value differed significantly ($\alpha = 0.01$) from those revealed 5 and 20 minutes after administration of the lower dose and 5 minutes after the administration of the same dose of acetylcholine.

### 4. The ultrastructure of the epithelium of terminal bronchioles in rabbits 5 minutes after i.v. administration of 0.1 mg acetylcholine

Five minutes after administration of 0.1 mg acetylcholine, the degree of alteration of the epithelium of rabbits' terminal bronchioles was mild.

In the cytoplasm of ciliated cells, only a small increase in number of lysosomes and tiny vacuoles was observed. Slightly dilated tubules of smooth endoplasmic reticulum represented the only signs of pathological alteration of the cytoplasm of Clara cells.

The epithelium comprised 44.1 ± 0.9% and 55.9 ± 0.9% of ciliated and Clara cells (Fig. 1). In 84 ± 11.5% of Clara cells, secretory granules were observed (Fig. 2). This value differed significantly ($\alpha = 0.01$) from those found in controls and 20 minutes after the administration of 0.5 mg of acetylcholine.

### 5. The ultrastructure of the epithelium of terminal bronchioles in rabbits 20 minutes after i.v. administration of 0.1 mg acetylcholine

Twenty minutes after administration of 0.1 mg acetylcholine, the epithelium of terminal bronchioles revealed almost no signs of pathological alteration.

In the cytoplasm of ciliated cells, only individual lysosomes with heterogeneous contents were seen (Fig. 9). On their apical surfaces, isolated small cytoplasmic protrusions were developed. The Clara cells contained an intact cytoplasm and the contents of their secretory granules were discharged only occasionally (Fig. 10).

In the epithelium, 40.4 ± 8.1% of ciliated cells and 59.6 ± 8.1% of the Clara cells were found (Fig. 1); 78.2 ± 10.4% of Clara cells contained secretory granules in their cytoplasm (Fig. 2). This value differed significantly ($\alpha = 0.01$) from the lowest value found 20 minutes after administration of the higher dose of acetylcholine.

## Discussion

The influence of cholinergic stimulation on the structure and function of the secretory epithelial cells was described by several authors. Nevertheless, all these papers dealt with mucus-producing elements, usually the goblet cells in the intestinal or airway epithelium. Both *in vitro* and *in vivo*, Specian and Neutra (1980) and later also Phillips and Wilson (1993) demonstrated that the administration of acetylcholine on the intestinal mucous membrane of rats and rabbits resulted not only in very rapid evacuation of goblet

cells, but changed also their secretory mechanism. Roumagnac and Laboisse (1987) described an increase in mucus secretion in the organ culture of human intestinal carcinoma after the addition of acetylcholine to the culture medium. The effect of cholinergic stimulation on the secretion of goblet cells was studied by Tokuyama and his co-workers in the airways of guinea pigs (Tokuyama et al. 1990). Using a light microscope, they observed a decrease in the mucus content in the goblet cells of trachea and large bronchi after an electric stimulation of cervical vagal nerves. Kamijo with co-workers studied the exocytosis of the secretion from the goblet cells and glands in the rat nasal mucous membrane due to the administration of various neurotransmitters (Kamijo et al. 1993). The authors again demonstrated a pronounced stimulating effect of the acetylcholine administration.

The effect of acetylcholine administration on the ultrastructure of the rabbit tracheal epithelium was studied in our laboratory several years ago (Konrádová et al. 1996ab). The acetylcholine administration affected mainly goblet cells. In highly stimulated goblet cells, the mechanism of secretion was accelerated. In some secretory elements, the apocrine secretion and also chain exocytosis were recorded. The completely exhausted goblet cells usually did not take part in the next secretory cycles, but degenerated and were gradually expelled from the epithelium. After the administration of both doses of acetylcholine, more than 90% of tracheal goblet cells were stimulated. After the administration of 0.5 mg acetylcholine, the peak of the reaction was reached within 5 minutes. After the administration of 0.1 mg acetylcholine, the reaction was more prolonged. Twenty minutes after the administration of 0.5 mg acetylcholine, a marked increase in the number of differentiating secretory elements accompanied with changes in their distribution resulting in the formation of intraepithelial mucous glands was recorded.

The reaction of the epithelium of terminal bronchioles differed from that of the tracheal epithelium. The influence of acetylcholine was also concentrated on the secretory cells. Similar to the effects of administration of various toxicants (naphthalene, 4-ipomeanol, trichloroethylene) (Plopper et al. 1992ab, 1994a; Van Winkle et al. 1995; Lakritz et al. 1996; Giovanetti et al. 1998), inhalation of ozone (Harkema et al. 1993; Plopper et al. 1994b), or tracheobronchography (Uhlík and Tůma 1998), response of the Clara cells to the administration of acetylcholine resulted in pathological alteration of their cytoplasm. In the first phase post exposure, the secretion of the Clara cells was inhibited and the number of cells containing secretory granules slightly increased. Twenty minutes after administration of the higher dose of acetylcholine, the Clara cells were mildly stimulated to discharge their secretion. However, the quantitative evaluation did not show any significant changes in the distribution of the cells in any of the experimental groups compared to control groups.

