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Localization and release of lysozyme from ferret trachea: Effects of adrenergic and cholinergic drugs

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Summary. Lysozyme is a bacteriolytic enzyme found in respiratory tract fluid. In this study, immunocytochemistry was used to determine the cells of origin of tracheal lysozyme in the ferret. Lysozyme was found in secretory granules of serous but not mucous cells in the submucosal glands, and was absent from the surface epithelium, cartilage, and connective tissue. The exclusive presence of lysozyme in serous gland cells renders it useful as a biochemical marker of that cell type.

Measurements of lysozyme assayed from the incubating medium indicated that bethanechol stimulated lysozyme release by $260 \pm 80.9\%$ (mean $\pm$SE), phenylephrine by $80 \pm 16.4\%$, and terbutaline by $25 \pm 10.2\%$. Electron-microscopic and immunocytochemical analysis of incubated tissues revealed loss of serous granules and lysozyme immunoreactivity in response to the drugs. Atropine, propranolol, and phentolamine blocked the stimulatory effects of bethanechol, terbutaline, and phenylephrine, respectively.

These findings establish the usefulness of lysozyme as a serous-cell marker and demonstrate that secretory responses of different magnitude are evoked by equimolar concentrations of alpha- and beta-adrenergic and cholinergic drugs.

Key words: Lysozyme – Trachea – Serous cell – Bethanechol – Phenylephrine – Terbutaline

Lysozyme is a bacteriolytic enzyme with a molecular weight of 14,500. It has been localized in cells in a variety of tissues and is presumed to play a major defensive role at epithelial surfaces such as those of the lumina of the gastrointestinal tract and airways.

Small, but significant, amounts of lysozyme are present in respiratory

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tract fluid, which consists primarily of water, ions, and glycoproteins. One cellular source of tracheal lysozyme has been shown immunocytochemically to be the submucosal glands (Klockars and Reitamo 1975; Mason and Taylor 1975; Bowes and Corrin 1977; Spicer and Frayser 1977). In humans, serous gland cells have been identified by immunocytochemistry as the sole source of tracheal lysozyme (Klockars and Reitamo 1975; Mason and Taylor 1975; Bowes and Corrin 1977), although biochemically, lysozyme has been demonstrated in surface epithelium as well (Konstan et al. 1981). In rodents, lysozyme is found in mucous cells and on the epithelial surface (Spicer and Frayser 1977).

Because tracheal glands consist of both serous and mucous cells, it is necessary to consider the regulation of secretion from each cell type. Until now, this has been difficult, except by use of tedious morphometric techniques (Basbaum et al. 1981). Isolation of glands and separation of the cell types have not yet been achieved. Therefore, the existence of an apparent marker for serous cells provides a tool for studying their regulation.

In this study, we establish the presence of lysozyme in serous but not mucous cells of the ferret trachea and show that tracheal lysozyme is released from serous cells differentially by adrenergic and cholinergic agonists.

Materials and methods

Animals and tissue preparation

Castrated male ferrets (Marshall Farms, NY) weighing about 1.5 kg were anesthetized with sodium phenobarbital (35 mg/kg, ip). Ferrets were used because their tracheas contain abundant submucosal glands and ferrets are readily available from colony breeders. Tracheas were removed, cleaned of excess connective tissue, and divided into segments 1 cm in length. Segments were placed either into (1) Bouin's fixative for 24 h at room temperature or (2) 0.2% glutaraldehyde in 0.08 M cacodylate buffer (pH 7.5) for 1 h at 4° C. Those samples fixed in Bouin's were dehydrated in graded ethanol solutions, cleared in alpha-terpineol, and embedded in paraffin; paraffin-embedded tissues were sectioned at 7 μm using a rotary microtome (American Optical). Those samples fixed in 0.2% glutaraldehyde were dehydrated in graded acetone solutions and embedded in araldite (Polysciences); araldite-embedded tissues were sectioned at 1 μm using an ultramicrotome (Porter-Blum MT-1).

Immunocytochemistry

Immunocytochemical staining was performed using the unlabeled antibody enzyme method of Sternberger (Sternberger 1974). Paraffin sections were deparaffinized and araldite sections were etched for 5 min with a solution of alcoholic sodium hydroxide and toluene. All sections were then treated for 30 min with 3% normal goat serum. This was followed by a 48-h (4° C) incubation with rabbit antihuman lysozyme (1:2500). This and all subsequent dilutions were made using phosphate-buffered saline containing 1% goat serum. Sections were then washed in phosphate-buffered saline and treated successively with (a) 3% normal goat serum (5 min), (b) goat antirabbit IgG (1:50, 30 min), (c) phosphate-buffered saline (15 min), (d) 1% normal goat serum (15 min), and (e) peroxidase-antiperoxidase (1:60, 30 min). Finally, sections were washed with 0.05 M tris-HCl (pH 7.4) and incubated in 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical) and 0.002% hydrogen peroxide, to localize the peroxidase. Sections were examined under a light microscope during the reaction to allow sufficient time for the reaction product to develop with minimal background staining. The optimal reaction time was determined to be 10 min. After the reaction, sections were washed in distilled water, counterstained with methyl green, dehydrated in graded alcohols, and cleared in xylene. Glass coverslips were mounted on the tissue sections with Permount.

