Histol Histopathol (1997) 12: 343-348

Histology and Histopathology

Ultrastructural changes in the synthetic and secretory patterns of pulmonary surfactant following pilocarpine *in vivo*

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Summary. Previous studies have demonstrated that the muscarinic agonist pilocarpine stimulates the secretion of pulmonary surfactant from mammalian alveolar type II cells. The results of the present study quantify, via ultrastructural stereologic analysis, this response through 24 hours. The cytoplasmic volume density of lamellar bodies decreases significantly at 0.5 and 4 hours post-injection. This value is increased significantly at 12 hours post-injection. Elements of the secretory apparatus increase significantly at many of the post-injection times. At 12 hours post-injection many of the type II cells are quite laden with lamellar bodies, with some appearing surprisingly large. This may be a useful model for continued study of the relationship between synthesis and secretion of pulmonary surfactant.

Key words: Type II cells, Pulmonary surfactant, Pilocarpine

Introduction

The synthesis, and particularly the secretion, of pulmonary surfactant by type II alveolar epithelial cells appears to be influenced, or controlled, by a number of factors (reviewed in Chander and Fisher, 1990; Wright and Dobbs, 1991). As regards the autonomic nervous system, ß-adrenergic agonists seem to have a direct effect on secretion, and probably synthesis (Abdellatif and Hollingsworth, 1980; Smith and Griffin, 1987). The mechanism of this, or other forms, of control is poorly understood. For example, it is not known if the synthesis of surfactant lipids and proteins is necessarily linked to the secretion of surfactant, in the form of lamellar inclusion bodies. There is some evidence that surfactant which is secreted following stimulation does not represent the entire pool of surfactant (Barr et al., 1988). Pilocarpine, a muscarinic agonist, has also been shown to affect the alveolar epithelium and the surfactant system. Maternal administration of pilocarpine, for example, has been demonstrated to accelerate the maturation of the fetal alveolar epithelium (Smith et al., 1979). In addition, administration of pilocarpine to adult animals has been shown to stimulate surfactant secretion (Goldenberg et al., 1969). However, there is evidence that the stimulatory effect of pilocarpine is mediated via indirect stimulation of the adrenal medulla, and subsequent adrenergic stimulation of the type II cells themselves (Massaro et al., 1982). The morphologic changes occurring within type II cells have been quantified at 0.5 hours after treatment (Pokrovskaya and Romanova, 1989) and through 4 hours post-treatment (Shimura et al., 1985). At these times, an increase in the volume density of lamellar inclusion bodies has been described, as compared to controls. In addition, Shimura et al. (1985) described an increase in what they termed "bar-like structures" in the rough endoplasmic reticulum in pilocarpine-treated cells. This may imply that the stimulation of surfactant secretion, and perhaps synthesis, may be qualitatively different than that seen in cells stimulated via ß-adrenergic agonists administered *in vivo*. The present study was designed to quantify, via ultrastructural stereologic analysis, the intracellular changes occurring in type II cells through 24 hours post-pilocarpine stimulation.

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Materials and methods

Young adult (300-350 g) male Sprague-Dawley rats (Charles River Labs, Wilminton, MA) were utilized for all experiments. They were housed singly, maintained on a 12-12 light-dark cycle and fed standard lab chow ad libitum. Some rats were utilized as uninjected controls, and thus were not handled prior to anesthesia and sacrifice, as detailed below. Other groups of animals were treated with a bolus subcutaneous injection of 150 mg/kg pilocarpine HCl in saline, and sacrificed at 0.5, 1, 2, 4, 6, 8, 12, 16 and 24 hours post-injection. Another group of animals received 10 mg/kg atropine (a specific muscarinic antagonist) intra-peritoneally, followed 15 minutes later by 150 mg/kg pilocarpine HCl sub-

cutaneously. Each group of animals at each time point, consisted of at least five animals. All animals were injected so as to make the time of death between 2:00 and 4:00 pm.

At the appropriate time post-injection, the animals were killed with an overdose (2 ml) of pentobarbital sodium. The thorax was opened and the apical lobe of the right lung was removed, immersed in 2% glutaraldehyde in 0.2M phosphate buffer and chopped, by hand, into approximately 1 mm pieces. The lung tissue was degassed in a gentle vacuum, and fixed for one hour at 4 ºC. The tissue was then processed for electron microscopy utilizing standard techniques which includes post-fixation in 1% osmium tetroxide for two hours, dehydration in a graded ethanol series and propylene oxide, infiltration in increasing concentrations of epon (polybed 812, Polysciences Inc.) and embedded in fresh epon. Following polymerization semi-thin (0.5 μm) sections were obtained from at least two blocks from each animal, selected at random. These sections were mounted, stained with toluidine blue, and studied by bright field light microscopy.

