Rabbit Lacrimal Acinar Cells in Primary Culture: Morphology and Acute Responses to Cholinergic Stimulation

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Purpose. The rabbit lacrimal gland yields large numbers of viable acinar cells that, when exposed to carbachol, respond with accelerated protein release, fluid phase endocytosis (Lucifer yellow uptake), and Na/H antiport activation. The current study was undertaken to determine whether such cells exhibit similar responses after having been maintained in primary culture.

Methods. Cells were isolated from 2-kg, juvenile male New Zealand White rabbits and maintained in a supplemented DMEM/Ham’s F-12 medium for up to 72 hours.

Results. Electron microscopy showed the organization of freshly isolated cells to be highly polarized, with secretory vesicles at one pole and nucleus at the other; vesicles were heterogeneous in size and in the electron density of their contents. The cells remained polarized after overnight culture, but the secretory vesicle population was more homogeneous in size and content, and the cells tended to aggregate. After 72 hours, roughly half the cells retained good morphology and cytoplasmic polarization, but the vesicles were enlarged and their contents less electron dense. Cells that had been maintained overnight responded to the addition of 10 μM carbachol with a 32.2% ± 15.5% (n = 12, P < 0.04) increase in the total amount protein released during a standard 20-minute incubation. This represented a mean 125% increase in the temperature-dependent component of protein release. The protein secretory response was decreased to 14.6% ± 6.1% (n = 3, P < 0.07) for cells that had been maintained for 72 hours. In the same samples, carbachol increased fluid phase endocytosis by 38.3% ± 8.1% (P < 0.01) and 70.9% ± 13.4% (P < 0.025), respectively. The protein secretory response was partially, and the endocytic response fully blocked by 1 mM atropine.

Conclusions. This model could be useful as a simplified system in which to study regulation of acinar cell function, rather than hours, as is required in fresh tissue models. Invest Ophthalmol Vis Sci. 1994;35:1176–1183.

The aqueous tears produced by the lacrimal glands provide lubrication, protection, nutrition, and optical uniformity of the ocular surface epithelium and are essential for the maintenance of a healthy eye. Several different experimental approaches have contributed to our understanding of the normal secretory physiology of the lacrimal gland. Cannulation of the excretory ducts of rat1 and rabbit2–4 lacrimal glands has led to documentation of the ionic composition of lacrimal gland fluid, and has provided general information about short-term neural and hormonal control of lacrimal secretion. More detailed information about lacrimal stimulus–secretion coupling has been obtained through studies of gland fragments, acini, and acinar cells isolated from rat exorbital lacrimal glands.5

It is becoming clear that, in addition to acute control exerted by autonomic secretomotor neurotransmitters and neuropeptides, the secretory function of the lacrimal glands is subject to long-term regulation by hormones of the hypothalamic–pituitary–gonadal axis.6 Hann and coworkers7 developed procedures for maintaining rat lacrimal acinar cells in primary culture.
as a simplified experimental preparation for studies of long-term regulation of lacrimal function. Kelleher et al. have found that androgens, as well as various hormones, cytokines, neurotransmitters, and neuropeptides modulate the synthesis of secretory component (the polymeric IgA receptor) in these cells. Such preparations, however, appear to lose the ability to release secretory proteins in response to acute exposure to cholinergic agonists.

We have found that it is feasible to isolate acini and acinar cells from rabbit lacrimal glands. Cholinergic stimulation of freshly isolated preparations appears to elicit several responses related to secretory function, including release of protein, activation of Na+ influx via Na/H antiporters, and acceleration of membrane recycling traffic. We also have found that it is possible to maintain rabbit lacrimal acinar cells in primary culture for at least 72 hours. Therefore, we undertook the current study with the goal of determining whether acinar cells from the rabbit lacrimal gland retain functions related to the processes of protein, fluid, and electrolyte secretion after they have been maintained in primary culture. We kept rabbit lacrimal acinar cells for up to 72 hours in a simple, serum-free culture medium. We then examined their morphology with transmission electron microscopy, and we evaluated the ability of the muscarinic cholinergic agonist, carbachol, to elicit protein release, cytoplasmic alkalization (a marker for Na/H antiporter activation), and accelerated fluid phase endocytosis (an index of membrane recycling traffic).