Staining controls consisted of sections processed as described above, except that rabbit antihuman lysozyme was omitted and replaced by normal rabbit serum. Controls for the specificity of the antibody consisted of sections reacted with rabbit antihuman lysozyme that had been pre-absorbed with 0.1 μg/ml of purified human lysozyme (obtained through the courtesy of Dr. E.F. Osserman, Columbia University, NY) before staining.

Light microscopy

Adjacent serial paraffin sections were stained either for lysozyme or with a polychrome stain (basic fuchsin, 1%; methylene blue, 1%) to distinguish serous from mucous cells. Adjacent sections, each stained by one of the two methods described, were examined and compared to determine the gross cellular localization of lysozyme.

Araldite sections (1 μm) were stained for lysozyme only and were examined using both regular bright field and phase optics to determine whether reaction product was associated subcellularly with secretory granules or cytoplasm.

Organ culture

In separate experiments, adrenergic and cholinergic agonists were tested for possible effects on lysozyme secretion. Appropriate concentrations of each agonist were identified as those concentrations producing maximum lysozyme release in dose-response experiments. Optimal doses were found to be between 10^{-6} M and 10^{-5} M for all three agonists.

The following drugs were used: bethanechol chloride (Merck, Sharpe & Dohme), phenylephrine hydrochloride (Winthrop) terbutaline sulfate (Astra), atropine sulfate (Elkins-Sinn) phentolamine (CIBA), and propranalol hydrochloride (Ayerst).

Ferrets were kept anesthetized with phenobarbital (30 mg/kg i.p.) for 2 h prior to removal of the tracheas. This step was found to reduce variability in baseline secretion. After 2 h, the trachea was removed, cleaned of excess connective tissue, and cut into segments 2-cm long. For each experiment, one tracheal segment was immediately placed in Bouin's fixative for histology, while the others were placed in small beakers, each containing 15 ml of Ham's F-12 culture medium, 100 units of penicillin, and 100 μg of streptomycin/ml. All beakers were incubated at 37° C in a Dubnoff metabolic shaker and oxygenated with a humidified mixture of 95% O_2 and 5% CO_2. Before exposure to drugs, samples were rinsed for 3 h to remove free lysozyme released during dissection and handling. Samples were then transferred to individual beakers containing control medium or one of the pharmacological antagonists at 10^{-4} M (Period 1). After 30 min, samples were transferred to another set of beakers containing (a) control medium, (b) agonist at 10^{-5} M, or (c) agonist (10^{-5} M), plus the appropriate antagonist (10^{-4} M) (Period 2). After 30 min, perfusates from all beakers in Periods 1 and 2 were collected, dialyzed against six changes of distilled water and lyophilized. Tissue samples were collected and blotted on filter paper before weighing. They were then placed in fixative and prepared for electron microscopy (see below).

In one experiment, the protocol was modified to allow 3-h exposure to bethanechol (10^{-5} M), the most potent lysozyme-releasing agent, to examine glands for signs of depletion of immunoreactive lysozyme. In this experiment, samples were fixed in Bouin's mixture immediately after 3 h in bethanechol or after a similar incubation in medium alone, and then were prepared for immunocytochemistry.

Electron microscopy

For electron microscopy, tracheal segments obtained from the experiments described above were placed in fixative containing 2.5% glutaraldehyde, 0.08 M cacodylate, and 5 mM $CaCl_2$. Fixation was begun at 21° C and continued for 18 h at 4° C. Samples were then further dissected into rectangular blocks of tissue from the anterior (gland-containing) region of the tracheal ring. These were placed in 1.5% OsO_4 buffered to pH 7.4 with 30 mM veronal acetate (4 h, 4° C). They were then dehydrated in ethanol, passed through propylene oxide and embedded in araldite. Thin sections were collected on parlodion coated 200 mesh copper grids, stained with 5% uranyl acetate, 0.4% lead citrate and viewed in a Zeiss EM-10 electron microscope at 80 KV.

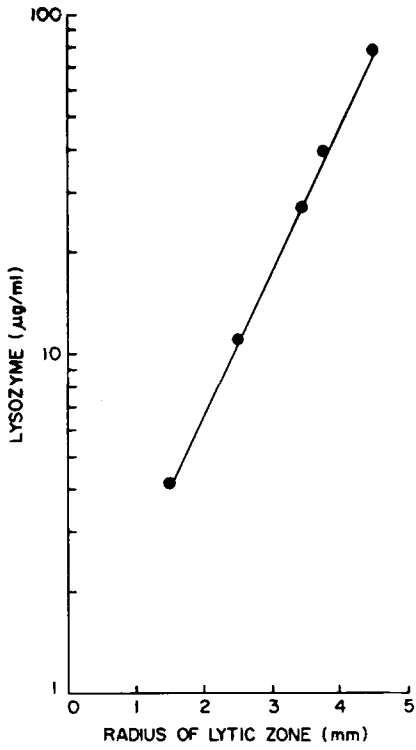


Fig. 1. Radial diffusion plate assay for lysozyme: standard curve. The radius of lytic zone is plotted against the log concentration of each standard, expressed as µg/ml