One block from each lobe, of each animal, was then randomly selected for electron microscopic studies. Thin (approximately 60 nm by interference color) sections were obtained, using a diamond knife and a Sorvall mt-5000 ultramicrotome, mounted on 200 mesh, thin-bar copper grids, and stained with uranyl acetate and lead citrate. These were then examined in either a Zeiss EM-9, or a Phillips CM-10 electron microscope, for qualitative interpretation of ultrastructural features. Each grid was then utilized for the stereologic analysis of type II cell cytoplasmic volume densities. In this procedure, each grid was scanned, and the first 10 type II cells encountered were photographed at a standard magnification of 4900. The microscope was calibrated periodically in order to maintain uniformity. Only those cells in which the plane of section passed through the nucleus were photographed, although this excluded few visible cells. This procedure resulted in photographs of at least 50 cells from each time point in each group. These were enlarged to a standard print magnification of 15,500x. The photographs were then analyzed as detailed previously (Smith and Griffin, 1987). Briefly, a 0.25 inch grid was randomly applied to each photograph, and the intersections of the grid lines were used as test points. Using the equations of Weibel (Weibel, 1963, 1972) cytoplasmic volume densities were obtained for lamellar inclusion bodies, Golgi apparatus, rough endoplasmic reticulum (RER) and mitochondria. All