MATERIALS AND METHODS

Materials

Ham's nutrient mixture F-12, DME (low-glucose Dulbecco's modified Eagle's medium), and Pen-Strep-Glu were purchased from Irvine Scientific (Irvine, CA). Hanks' balanced salt solution (Ca2+- and Mg2+-free), DNase I (type II), soybean trypsin inhibitor, linoleic acid, and bovine serum albumin (BSA) were from Sigma Chemical Co. (St. Louis, MO). Lucifer yellow (Lucifer yellow-Ch) and 2',7'-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) were purchased from Molecular Probes (Eugene, OR). Collagenase was obtained from Gibco (Grand Island, NY), and hyaluronidase was from Worthington (Freehold, NJ). All other chemicals were reagent grade and were obtained from standard suppliers.

Solutions

Hanks' balanced salt solution, Ca2+ and Mg2+ free, was supplemented with ethylenediamine tetraacetic acid (EDTA; 0.76 mg/ml) and HEPES (10 mM), and brought to pH 7.6 with 1 M NaOH (s-Hanks'). Ham's F-12 medium was supplemented with BSA (0.5%), soybean trypsin inhibitor (0.05 mg/ml), penicillin (100 U/ml), streptomycin (0.1 mg/ml), glutamine (2 mM), linoleic acid (0.084 μg/ml), sodium butyrate (2 mM), and HEPES (10 mM), and brought to pH 7.6 with 1 M NaOH (s-Ham's). Before use, both solutions were equilibrated at 37°C and gassed with 95% O2/5% CO2. The pH was checked both before use and during the cell isolation procedure.

Collagenase (200 U/ml), DNase (10 U/ml), and hyaluronidase (698 U/ml) were dissolved in Ham's medium (Collagenase DNase Hyaluronidase-Ham's, or CDH-Ham's) just before use for acinar cell isolation. Culture medium consisted of Ham's F-12 (500 ml), and DME (500 ml), supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml), glutamine (2 mM), linoleic acid (0.084 μg/ml), sodium butyrate (2 mM), hydrocortisone (5 nM), transferrin (5 μg/ml), insulin (5 ng/ml), and EGF (10 ng/ml).

The physiological saline for cytoplasmic pH measurements consisted of NaCl (130 mM), K2HPO4 (2.5 mM), KCl (1 mM), glucose (5 mM), and HEPES (5 mM); pH was adjusted to 7.1 with NaOH. A stock solution of Lucifer yellow (10 mg/ml) was prepared in Hanks' balanced salt solution, filtered through a 20-μm Millipore filter, and kept frozen until use.

Isolation and Culture of Acinar Cells

The studies described herein conformed to the ARVO Resolution on the Use of Animals in Research, and animals were maintained in facilities fully accredited by the American Association of Laboratory Animal Science. Single cells were isolated by a modification of previously described procedures. Typically, six male New Zealand rabbits (Irish Farms, Norco, CA), weighing approximately 2 kg, were anesthetized by an intramuscular injection of 10 mg/kg xylazine HCl and 40 mg/kg ketamine HCl, then euthanized by intravenous administration of 65 mg/kg sodium pentobarbital. The eyes were removed and the lacrimal glands were harvested from a beaker containing 25 ml of s-Ham's. The glands were rinsed with s-Ham's, placed in 5-ml pools of s-Ham's on ultraviolet-irradiated dental wax sheets, cut into 1-mm3 pieces with a pair of scalpels, and transferred to two 100-ml polypropylene Erlenmeyer flasks. The fragments were allowed to settle and the supernatant was aspirated off and replaced with 30 ml of s-Ham's per flask. The fragments were then washed around, then allowed to settle, and the supernatant was removed. This procedure was repeated once with s-Ham's and once with s-Hanks' before the fragments were suspended in 30 ml of s-Hanks'. The flasks were gassed with 95% O2/5% CO2 for 5 seconds, capped,
and placed in a shaking water bath set at 37°C and 120 oscillations/minute.