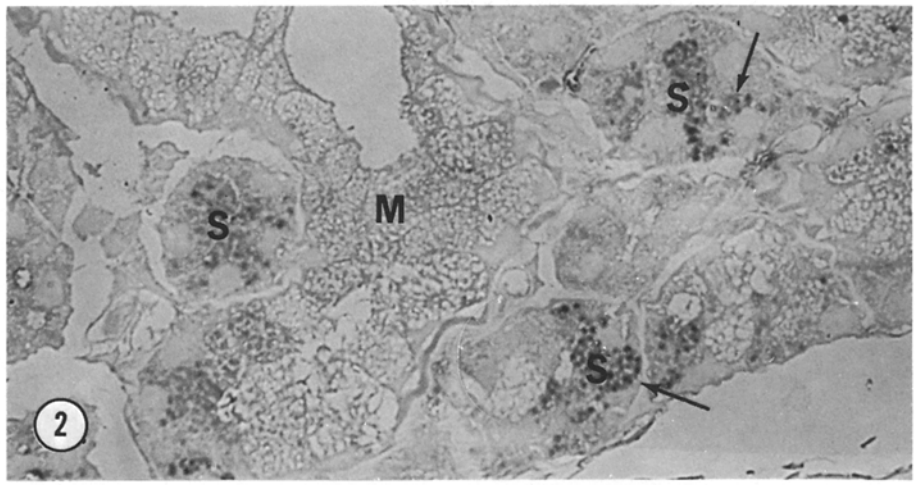
Lysozyme assay

A modification of the radial-diffusion plate method (Osserman and Lawlor 1966) was used to measure lysozyme. Briefly, 30 mg of *Micrococcus lysodeikticus* (Millipore) was suspended in 1% melted agarose (Cal-Biochem) in 0.05 M tris-HCl (pH 7.0). A fixed volume of this medium was poured into Petri dishes (15 × 20 mm) and allowed to set. Using an LKB gel puncher, 3-mm wells were made in the plates. The wells were filled with 3 µl of either the unknown sample or reference standard of human lysozyme (4.2–79 µg/ml, Kallestead Lab). The plates were then incubated at 37° C for 20 h after which zones of lysis appeared. A standard curve was obtained by measuring the radii of these lytic zones and plotting the radius against the semi-logarithmic concentration of reference standard (Fig. 1). In 12 assays ($n = 48$ samples), the within-assay variability was 0.3% and the between-assay variability was 7% (Rodbard 1974). Concentrations of unknown samples were calculated from the standard curve. Paired t tests were used to determine statistical significance (Zar 1974).

Fig. 2. Section of submucosal gland of ferret trachea stained with rabbit antihuman lysozyme (1:2500). *Arrows* indicate staining associated with serous (*S*) cell granules. Mucous (*M*) cells are devoid of staining. Araldite (1 µm). × 350

Fig. 3. Section of submucosal gland of ferret trachea stained with normal rabbit serum (1:2500). Note the absence of staining in both serous (*S*) and mucous (*M*) cells. Araldite (1 µm). × 350

Fig. 4. Section of submucosal gland of ferret trachea stained with rabbit antihuman lysozyme preabsorbed with purified human lysozyme (1:2500). No staining is seen in serous (*S*) or mucous (*M*) cells. Araldite (1 µm). × 350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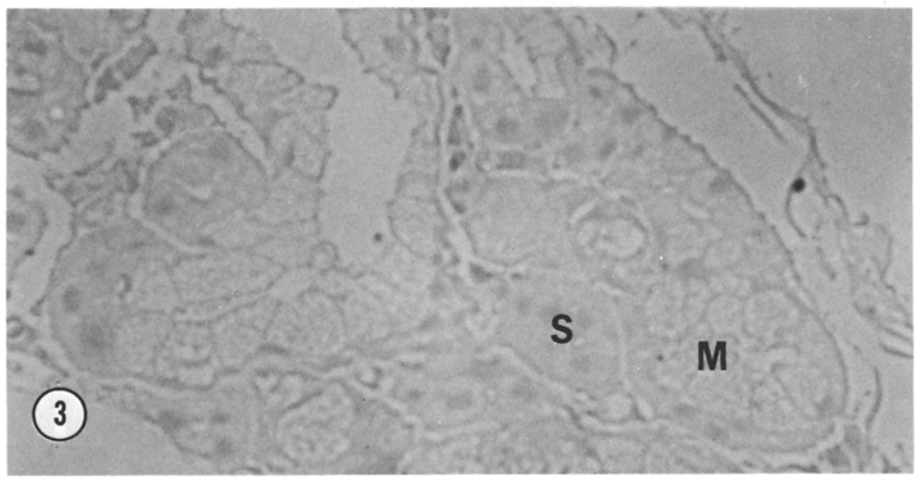
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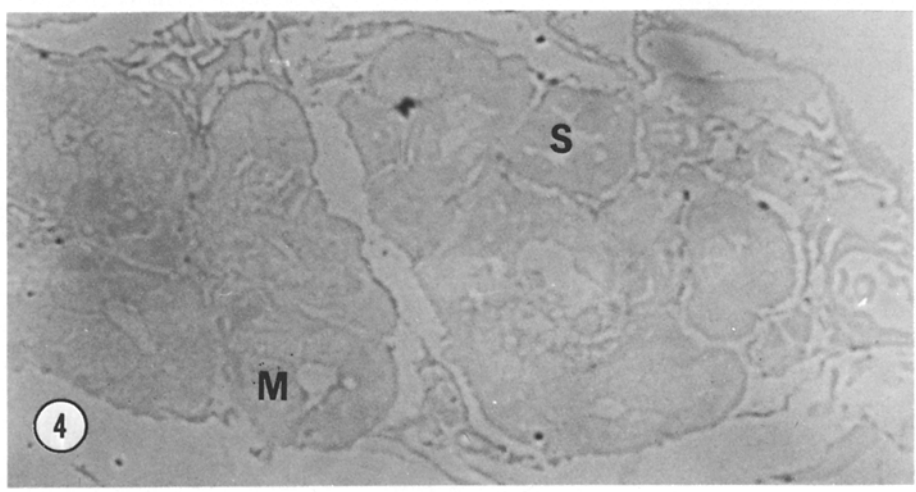
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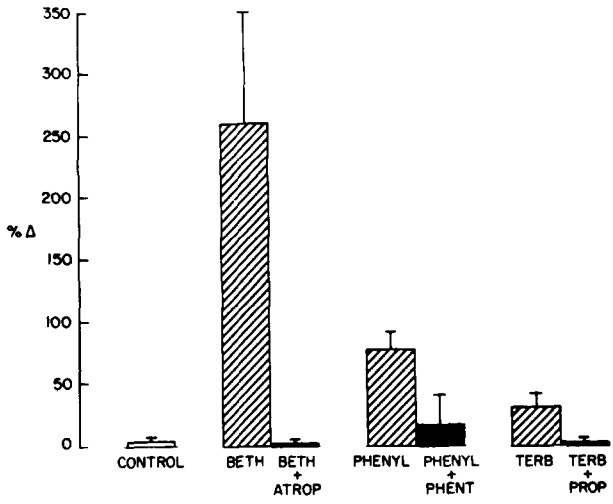