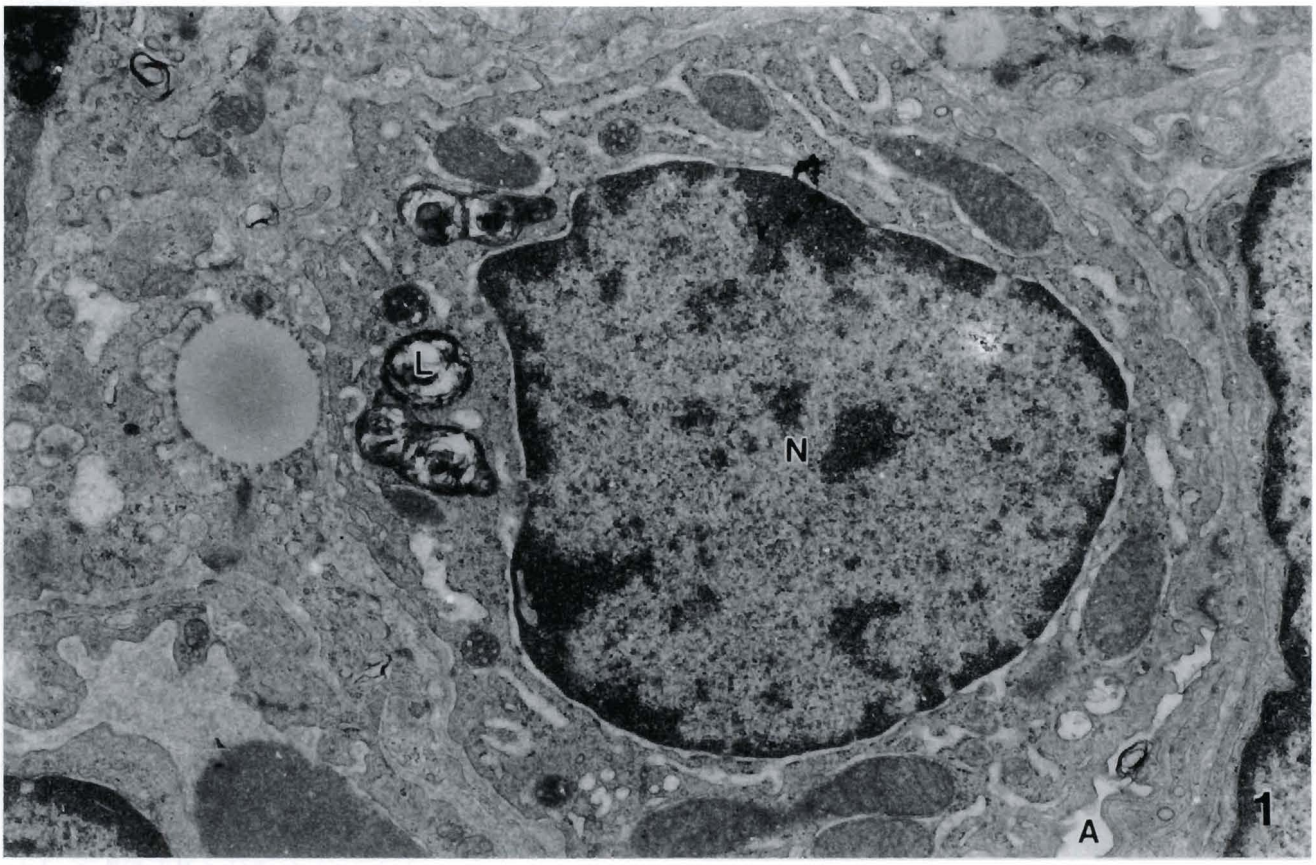

Fig. 1. Electron micrograph of a type II alveolar epithelial cell from a control animal. Note lamellar inclusion bodies (L). A: alveolar space; N: nucleus. x 9,900

data were analyzed using student's t-test.

Results

The administration of the pilocarpine had very noticeable effects on the animals, in that they responded with lacrimation, salivation, and defecation. These responses were completely abolished in those animals receiving atropine in addition to the pilocarpine. The gross appearance of the lungs of all animals in the study was normal.

By light microscopy, the overall architecture of the lungs appeared normal in all cases. The alveolar epithelial cells were, clearly visible, and the lamellar inclusion bodies were except perhaps for the smallest, also easily observed. The type II cells from animals treated with pilocarpine alone at the early time points (0.5-4 hours) appeared to contain somewhat fewer, and smaller lamellar bodies. At the 12 hour time point, however, many of the type II cells contained large, very noticeable, lamellar inclusion bodies. It is appropriate to note that this was not true of every type II cells from every animal. In some cases the cells contained extraordinarily large inclusions, which were assumed to be abnormally enlarged lamellar inclusion bodies. These were not seen in the animals killed 16-24 hours post-injection. In addition to the larger alveolar epithelial cells, there also seemed to be a great many macrophages in the alveolar lumina in the pilocarpine-treated animals from 8-16 hours post-injection, and particularly at 12 hours.

The impressions gained by light microscopic observation were confirmed and expanded by electron microscopy. Type II cells from control animals appeared normal, with evident lamellar bodies and secretory apparatus (Fig. 1).

There was some variation in the response of the type II cells in the pilocarpine-treated group. Some cells appeared to respond strongly, while others appeared much like those of control animals. In general, the trend agreed with the light microscopic observations. Type II cells from animals sacrificed at 0.5-4 hours post-injection seemed to have fewer, and smaller lamellar inclusion bodies. Occasionally, lamellar bodies could be observed during the secretory process (Fig. 2). At 12 hours, many cells had numerous, large lamellar bodies. In some instances, this reached phenomenal proportions, with lamellar bodies so huge that one or two occupied most of the cell's cytoplasm (Figs. 3, 4).