The supernatant was aspirated off after 15 minutes of incubation, and the fragments were washed once with s-Ham's, placed in 30 ml CDH-Ham's, and incubated as above. Cells were sedimented by centrifugation for 5 minutes at 101g in a centrifuge equipped with a swing-out rotor. The supernatant was discarded and the pellet suspended in 30 ml of s-Hanks'. This suspension was repelleted and suspended once more, transferred back to the flask and incubated for another 15 minutes, followed by another CDH-Ham's treatment for 25 minutes. The cell suspension was washed twice by centrifugation as above, with the addition of 10 ml of s-Ham's, and filtered through a 500-μm nylon mesh, followed by a 25-μm mesh. The filtrate was carefully layered on top of a gradient consisting of 10 ml each of 4%, 3%, and 2% Ficoll in s-Ham's and centrifuged at 50g for 15 minutes. The pellet was suspended in s-Ham's, and then washed once with s-Ham's and once with culture medium. After the wash, the pellet, containing cells from six rabbits, was suspended in 40 ml of culture medium.

A 1-ml aliquot containing roughly 7 × 10^6 resuspended cells and a 5-ml aliquot of culture medium were placed in each 100-mm plastic tissue culture plate (Corning Glassware, Corning, NY); 0.1% BSA was added to the culture media in some experiments, but had no effect on the results. The entire procedure described above was done under sterile conditions. The viability of the cells was checked with trypan blue. The supernatant was aspirated off after 15 minutes of incubation, and the fragments were washed once with s-Ham's, placed in 30 ml CDH-Ham's, and incubated as above. Cells were sedimented by centrifugation for 5 minutes at 101g in a centrifuge equipped with a swing-out rotor. The supernatant was discarded and the pellet suspended in 30 ml of s-Hanks'. This suspension was repelleted and suspended once more, transferred back to the flask and incubated for another 15 minutes, followed by another CDH-Ham's treatment for 25 minutes. The cell suspension was washed twice by centrifugation as above, with the addition of 10 ml of s-Ham's, and filtered through a 500-μm nylon mesh, followed by a 25-μm mesh. The filtrate was carefully layered on top of a gradient consisting of 10 ml each of 4%, 3%, and 2% Ficoll in s-Ham's and centrifuged at 50g for 15 minutes. The pellet was suspended in s-Ham's, and then washed once with s-Ham's and once with culture medium. After the wash, the pellet, containing cells from six rabbits, was suspended in 40 ml of culture medium.

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Microscopy

For ultrastructural studies, cells were resuspended in fixative (1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.6) and allowed to stand for 60 minutes. They were then washed three times in cold 0.1 M cacodylate buffer, pH 7.6, and postfixed in 2% aqueous osmium tetroxide for 30 minutes. The cells were subsequently rewashcd in cold buffer, dehydrated in a graded series of ethanol, and embedded in Epon 812 (Ladd Research Industries, Burlington, VT). Thin sections were cut with a Sorvall MT2B Ultramicrotome (RMC, Inc., Tucson, AZ), stained with uranyl acetate and lead citrate, and viewed under a JEOL 1200CX transmission electron microscope (Peabody, MA). In addition, fresh lacrimal tissue was taken at the time of sacrifice and processed in an identical manner.