Fig. 5. Agonist-induced release of lysozyme, expressed as percent of control values. *Open bar* represents control tissues in which no agonists were added ($n=17$). *Hatched bars* represent tissues treated with agonists (10^{-5} M); bethanechol ($n=8$), phenylephrine ($n=10$), and terbutaline ($n=8$). Opaque bars represent tissue treated with agonists (10^{-5} M) and antagonists (10^{-4} M); bethanechol + atropine ($n=6$), phenylephrine + phentolamine ($n=10$), and terbutaline + propranolol ($n=6$)

Results

Immunocytochemistry

Examination of immunocytochemically stained sections through tracheal glands showed lysozyme immunoreactivity to be associated with submucosal gland demilunes and other cells aggregated in small round acini. By examining adjacent polychrome-stained sections, these cells could be identified as serous cells. The epithelium, lamina propria, and mucous cells were devoid of immunoreactivity. Staining in the cartilage proved to be nonspecific (see below).

Araldite sections (1 μm) provided sufficient resolution to observe that lysozyme was associated with secretory granules, rather than with the cytoplasm of serous cells. As in paraffin sections, mucous cells were devoid of immunoreactivity (Fig. 2). Sections stained with normal rabbit serum (Fig. 3) showed negligible staining. The staining that did occur appeared along the apical surface of the mucosa and in the cartilage.

Preabsorption of the antibody with purified human lysozyme (Fig. 4) abolished serous cell reactivity. In contrast, staining in the cartilage was not eliminated by absorption. On this basis we regarded staining in the cartilage to be nonspecific. Elimination of staining in the serous cells demonstrated antibody specificity and cross-reactivity between ferret and human lysozyme. Without amino acid analyses, we cannot be certain that human lysozyme and ferret lysozyme are chemically identical; however, they may share homologous sequences.