The results of the stereologic analysis of cytoplasmic

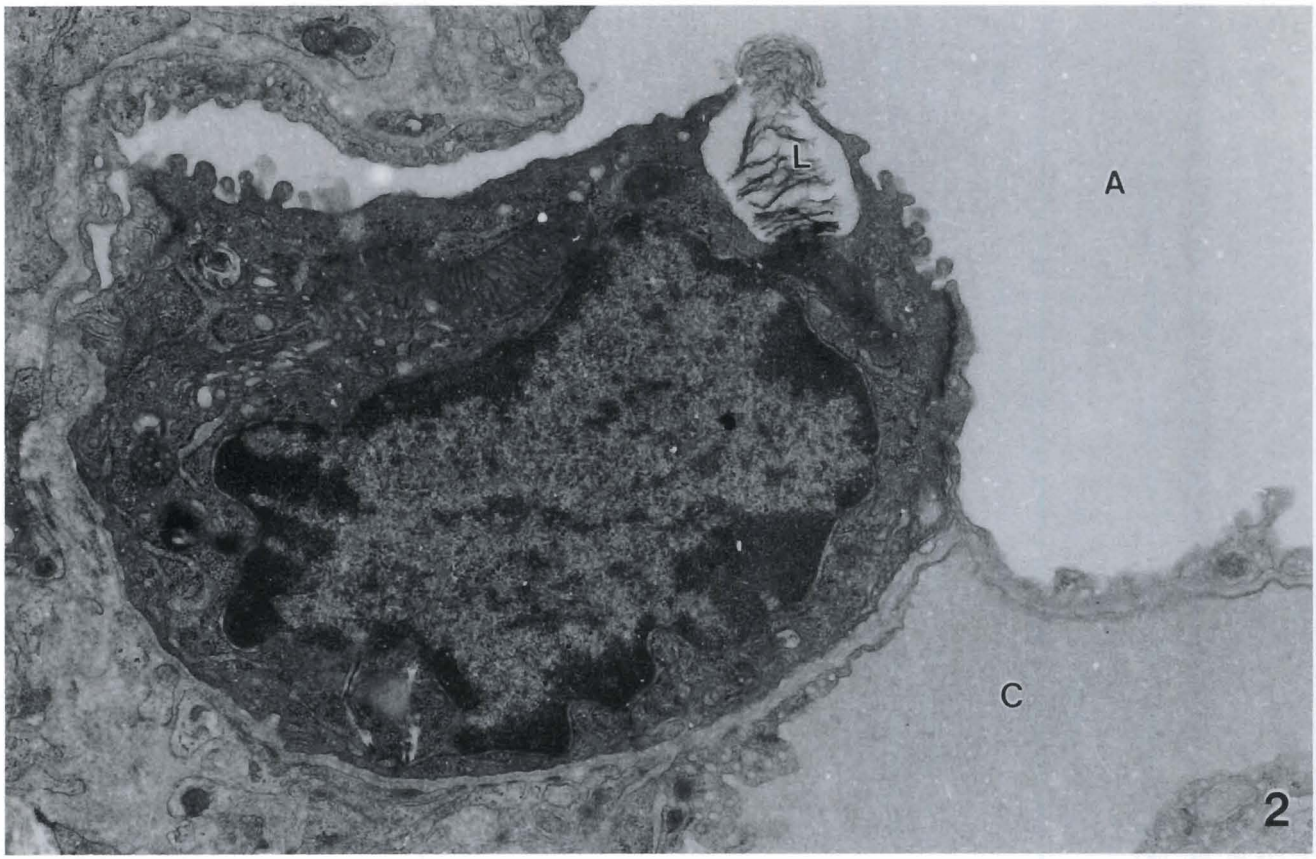

Fig. 2. Type II cell from an animal treated with pilocarpine, at 2 hours post-injection. Note relative lack of lamellar inclusion bodies, with the single mature inclusion present being secreted into the alveolar space (L). A: alveolar space; C: capillary lumen. x 9,700

components of the type II cells are presented in figure 5. There was a sharp decrease in the lamellar body volume density from 0.5-4 hours, with statistically significant decreases at 0.5 and 4 hours. This was followed by a return to control values at 6 and 8 hours, and a significant increase at 12 hours. RER volume density was significantly increased at 0.5, 1, 2, and again at 12 and 16 hours post-injection. The volume density of the Golgi apparatus was decreased at 0.5 and 1 hour post-injection, while it was increased at 2, and again at 8 hours after pilocarpine treatment. In the group which received atropine and pilocarpine, there were no significant differences in any of the structures studied through 24 hours post-treatment.

Discussion

The results of the present study indicate that pilocarpine administration causes a marked increase in the secretion of pulmonary surfactant through at least four hours post-injection. This is evidenced by a significant decrease in the cytoplasmic volume density of lamellar inclusion bodies. This treatment also seems to increase the production of surfactant, as inferred by the increase in the morphologic counterparts of biochemical activity, namely the RER and the Golgi apparatus. This increase in elements of the synthetic apparatus is followed by a dramatic increase in the volume density of lamellar inclusion bodies at twelve hours post-injection, with a decline back to control levels through 16-24 hours. More dramatic than the overall increase in lamellar body volume density, was the excessive accumulation of lamellar body material within some of the type II cells, sometimes to the point of the severe compression of other cellular elements.