In contrast to the stimulation, degeneration and consecutive differentiation of tracheal goblet cells, the reaction of the secretory elements of terminal bronchioles on the acetylcholine administration was less pronounced. Nevertheless, significant changes in the secretory activity of Clara cells were recorded.

**Ultrastruktura epitelu terminálních bronchiolů králíků po aplikaci acetylcholinu**

Sledovali jsme reakci epitelu terminálních bronchiolů králíků vyvolanou intravenózním podáním dvou různých dávek acetylcholinu. Materiál pro elektronově mikroskopické vyšetření jsme odebírali 5 a 20 minut po aplikaci zkoumané látky. Aplikace 0,5 mg acetylcholinu vyvolala v epitelu terminálních bronchiolů vznik známek patologické alterace cytoplazmy buněk, které byly patrné i po 20 minutách. Vylučování sekretu Clara buňkami bylo v první fázi utlumeno, 20 minut po aplikaci acetylcholinu však docházelo k mírné stimulaci sekrece. Po aplikaci 0,1 mg acetylcholinu byly známky patologické alterace

cytoplazmy epitelových buněk méně vyjádřeny, po 20 minutách se již nálezy prakticky nelišily od nálezů u kontrolních zdravých králíků. Po počátečním útlumu sekrece Clara buněk docházelo v druhé fázi k návratu sekreční aktivity na úroveň kontrolních zvířat. V žádné experimentální skupině jsme nenalezli známky zvýšené proliferace epitelových buněk.

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Plate V
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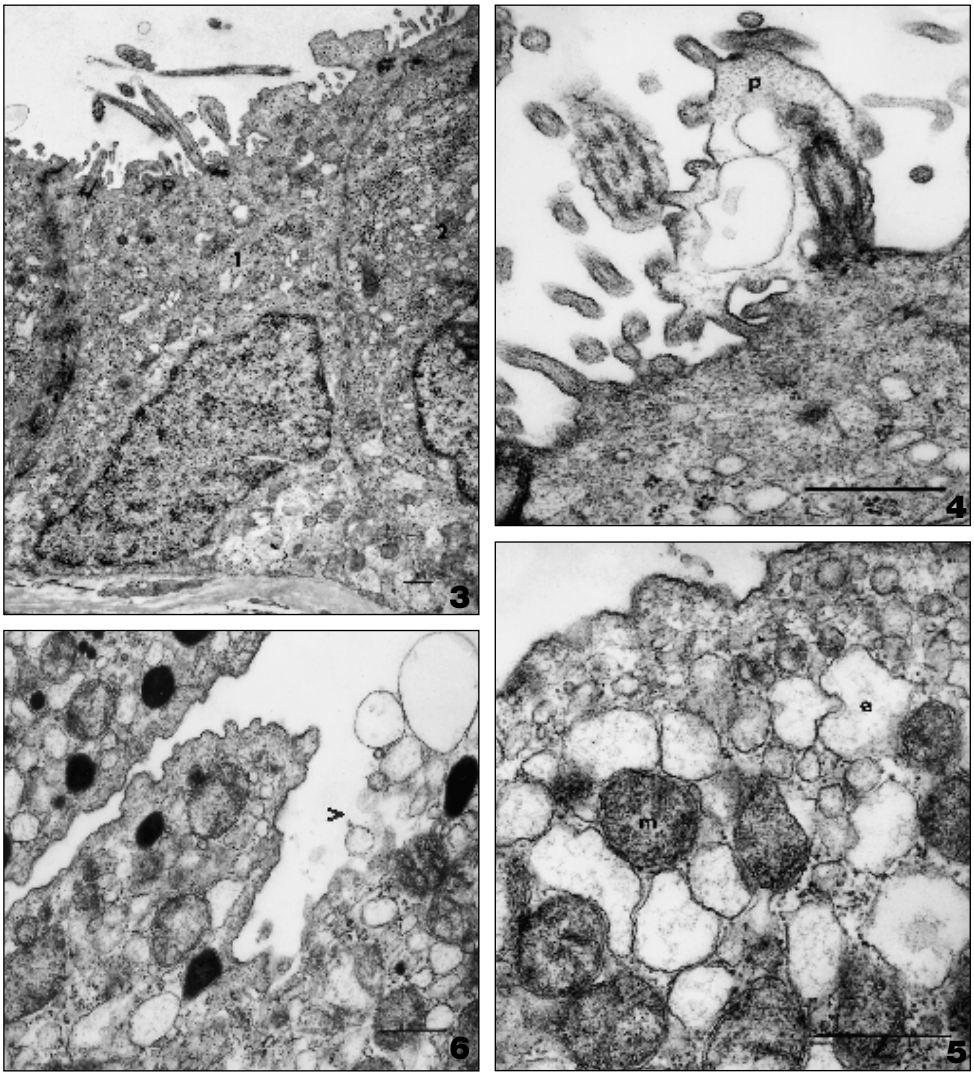


Fig. 3: A portion of the altered epithelium of a terminal bronchiole. The ciliated (1) and Clara (2) cells reveal signs of the pathological alteration of their cytoplasm. Five minutes after the administration of 0.5 mg acetylcholine

Fig. 4: A small cytoplasmic protrusion (p) containing an axoneme of a cilium and vacuoles on the apical surface of the ciliated cell. Five minutes after the administration of 0.5 mg acetylcholine.