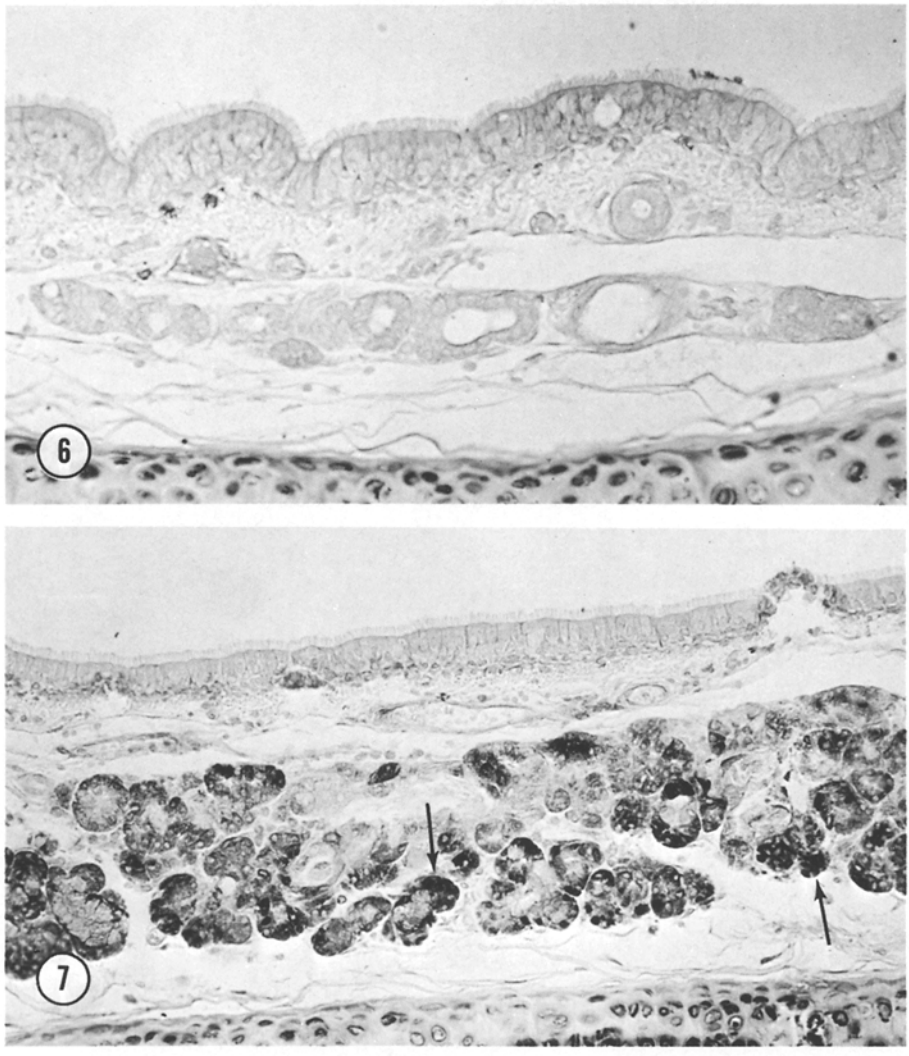
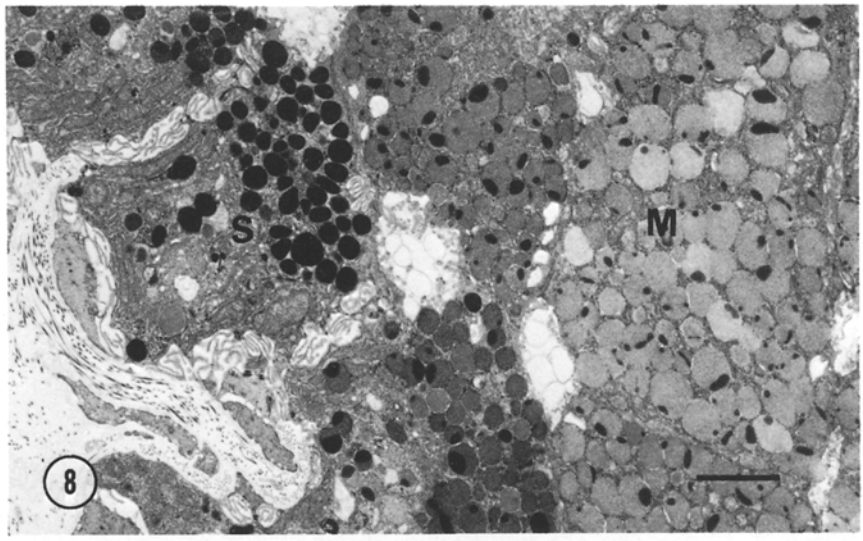


Fig. 6. Light micrograph illustrating the appearance of ferret trachea after incubation for 3 h in 10^{-5} M of bethanechol. Section was stained with rabbit antihuman lysozyme (1:2500). × 300

Fig. 7. Light micrograph illustrating the appearance of ferret trachea after incubation for 3 h in control medium. Section was stained with rabbit antihuman lysozyme (1:2500). *Arrows* indicate immunoreactive lysozyme. × 300