Previous studies have demonstrated that pilocarpine administration results in an increase in the secretion of surfactant. Goldenberg et al. (1969) described this stimulation qualitatively, and stated that it peaked at 2-4 hours post-injection. Shimura et al. (1985) described a slight dilatation of the RER at 0.5-4 hours post-pilocarpine treatment. The results of the present study confirm these observations, and demonstrate quantitatively a significant increase in the cytoplasmic volume density of the RER 0.5-2 hours post-injection. In addition, the present results show a significant increase in the cytoplasmic volume density of the Golgi apparatus at one and eight hours post-injection. This would argue for a stimulation of surfactant synthesis following pilocarpine treatment. An interesting question is whether

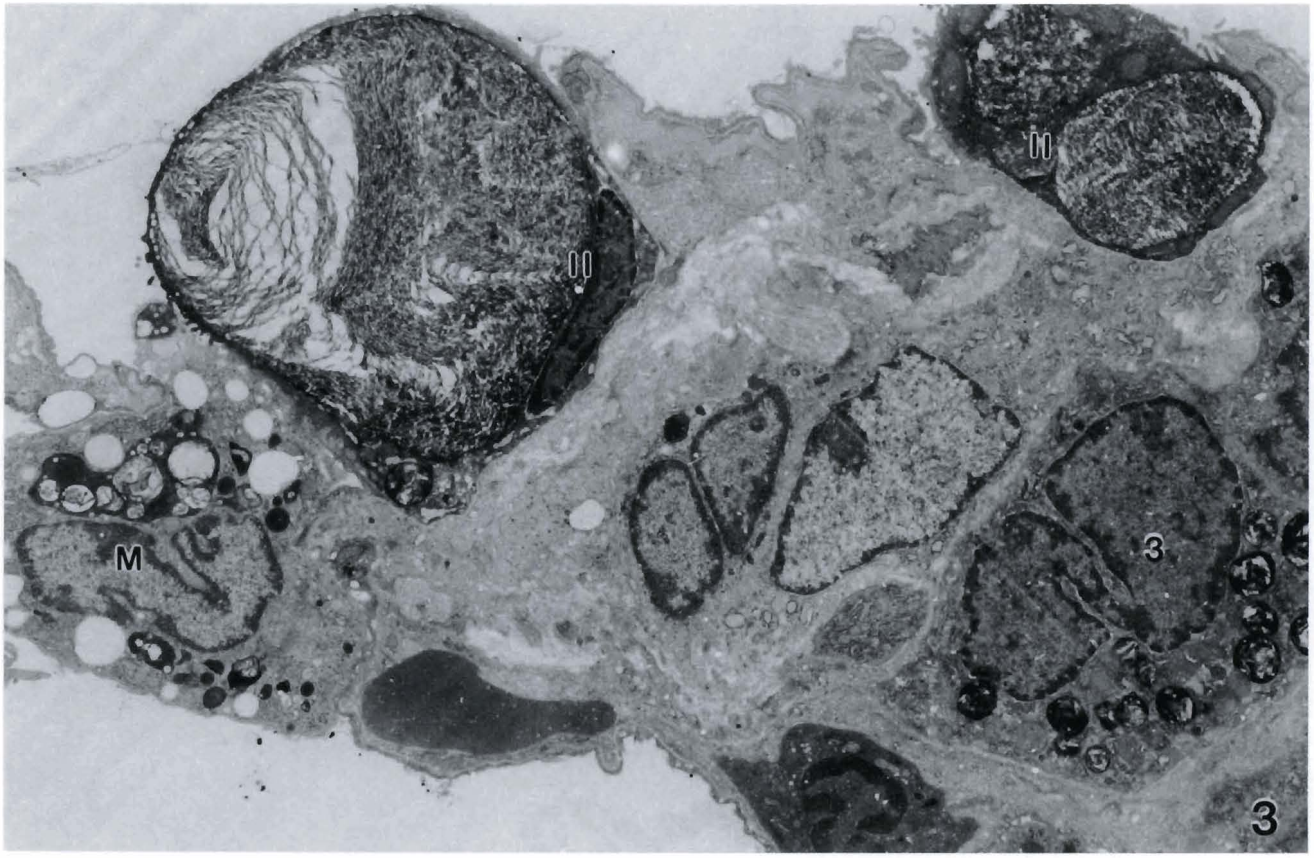

Fig. 3. Electron micrograph of an alveolar septum from an animal treated with pilocarpine, at twelve hours post-injection. Note abnormally large lamellar inclusion bodies in two of the type II cells (II). A third type II cell (#3) has numerous, but otherwise normal appearing inclusion bodies. M: macrophage. x 3,200

this stimulation of synthesis is a result of the prior secretory activity of the cell, or whether, as argued by Mettler et al. (1981), secretion and synthesis of surfactant are independent processes. The accumulation of lamellar inclusion bodies at 12 hours post-injection, in the present study is very intriguing regarding this question.

