Cytopathology and Exocrine Dysfunction Induced in Ex Vivo Rabbit Lacrimal Gland Acinar Cell Models by Chronic Exposure to Histamine or Serotonin

Michelle L. McDonald,1 Yanru Wang,1 Shibaram Selvam,2,5 Tamako Nakamura,1 Robert H. Chow,1,4 Joel E. Schecter,5 Samuel C. Yiu,1,2 and Austin K. Mircheff1,3

PURPOSE. Lacrimal immunohistopathology has diverse clinical presentations, suggesting that inflammatory mediators exert diverse influences. Chronic exposure to agonistic acetylcholine receptor autoantibodies has been studied previously; the present work addressed mediators that signal through other G protein–coupled receptors.

METHODS. Acinus-like structures and reconstituted acinar epithelial monolayers from rabbit lacrimal glands were exposed to varying concentrations of histamine or 5-hydroxytryptamine (5-HT) for 20 hours. Net and vectorial β-hexosaminidase secretion, cytosolic Ca2+ (Ca2+) elevation, apical recruitment of p150GluRed, actin microfilament meshwork organization, and ultrastructure were assessed.

RESULTS. Histamine and 5-HT acutely stimulated β-hexosaminidase secretion at lower but not higher concentrations. Neither of them acutely elevated Ca2+ levels. Both recruited p150GluRed at concentrations that failed to induce secretion. Chronic exposure to 10 mM histamine inhibited carbachol (CCh)-induced β-hexosaminidase secretion and prevented the formation of continuous monolayers; 1 mM 5-HT partially inhibited secretion at the apical medium. Neither altered secretion to the basal medium. Chronic exposure to histamine or 5-HT partially decreased CCh induced Ca2+ elevations and p150GluRed recruitment, even at concentrations that did not inhibit secretion. Both expanded acinar lumina and thickened microfilament meshworks, and both caused homotypic fusion of secretory vesicles and formation of aqueous vacuoles in the apical and basal cytoplasm. Chronic exposure to forskolin, which activates adenylyl cyclase, induced similar cytopathic logic changes but impaired secretion modestly and only at the highest concentration tested.

CONCLUSIONS. Inflammatory mediators that signal through G protein–coupled receptors cause acinar cell cytopathology and dose-dependent reductions of CCh-induced β-hexosaminidase secretion. Although agonistic acetylcholine receptor autoantibodies may cause pervasive functional quiescence, inflammatory mediators may cause varying degrees of exocrine dysfunction. (Invest Ophthalmol Vis Sci. 2009;50:3164–3175) DOI:10.1167/iovs.08-2768

Ocular surface fluid provides a microenvironment, a milieu extérieur, for the living, nonkeratinized epithelial layers of the cornea and conjunctiva, buffering them from the desiccating influence, infectious agents, and chemical and particulate irritants of the atmosphere and lubricating the lid-globe interface. Ocular surface tissue and lacrimal glands maintain homeostasis largely by regulating the production of fluid, and failure of this homeostasis usually becomes evident symptomatically, with sensations of dryness, burning, or grittiness. Dry eye disease is common; according to one estimate,1 it affects more than 14 million Americans.

Some cases of dry eye are clearly associated with robust immunopathophysiological processes in the lacrimal gland, as occur in Sjögren’s syndrome, graft-versus-host disease, Wegener granulomatosis, sarcoidosis, and diffuse infiltrative lymphocytosis syndrome.2 The process in Sjögren’s syndrome, which has been studied most extensively, is characterized by the organization of CD4+ T cells, IgG+ B cells, and dendritic cells into foci near venules and interlobular ducts. A large volume of parenchymal tissue may coexist with the infiltrating lymphocytes, but, though the epithelial cells appear grossly intact, their exocrine functions are quiescent.3–5

Many more cases of dry eye occur without signs of systemic autoimmune disease, but they may be associated with local inflammatory processes. Postmortem studies have documented age-related increases in diffuse lymphocytic infiltration, interstitial fibrosis, acinar atrophy, and ductal stenosis in human lacrimal glands.6–10 Strikingly, though, age-related histopathology, which occurs in 65% of women 75 years of age and older, is more frequent than symptomatic dry eye disease. This disparity suggests that different immunopathophysiological processes can occur with similar histopathologic appearance but different impacts on exocrine function. This conundrum is reinforced by the finding that accumulation of extensive ectopic lymphoid tissue in the lacrimal glands of patients with Mikulicz’ disease can have little impact on exocrine function.11 Evidence indicates that the inflammatory cytokines TNF-α and IL-1β can cause exocrine quiescence. In ex vivo models of mouse lacrimal gland, these cytokines impair the release of neurotransmitters from secretomotor nerve endings and exocrine responses to pharmacologic secretagogue stimulation.11

Another studies have implicated agonistic IgG autoantibodies to the M3 muscarinic acetylcholine receptor (M3ACHR),12 which mediates parasympathetic control of lacrimal secretion. Chronic exposure of ex vivo acinar cell models from rabbit lacrimal gland to a half-maximal dose of the M3ACHR agonist, carbachol (CCh), induces exocrine quiescence associated with a number of cytologic, biochemical, and functional changes,13 including downregulation of protein kinase Ca2+, γ-adaptin, and

From the Departments of 1Physiology and Biophysics, 2Ophthalmology, and 3Cell and Neurobiology and the 4Zilkha Neurogenetics Institute, Keck School of Medicine, Los Angeles, California; and the 5Mork Family Department of Chemical Engineering and Materials Science, Viterbi School of Engineering, University of Southern California, Los Angeles, California.