Fig. 5: An apical portion of the Clara cell containing moderately altered mitochondria (m) and extremely dilated tubules of the smooth endoplasmic reticulum (e) in its cytoplasm. Five minutes after the administration of 0.5 mg acetylcholine.

Fig. 6: An impairment of the apical plasma membrane of the altered Clara cell resulting in the liberation of remnants of its degenerated cytoplasm into the lumen (arrowhead). Five minutes after the administration of 0.5 mg acetylcholine.

Plate VI

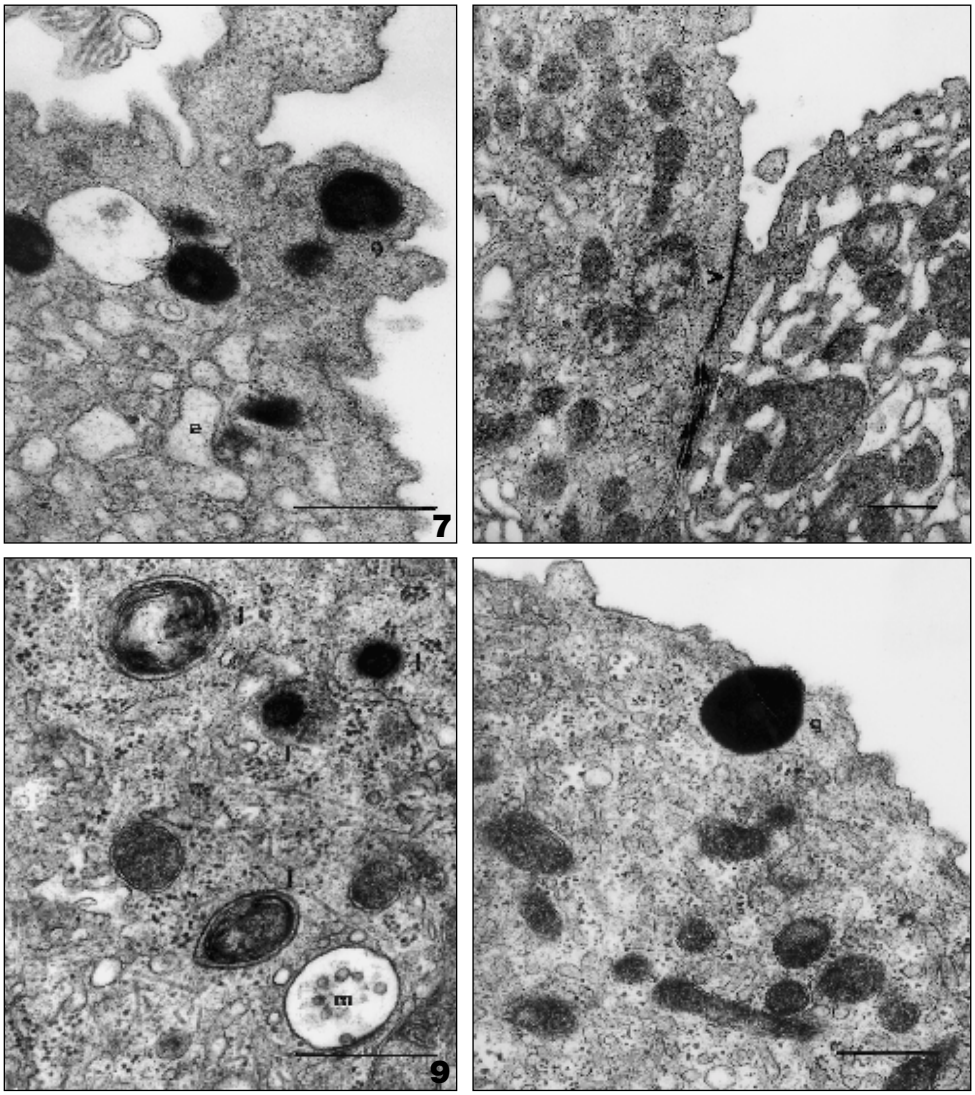


Fig. 7: An apical cytoplasmic protrusion containing a secretory granule (g) on the apical surface of the Clara cell. The cytoplasm contains dilated tubules of the smooth endoplasmic reticulum (e). Five minutes after the administration of 0.5 mg of acetylcholine.

Fig. 8: An intact apical junctional complex (arrowhead) between two Clara cells revealing different signs of the pathological alteration of their cytoplasm. Twenty minutes after the administration of 0.5 mg acetylcholine.

Fig. 9: Lysosomes (l) and a multivesicular body (m) in the cytoplasm of the ciliated cell. Twenty minutes after the administration of 0.1 mg acetylcholine.

Fig. 10: A secretory granule (g) situated in the close vicinity to the apical surface of the Clara cell with the intact cytoplasm. Twenty minutes after the administration of 0.1 mg acetylcholine.