# LOCALIZATION OF PHOSPHATIDIC ACID PHOSPHATASE ACTIVITY IN GRANULAR PNEUMONOCYTES

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The alveoli of the mammalian lung are lined by a film of surface-active material (surfactant) which is commonly believed to be a phospholipid (1, 5, 8, 11). A considerable body of evidence has accumulated which suggests that surfactant is synthesized by the granular pneumonocytes (type II cells) of the alveolar epithelium (1, 9, 10). Although a number of acid hydrolases have been demonstrated in the inclusion bodies of granular pneumonocytes (4, 6), no attempt has been made to localize enzymes specifically concerned with phospholipid metabolism. Phosphatidic acid phosphatase is of particular interest because of its key role in the biosynthesis of phosphatides. Unfortunately, this enzyme cannot be demonstrated by conventional histochemical methods because of the insolubility of the substrate (phosphatidic acid) in water. Stable emulsions of phosphatidic acid can be prepared but these do not diffuse into cells. One method of overcoming this difficulty is to inflict minor localized damage on cells and expose the organelles to an emulsion of phosphatidic acid in a suitable medium.

A method of producing minor trauma by osmotic forces has been developed to enable the localization of phosphatidic acid phosphatase to be studied in granular pneumonocytes.

## MATERIALS AND METHODS

Young adult hamsters of 150–200 g body weight were killed by cervical dislocation, and small blocks of lung tissue were excised and fixed for 2 hr in ice-cold 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 7.5% sucrose. After fixation the blocks were washed for 20 min in 0.1 M cacodylate buffer (pH 7.2) containing 7.5% sucrose, and then 0.5 mm slices were cut freehand. The tissue slices were incubated at 37°C for 15–90 min in a freshly prepared medium containing phosphatidic acid as substrate. The medium consisted of 3 ml of 0.1 M acetate buffer (pH 5.1) containing 3% sucrose, 5 ml of distilled water, 1 ml of 5% lead nitrate solution, and 1 ml of 2% phosphatidic acid (Sigma London Chemical Co., London, England) emulsion prepared by the method of Clark and Hübscher (2). The medium was preheated to 37°C before use. Control sections were incubated in substrate-free media or in complete media containing 0.01 M sodium fluoride. After incubation the tissues were washed briefly in cacodylate buffer, postfixed in cacodylate buffer–$OsO_4$ (1%), dehydrated in a series of graded alcohols, and processed through propylene oxide to Araldite CY212. Ultrathin sections were examined

unstained in an AEI801 electron microscope operated at 40 or 60 kv.

## RESULTS

In incubated tissue the inclusion bodies of granular pneumonocytes are dilated and there is a considerable loss of the lamellated contents. In routine micrographs the walls of the inclusion bodies appear intact, but in serially-sectioned material a large proportion of the inclusions are found to have small defects in the superficial aspects of their walls (Figs. 1, 2). Such defects establish communications between the inclusion vesicles and the alveolar lumen. Other cell organelles, in particular the mitochondria, are not obviously affected by the incubation procedure.

Phosphatidic acid phosphatase activity is prominent in the granular pneumonocytes of tissue incubated for 40 min or longer (Fig. 3). The lead phosphate deposit is localized in the lining membrane of the inclusions; little reaction is observed in the residual lamellated contents of the inclusions (Fig. 4). The majority of inclusions situated in the apical cytoplasm of any granular pneumonocyte show enzyme activity. Dilated inclusions are frequently seen in a partially fused state. In such cases reaction product is absent from the fused areas of the inclusion walls (Fig. 5). Enzyme activity also occurs in multivesicular bodies where these structures are adjacent to inclusion bodies (Fig. 6). It is probable that stained multivesicular bodies communicate directly with inclusion vesicles as envisaged by Sorokin (10), although the exact morphological relationship between these two types of organelle is difficult to establish because of the low contrast of membranous structures in incubated tissues.

Nonspecific deposition of lead occurs to a slight degree in the apical plasma membranes and nuclear membranes of the granular pneumonocytes. This deposition becomes more pronounced if the pH rises abofe 5.1. Control sections incubated in substrate-free media or in media containing sodium fluoride as inhibitor are always negative.

## DISCUSSION

The technique described appears to produce relatively minor damage to the pulmonary epithelial cells. Preliminary studies showed that the use of media containing less than 3% sucrose results in unacceptable tissue damage. It must be stressed that this method, at least in its present form, is not suited for use in other tissues; the method depends basically on the peculiar osmotic properties of the granular pneumonocyte inclusions.

The reaction is considered to indicate phosphatidic acid phosphatase activity as biochemical studies (3) show that the substrate (phosphatidic acid) is not hydrolysed by nonspecific phosphomonesterase. Moreover, the staining obtained with $\beta$-glycerophosphate substrate in glutaraldehyde-fixed lung (6) differs considerably from the staining observed in the present study with phosphatidic acid substrate. The absence of reaction after incubation in substrate-free media eliminates the possibility of nonenzymatic lead deposition.

Recent biochemical studies have demonstrated that dipalmityl lecithin forms the chief component of pulmonary surfactant (7). According to Coleman and Hübscher (3), phosphatidic acid phosphatase is involved in an important step of the synthesis of dipalmityl lecithin, namely the conversion of 1,2-dipalmityl phosphatidic acid to 1,2-dipalmityl glyceride. The demonstration of phos-

FIGURE 1 Granular pneumonocyte incubated in substrate-free medium. No reaction product in inclusion vesicle. Bar, 0.5 $\mu$. × 31,250.