Cytoplasmic pH

Cells were suspended in physiological saline, then loaded with the BCECF-AM for 15 to 20 minutes. Excess dye was removed by several wash/spin cycles. For each measurement, a sample of BCECF-loaded cells was placed in a fluorometer cuvette containing 2 ml of physiological saline. The ratio of fluorescence emission at 530 nm with excitation at 500 nm to fluorescence with excitation at 440 nm was recorded in a Perkin-Elmer LS 5B fluorometer (Norwalk, CT). The ratios were then converted to pH values by using a calibration curve generated by the nigericin pH clamp technique.14

Fluid Phase Internalization and Protein Release

Harvested cells were washed once with s-Hanks' (without EDTA), then resuspended in 700 μl of ice-cold s-Hanks' (without EDTA). Ninety-microliter aliquots containing roughly 10^7 cells were added to siliconized tubes containing 10 μl of Lucifer yellow and buffer (s-Hanks' without EDTA), either with or without carbachol. The uptake and release processes were re-started by placing the tubes in a shaking water bath at 37°C. Background values were obtained from parallel incubations at 4°C. After 20 minutes, 1-ml aliquots of ice-cold buffer were added to quench the reactions. A 100-μl sample was removed from each tube for determination of total protein, and the remaining cells were sedimented at 101g for 5 minutes in a centrifuge refrigerated to 4°C. The supernatant was saved for measurement of released protein. The pellet was washed three times with 4 ml of ice-cold buffer. The pellet was resuspended in 1 ml of 0.1% Triton X-100 and transferred to a plastic centrifuge tube. A 100-μl aliquot of 0.15% sodium deoxycholate was added, and the tubes were incubated at room temperature for 15 minutes before the addition of 150 μl of ice-cold 50% trichloroacetic acid (TCA). The tubes were placed on ice overnight, and the precipitate was discarded after centrifugation at 10,000 rpm in a GSA rotor (Sorvall, Wilmington, DE) for 15 minutes. The supernatant was collected and diluted with 1 ml of s-Hanks' (without EDTA). Fluorescence was measured in the Perkin-Elmer fluorometer at an excitation wavelength of 429 nm and an emission wavelength of 535 nm. Total cellular and released protein were determined by the method of Lowry et al15 after precipitation in 50% TCA in the presence of 0.15% deoxycholate. Lucifer yellow internalization and protein release data were normalized to the values obtained at 37°C in the ab-
sence of carbachol. Carbachol-induced differences were analyzed with Student's t test for paired samples.

RESULTS

Microscopy
As illustrated in Figure 1, rabbit lacrimal acinar cells in situ are highly polar, with the apical cytoplasm containing numerous secretory vesicles. These vesicles vary in size and in electron density. The basal cytoplasm contains nuclei, prominent endoplasmic reticulum, and well developed Golgi apparatus. Freshly isolated acinar cells were similar in morphology (Fig. 2), although the rough endoplasmic reticulum tended to be relatively more prominent, perhaps due to swelling, giving the cytoplasm a denser appearance. After 24 hours in primary culture, the cytoplasm remained polarized. The secretory vesicle population, however, appeared more homogeneous in size and content (Fig. 3A, B). Aggregates of two to three cells were common. In some cells (Fig. 3B), smaller vesicles appeared to have coalesced and emptied their contents into larger vesicles. After 72 hours, most cells were associated in multicellular aggregates containing both intact cells, which remained highly polarized, and atretic cells (Fig. 4). Secretory vesicles appeared to be enlarged, with less electron-dense contents.

Carbachol-Induced Cytoplasmic Alkalinization
Cytoplasmic pH of cells after overnight culture was 6.9 ± 0.21. Stimulation with 10 μM carbachol caused a 0.10 ± 0.01 unit increase in pH (Fig. 5), a change that was almost identical to the change of 0.11 ± 0.02 pH unit seen in freshly isolated cells. Several attempts were made to obtain corresponding measurements on cells that had been maintained in culture for 72 hours, but it proved difficult to load them with sufficiently high concentrations of BCECF.