Lysozyme assay

The biochemical findings are shown in Fig. 5. In control medium, there was no change or a slight decrease in the amount of lysozyme released between Periods 1 and 2. In contrast, the presence of each of the three agonists caused a significant increase in release of lysozyme during Period 2.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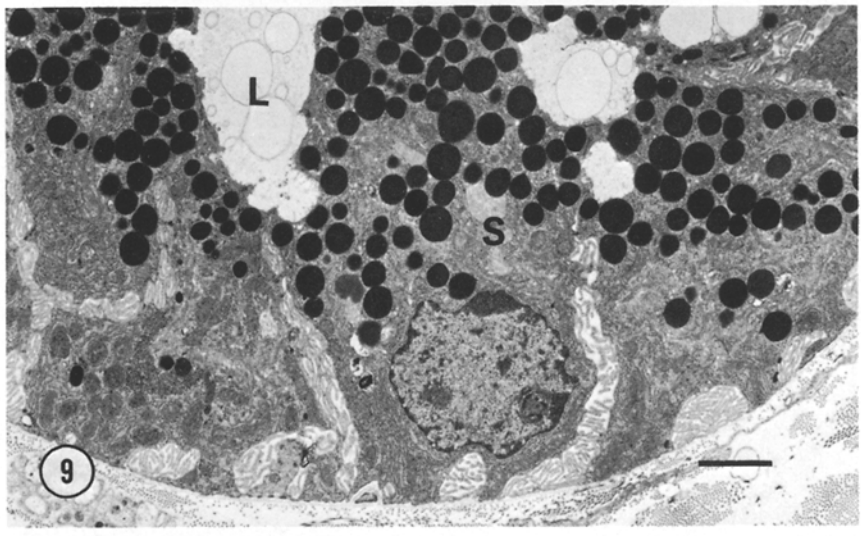
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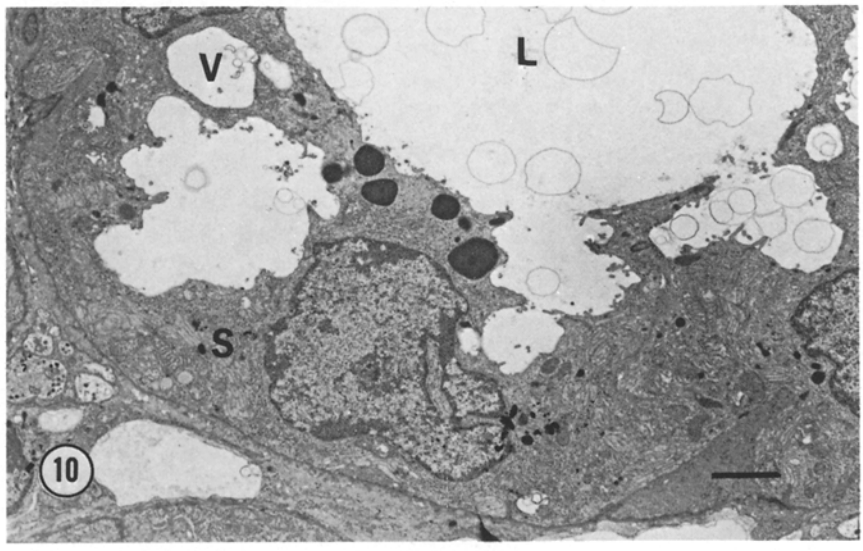
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Bethanechol caused an increase of 260 ± 80.9% (mean ± SE, $n=8$); phenylephrine 80 ± 16.4% ($n=10$); and terbutaline 25 ± 10.2% ($n=8$).

Atropine and propranolol abolished the stimulatory effects of bethanechol and terbutaline, respectively. Phentolamine inhibited but did not completely abolish the stimulatory effects of phenylephrine. The amount of lysozyme released during phentolamine blockade was not, however, significantly different from control values.

Analysis of variance and the Neuman-Keuls comparison test (Zar 1974) indicated that the magnitude of the stimulatory effects of bethanechol, phenylephrine and terbutaline were significantly different from each other, bethanechol > phenylephrine > terbutaline.

Histology

Histological examination of tissues taken before and after incubation confirmed that submucosal glands and epithelium remained intact throughout the experiments. Tissues exposed to bethanechol for 3 h showed a marked loss of immunoreactive lysozyme from serous cells and large increases in the luminal diameters (Fig. 6). As would be predicted, nonspecific staining in the cartilage was not affected by stimulation. Tissues incubated in control medium for the same time period showed no significant loss of immunoreactive lysozyme (Fig. 7).

In the submucosal glands fixed immediately after removal from the animal, the mucous cells contained secretory granules that were electron transparent, but with eccentric-dense cores (Fig. 8). Serous cells contained secretory granules that were uniformly electron dense. In both cell types, secretory granules occupied a large proportion of the cell cytoplasm.

After 30-min incubation in control medium, the cells, as in fresh tissue, were filled with dense secretory granules (Fig. 9). Lumens were small and regular in contour.

Electron-microscopic examination of tracheal segments exposed to bethanechol or phenylephrine during Period 2 showed serous cells to be markedly depleted of secretory granules (Figs. 10, 11). Apical membranes of many stimulated cells were convoluted and contained exocytotic figures (Fig. 12). In tracheal segments stimulated with terbutaline, serous cell morphology was similar to that of control tissues (Fig. 13) though some degree of deple-

Fig. 8. Electron micrograph illustrating the appearance of serous (*S*) and mucous (*M*) cells in submucosal glands of ferret trachea. Note differences in density and size of secretory granules in the two cell types. × 4800, Scale bar = 2 μm

Fig. 9. Electron micrograph illustrating the appearance of serous (*S*) cells after incubation for 30 min in control medium; *L* lumen. × 4200, Scale bar = 2 μm

Fig. 10. Electron micrgraph illustrating the appearance of serous (*S*) cells after incubation for 30 min in bethanechol (10^{-5} M). Note marked depletion of secretory granules, presence of vacuoles, and convoluted appearance of gland lumen. *V* vacuole, *L* lumen. × 4200, Scale bar = 2 μm