The massive accumulation of lamellar body material in some cells, to the point of nearly obliterating the remainder of the cytoplasm, is particularly interesting. This short of massive over-accumulation has been described in beige mice by Chi et al. (1976), who presumed it to be due to the lysosomal storage effects of the Chediak-Higashi syndrome. The cause of the accumulation in the present study may, similarly, be of lysosomal origin, in that it could be due to massive re-uptake of previously secreted surfactant. On the other hand, it could be due to a suppression of secretion, while synthesis remains high. This suppression of secretion could conceivably be due to the presence of large

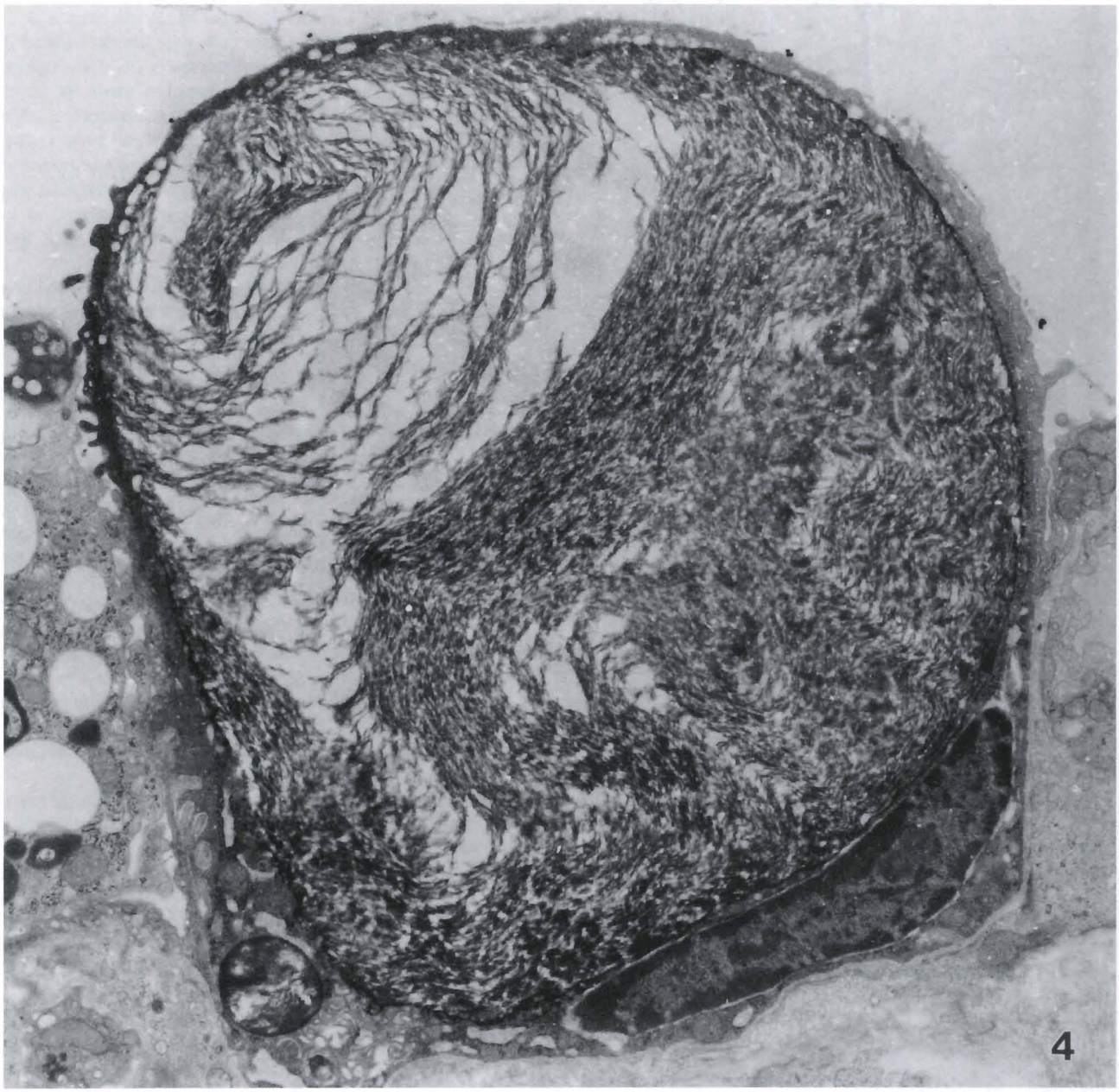

Fig. 4. Electron micrograph of a type II cell from an animal treated with pilocarpine, 12 hours post-injection. This is one of the most bizarre appearing type II cells observed in this group of animals. Note than the lamellar inclusion body is so large, as to have displaced the remainder of the cytoplasm of the cell. Otherwise it appears fairly normal. x 9,000