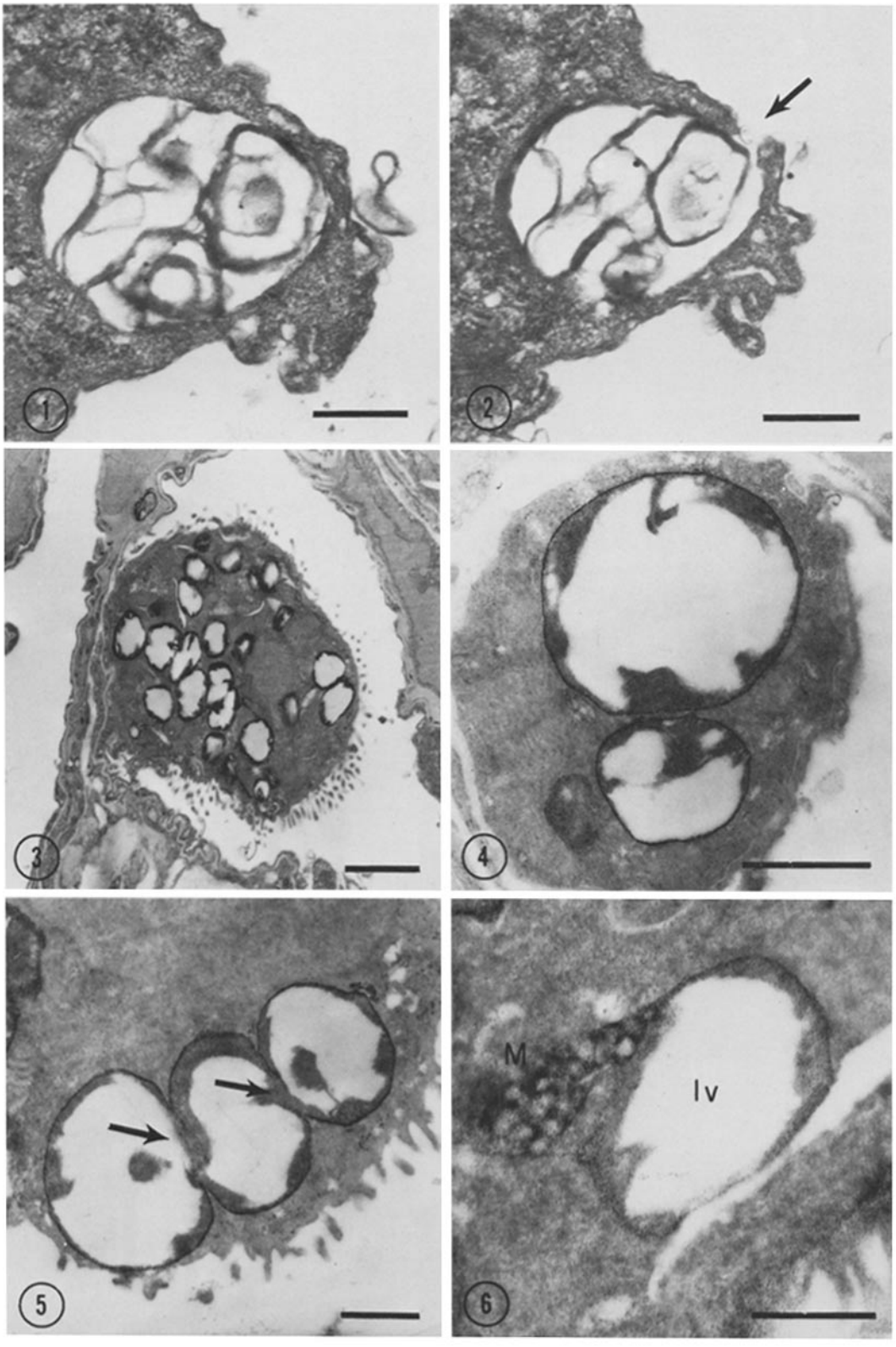
FIGURE 2. Serial section through the same inclusion as in Fig. 1. A small communication (arrow) exists between the inclusion vesicle and the alveolar lumen. Bar, 0.5 $\mu$. × 31,250.

FIGURE 3 Granular pneumonocyte incubated in complete medium (40 min). A number of inclusion bodies contain reaction product. Bar, 2 $\mu$. × 7800.

FIGURE 4 Inclusion bodies showing reaction product localized in lining membranes. Inclusion contents are nonreactive. Incubation period, 60 min. Bar, 1 $\mu$. × 20,000.

FIGURE 5 Partially fused inclusion bodies in apical cytoplasm of granular pneumonocyte. Reaction product is absent from fused areas (arrows). Incubation period, 60 min. Bar, 0.5 $\mu$. × 23,750.

FIGURE 6 Reaction product localized in a multivesicular body (*M*) lying near an inclusion vesicle (*Iv*). Incubation period, 45 min. Bar, 0.3 $\mu$. × 62,500.



phatidic acid phosphatase in the inclusion bodies of granular pneumonocytes therefore provides strong evidence of this cell type being involved in the production of pulmonary surfactant.

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