Protein Release and Fluid Phase Endocytosis
As depicted in Figure 6, cells that had been kept in culture overnight responded to 10 μM carbachol by increasing protein release and by increasing internalization of extracellular fluid. The background values measured at 4°C accounted for 78% of the protein release and 65% of the Lucifer yellow internalization observed at 37°C. These values probably represented leakage of protein out of, and fluid phase marker into, damaged cells. Despite these background values, it appears that protein release and fluid phase internalization proceeded at significant rates in the absence of carbachol. In paired analyses of 12 separate preparations, 10 μM carbachol increased protein release by 32.2% ± 15.1% (P < 0.04); because the background protein release was 74.3% ± 5.6% of the value at 37°C, the carbachol-induced change represented a 125% increase in the temperature-dependent component of the total protein release. In the same 12 preparations, carbachol increased Lucifer yellow internalization by 38.3% ± 8.1% (P < 0.01), representing a 119% increase in the temperature-dependent component of the total fluid phase marker internalization. These carbachol-induced changes were somewhat larger than the 50% increase in temperature-dependent protein release and 75% increase in temperature-dependent fluid phase internalization noted in freshly isolated rabbit lacrimal acinar cells. Although the variances for protein release and Lucifer yellow internalization

FIGURE 1. Transmission electron micrograph of rabbit lacrimal cells in situ (original magnification X3636).

FIGURE 2. Transmission electron micrograph of freshly isolated rabbit lacrimal acinar cells (original magnification X4545).
FIGURE 3. Transmission electron micrographs of cells after overnight primary culture. (A) A cell with several large vesicles. (B) A cell with smaller secretory vesicles appearing to fuse and release their contents into larger vesicles (original magnifications: A, ×4725; B, ×4524).

were similar in the absence of carbachol, the variance for carbachol-stimulated protein release was more than twice the variance of carbachol-stimulated Lucifer yellow internalization. In two preparations, 1 mM atropine sulfate inhibited approximately 50% of the carbachol-dependent protein release and 100% of the carbachol-dependent Lucifer yellow uptake. We are not at this time able to offer an explanation for the apparent atropine-independent component of carbachol-induced protein release.

FIGURE 4. A montage of transmission electron micrographs illustrating cells after 72 hours in primary culture that have aggregated into a larger assembly (original magnification ×2085).

When cells were maintained in culture for 72 hours, the protein secretory response to carbachol was blunted, whereas the endocytic response was enhanced (Fig. 7). In paired analyses of three separate preparations, the amount of protein released in the presence of 10 μM carbachol was 14.6% ± 6.1% greater than in the absence of carbachol (P < 0.07). In contrast, at this concentration carbachol increased total Lucifer yellow internalization by 70.9% ± 13.4% (P < 0.02). This represented a greater than sevenfold increase in the temperature-dependent component of the fluid phase internalization rate.

DISCUSSION

The ability of lacrimal acinar cells to release secretory proteins in response to neurotransmitters and neuropeptides has been documented in numerous studies of stimulus–secretion coupling mechanisms. It also is widely appreciated that secretagogues activate several different acinar cell ion transport pathways, including Cl− channels, K+ channels, and Na/H antiporters. Much more recently, it has become apparent that cholinergic stimulation accelerates a bulk membrane recycling traffic between the acinar cell basal–lateral membranes and endocytic compartment, and induces a significant redistribution of Na,K-ATPase pump units from intracellular pools to the plasma membranes.

In addition to the short-term regulation mediated by autonomic secretomotor innervation, longer-term regulation of lacrimal function is exerted by hormonal interactions. As Sullivan has discussed in a recent, comprehensive review, a number of functional and morphologic features of lacrimal acinar cells, includ-
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FIGURE 5. Cytoplasmic alkalinization. Intracellular pH was allowed to reach a stable steady state for 15 minutes before the addition, at t = 0, of 10 μM carbachol. Results presented are mean changes above resting pH ± SEM for three separate preparations.