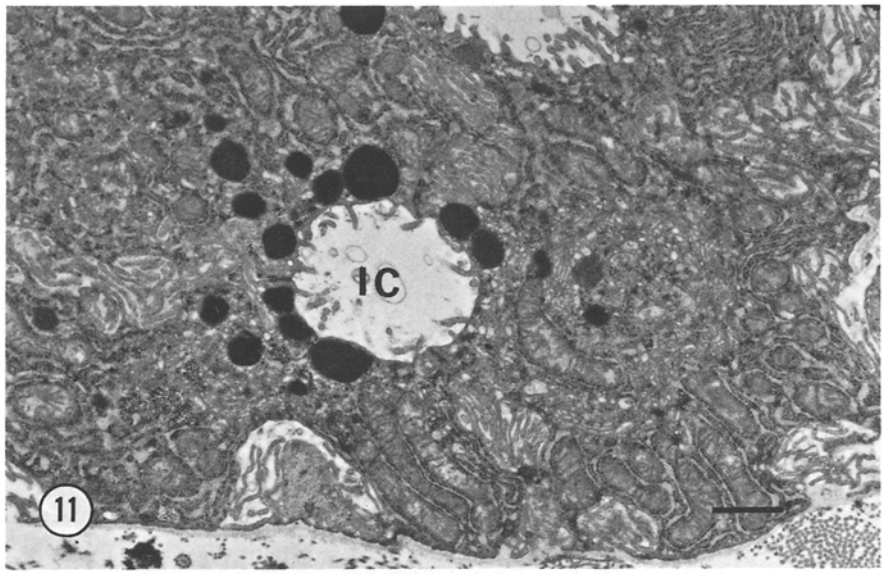

Fig. 11. Electron micrograph illustrating the appearance of serous cells after incubation for 30 min in phenylephrine (10^{-5} M). *IC* intercellular canaliculus. × 8660, Scale bar = 1 μm

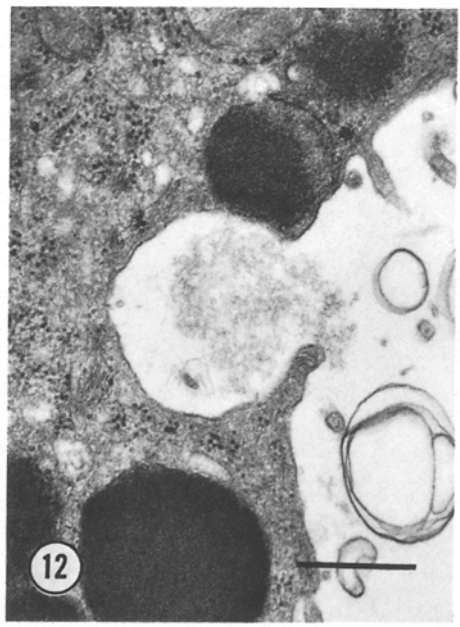

Fig. 12. Electron micrograph illustrating one of many exocytotic figures observed in serous cells after incubation for 30 min in bethanechol (10^{-5} M). × 35000, Scale bar = 0.4 μm

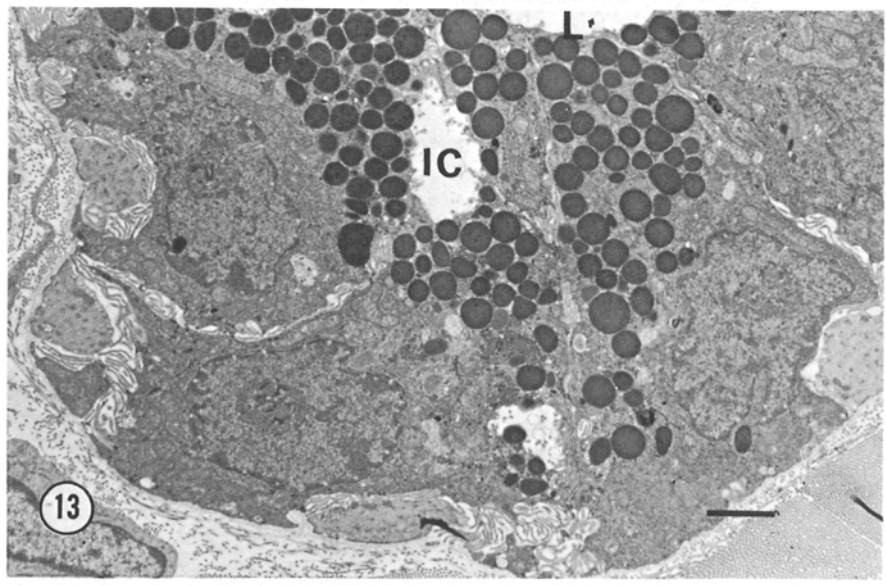

Fig. 13. Electron micrograph illustrating the appearance of serous cells after incubation for 30 min in terbutaline (10^{-5} M). *IC* intercellular canaliculus, *L* lumen. ×4480, Scale bar = 2 μm

tion was apparent. Similar changes have been described extensively and quantified in an earlier paper (Basbaum et al. 1981).

Discussion

We have shown that in the ferret, the primary cellular source of lysozyme is the serous cell of the submucosal gland. Although immunocytochemical studies were not done at the electron-microscopic level, observations made on araldite sections (1 μm) provided sufficient resolution to determine that lysozyme was associated with secretory granules. Knowing this, we postulated the existence of a relationship between secretory activity of serous cells and release of lysozyme. Using combined biochemical, electron microscopic and immunocytochemical methods, we confirmed the existence of this relationship by comparing the effects of pharmacologic stimulation on (a) lysozyme release, (b) depletion of secretory granules, and (c) loss of immunoreactive lysozyme from tissue sections. Those agents that caused release of lysozyme also depleted serous cells of secretory granules and, where tested, also depleted immunoreactive material from tissue sections. Terbutaline, which has a small effect on lysozyme release, was relatively ineffective in causing depletion of secretory granules. This appears to establish the usefulness of lysozyme as a protein marker for serous cells, at least in ferret trachea, where lysozyme is confined to that cell type.