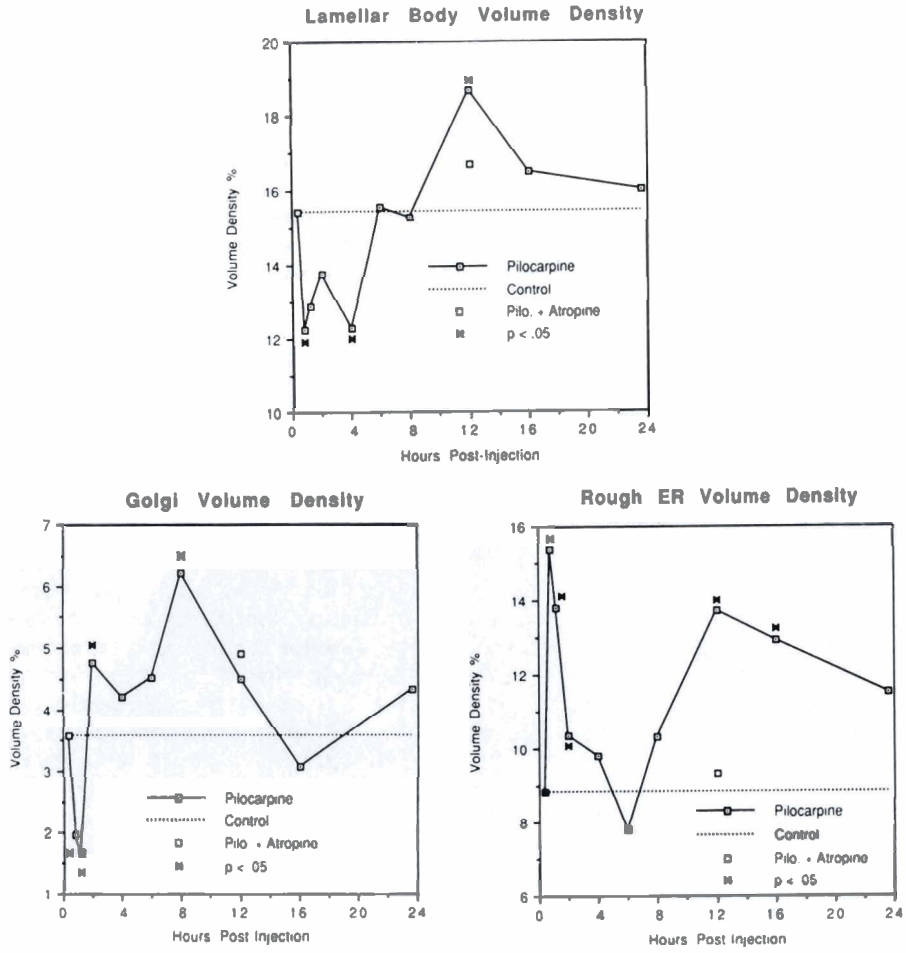

Fig. 5. Graphs illustrating the results of the determination of the cytoplasmic volume density of lamellar inclusion bodies (#1), Golgi apparatus (#2), and rough endoplasmic reticulum (#3).

amounts of surfactant in the alveolus, which has been demonstrated to inhibit secretion (Dobbs et al., 1979).

The results of the present study may indicate that the cell is capable of synthesizing and storing great amounts of surfactant, without the necessity of secreting it. It should be stressed that this certainly reflects a derangement of normal synthesis and secretion. However, it may be a useful tool for the further examination of the mechanisms of synthesis and secretion of pulmonary surfactant.

Acknowledgements. Supported by National Institutes of Health AREA grants R15 HL42597 and R15 HL 46296. The authors wish to thank Ann Sutherland and Karen Yokoo, who worked on aspects of this study while students at Wellesley College.

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Accepted October 14, 1996