ING their size and, most notably, the rate at which they synthesize secretory component, are regulated by hormones of the hypothalamic–pituitary–gonadal axis. Until recently, little attention has been devoted to the hormonal regulation of aspects of the acinar cell phenotype that are related either to the release of secretory proteins or to the transepithelial secretion of electrolytes. Preliminary reports, however, suggest that the levels at which lacrimal glands express several membrane-associated proteins, including Na,K-ATPase pumps, muscarinic cholinergic receptors, and β-adrenergic receptors, are modulated by the sex hormones.

It is beginning to appear that the rabbit lacrimal gland will be a particularly useful model for parallel in vivo and in vitro studies of the long-term regulation of lacrimal acinar cell functions. It is feasible to isolate large numbers of functionally responsive cells from rabbit lacrimal glands. Because the results described herein indicate that such cells retain several characteristic morphologic and functional features during maintenance in a simple, serum-free culture medium, it now should be possible to begin dissecting the mechanisms underlying hormonal regulation of these features.

During the first 24 hours in culture, the cells retain the ability to alkalinize their cytoplasm, release protein, and accelerate membrane recycling traffic in response to carbachol. The alkalinization response after overnight culture is similar to that observed in freshly isolated cells. The protein secretory response after overnight culture is similar to that observed in preparations of freshly isolated, intact acini, and both the protein-secretory and the fluid-phase internalization responses are somewhat larger than those observed in preparations of freshly isolated acinar cells. By 72 hours in culture, the protein-secretory response appears to be substantially attenuated, and it becomes difficult to load the cells with enough BCECF to provide accurate measurements of the cytoplasmic pH. The reasons for diminished loading with BCECF after the longer culture interval are unknown. One possible explanation for the time-dependent decrement in protein secretion is that the integrity of the pathway for assembly, storage, and release of secretory proteins depends on a continuous flux of material, which is interrupted when cells are isolated and no longer intermittently stimulated. Another possibility worthy of further consideration is that the organization of this pathway depends on minimal levels of intracellular mediators whose production decreases when cells are removed from their normal structural, paracrine, neural, and endocrine milieu. Preliminary reports suggest that a specific component of the extracellular matrix may be critical in this communication.

Contrasting with the difficulties of measuring protein secretion and cytoplasmic alkalinization after 72 hours in culture, the cultured acinar cells fully retain their ability to accelerate fluid-phase internalization in response to carbachol. The relative magnitude of the
FIGURE 6. Protein release and fluid phase internalization by cells that had been maintained in primary culture overnight. Washed and resuspended cells were incubated for 20 minutes at 4°C, 37°C, or 37°C in the presence of 10 μM carbachol (CCh). Data were normalized to the 37°C values, which averaged 0.393 ± 0.065 mg protein released/mg total protein and 36.2 ± 6.4 fluorescence units/mg total protein. Results presented are means ± SEM from 12 separate preparations.

The absolute values at 37°C were 0.538 ± 0.089 mg protein released/mg total protein and 12.7 ± 4.1 fluorescence units/mg total protein.

FIGURE 7. Protein release and fluid phase internalization by cells that had been maintained in primary culture for 72 hours. Values presented are means ± SEM from three separate preparations; other details are as in Figure 6. The absolute values at 37°C were 0.538 ± 0.089 mg protein released/mg total protein and 12.7 ± 4.1 fluorescence units/mg total protein.
their target membranes). That the acinar cells retain this response during several days of primary culture means that, in addition to studying long-term regulation of the levels of expression of individual cellular proteins, it will now be possible also to study the regulation of a complex process related to lacrimal secretory function.

**Key Words**
morphology, fluid phase endocytosis, protein secretion, Na/H antiport activation

**Acknowledgment**
The authors thank Kerry M. Zimmerman for her assistance with statistics and preparation of the manuscript and illustrations.

**References**