The serous cell has been established as the primary cell of origin for

lysozyme at many tissue sites in a variety of species. These include the human bronchus (Bowes and Corrin 1977), mouse laryngotracheal glands (Spicer and Frayser 1977), human salivary glands (Mason and Taylor 1975; Klockars and Reitamo 1975), and mouse salivary glands (Spicer and Frayser 1977). Other investigators have observed lysozyme in cartilage, macrophages (Klockars and Reitamo 1975; Mason and Taylor 1975), and on the cilia of the surface epithelium of the upper respiratory tract of the rat (Spicer and Frayser 1977; Konstan et al. 1981). The lack of immunoreactivity of these cells in our hands may reflect species differences, loss of antigenic determinants during tissue processing, or lack of adequate sensitivity to detect small quantities of lysozyme in certain cell types. Data reported by Konstan et al. (1981) indicate that despite the lack of immunochemical staining for lysozyme, explants of human tracheal epithelium release considerable amounts of lysozyme. This release occurs spontaneously in culture and the cellular source is unknown. Since lysozyme is a highly basic protein having enzymatic activity, its presence in cytoplasm might be expected to have a deleterious effect on the internal cellular milieu. Consistent with this, immunocytochemical studies (Bowes and Corrin 1977) have demonstrated that lysozyme is confined to secretory granules within cells and does not occur in cell cytoplasm. The tracheal epithelium of the ferret has few or no granulated secretory cells (Jacob and Poddar 1981) and therefore is likely to secrete little or no lysozyme, although this has not been tested biochemically in isolated ferret tracheal explants. Further, if lysozyme were found to be secreted by ferret epithelium its secretion rate would be unlikely to be influenced by the drugs used in this study since, unlike tracheal glands, the epithelium appears unresponsive to neurotransmitter-like agents with respect to both secretion of macromolecules and stimulation of cAMP (Liedtke et al. 1982).

Our studies were performed using a heterologous (human) antibody. Cross-reactivity between lysozyme from humans and lysozyme from other species has been demonstrated previously (Glynn and Parkman 1964; Spicer and Frayser 1977). Immunologic cross reactivity has also been observed between mouse and rat lysozymes (Glynn and Parkman 1964), between lysozymes in different primates (Wilson and Prager 1974), and between lysozymes in various avian species (Arnon 1977). Our finding of immunocytochemical cross reactivity between human and ferret lysozyme suggests the existence of homologous immunoreactive sites in these species. Molecules of human and ferret lysozymes may not be cross-reactive in their native states, but rather may become cross-reactive when the molecules are chemically altered by fixation (Arnheim 1971). Fixation may also alter the steric conformation of lysozyme, resulting in the unfolding and exposure of antigen sites necessary for cross-reactivity to occur.

The data from the present study are fundamentally consistent with those obtained in morphometric studies in which depletion of serous granules was observed in response to both adrenergic and cholinergic agonists (Basbaum et al. 1981). However, in the former study, small effects of terbutaline did not reach statistical significance. In the present study, terbutaline

produced a small but significant effect. Our current interpretation is that beta receptors are present on serous cells but their role in mediating exocytosis is minor relative to that of alpha adrenergic and cholinergic receptors. In the present study, using the lysozyme assay, responses of thousands of cells could be monitored in a single assay requiring a relatively short period of time. The morphometric method could realistically provide samples of only several hundred cells, with data collection requiring months of work. Therefore, small differences, such as that seen following terbutaline stimulation, were better revealed using the assay.

Adrenergic effects on lysozyme release have not previously been described in the airways. Human bronchial explants released lysozyme in response to cholinergic but not to adrenergic agonists (Boat and Kleinerman 1975). More recent studies have shown that adrenergic agonists do evoke release of macromolecules from human bronchial explants (Phipps 1979; Phipps et al. in press), cat trachea (Ueki et al. 1980) and ferret trachea (Borson et al. 1980). However, the adrenergic receptors in human glands may mediate secretion from cells that do not secrete lysozyme.

Preliminary data from our laboratory (with B. Borson) indicate that stimulation by electrical field can evoke lysozyme release via excitation of axons present within the tissue. Such experiments confirm that neurotransmitter released by electrical stimulation is able to reach receptors on the glands in sufficient concentration to effect the release of lysozyme.

The application of lysozyme as a marker for a specific cell type has been previously demonstrated in the stomach, where it was used as a marker to identify neoplastic Paneth cells in gastric adenocarcinoma (Heitz and Wegmann 1980). In the airways, we tentatively propose that release of lysozyme can be used to identify neurotransmitters, neuropeptides and other mediators (e.g., histamine, prostaglandins, calcium, cyclic AMP) involved in regulation of secretion by serous cells. To confirm that small unstainable quantities of lysozyme are not being released by mucous or other cell types requires isolation and stimulation of these cells in vitro.

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