Supported by National Institutes of Health Grants EY005801 (AKM) and EY010550 (JES) and Digestive Diseases Core Grant P30 DK068522. Submitted for publication August 25, 2008; revised December 27, 2008, and February 5, 2009; accepted May 18, 2009.

Disclosure: M.L. McDonald, None; Y. Wang, None; S. Selvam, None; T. Nakamura, None; R.H. Chow, None; J.E. Schecter, None; S.C. Yiu, None; A.K. Mircheff, None.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked ‘advertisement’ in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Austin K. Mircheff, Department of Physiology and Biophysics, Keck School of Medicine, 1333 San Pablo Street, MMR 626, Los Angeles, CA 90089-9142; amirchef@usc.edu.
the polymeric immunoglobulin receptor (pIgR); redistribution of M3ACrR from intracellular compartments to the cell surface; increased content of total membrane phase-associated actin; thinning and punctate interruptions of the actin microfilament meshwork underlying the apical plasma membrane; thickening of the actin meshwork underlying the basal-lateral plasma membrane; and diminished abilities to elevate cytosolic calcium (Ca<sup>2+</sup>) and secrete β-hexosaminidase in response to stimulation with normally optimal doses of CCh. Many of these changes are reminiscent of changes that have been documented in biopsy samples from labial salivary glands of patients with Sjögren’s syndrome, and they are consistent with the effects anti-M3ACrR autoantibodies exert in other ex vivo models. In addition to altering the apical actin meshwork, chronic exposure to half-maximal CCh also prevents optimal CCh doses from inducing recruitment of the dynactin complex, marked by p150<sup>Glued</sup>, to the immediate subapical region, where the cortical actin microfilament meshwork normally is most well developed. This phenomenon is of interest because the dynactin complex links cargo vesicles to microtubules and the molecular motor, cytoplasmic dynein, which traffics toward microtubule minus-ends focused in the subapical actin microfilament meshwork.

Results of studies of the lacrimal glands of aging rodent models suggest that mast cell mediators also might cause exocrine quiescence. Age-associated histopathology in rats<sup>19,20</sup> and mice<sup>21</sup> is characterized by striking accumulations of mast cells. Functional experiments indicate that two mast cell mediators, histamine and serotonin (5-hydroxytryptamine [5-HT]), are secretagogues for ex vivo acinar cell models from rat lacrimal glands. M<sub>3</sub>AChR activates the heterotrimeric GTP-binding proteins G<sub>q</sub> and G<sub>11</sub>. There are several subtypes of receptors for histamine and 5-HT, some of which also activate G<sub>q</sub> and G<sub>11</sub>; some subtypes activate G<sub>q</sub>, some activate G<sub>q</sub> and G<sub>11</sub>, and some are ligand-gated channels. The histamine and 5-HT receptor subtypes in lacrimal acinar cells have not been characterized, though it has been established that G<sub>q</sub>, G<sub>q</sub>, G<sub>q</sub>, and G<sub>q</sub>/G<sub>11</sub> are present<sup>23,24</sup> and widely distributed through a system of endomembrane compartments.<sup>25</sup> Therefore, it seemed reasonable to pose the hypothesis that chronic exposure to histamine or 5-HT would have effects on exocrine function similar to the effects of chronic exposure to CCh.

**Materials and Methods**

**Animals**

Female New Zealand White (NZW) rabbits weighing approximately 2 kg each were obtained from Irish Farms (Norco, CA). All experiments were performed according to a protocol conforming to the ARVO Statement for the Use of Animals in Ophthalmic Research and the American Physiological Society’s Guiding Principles for the Use of Animals in Research and were approved by the Animal Care and Use Committee of the authors’ institution. Culture medium was from BD Biosciences (HepatoStim; Medford, MA). Forskolin, 5-HT, PGE<sub>2</sub>, histamine, bovine serum albumin (BSA), methylumbelliferyl-N-acetyl-[D]-glucosaminide, and rhodamine-phalloidin were from Sigma Chemical Company (St. Louis, MO). Materials for confocal microscopy, including anti-p150<sup>Gluced</sup> mouse monoclonal antibody, goat-anti–mouse secondary antibody conjugated to FITC, and mounting medium were purchased from Transduction Laboratories (Anti-fade; Lexington, KY). AM-Fura-2 and Alexa Fluor-phalloidin were from Invitrogen (Carlsbad, CA).

**Solutions**

Solutions and media for cell culture have been described in previous publications.<sup>26,47</sup>

**Acinar Cell Isolation and Reconstitution of Acinus-like Structures**

Superior and inferior lacrimal glands were removed at necropsy from rabbits weighing approximately 2 kg each. Acinar cells were isolated and maintained in conditions described in previous reports.<sup>15,20–25</sup> They were cultured on basement membrane matrix (Matrigel; BD Biosciences)-coated 24-well plates for secretion assays, on basement membrane matrix-coated #2 thickness coverslips in 12-well plates for confocal fluorescence imaging studies, on basement membrane matrix-coated #1 thickness coverslips in 24-well plates for Ca<sup>2+</sup> imaging studies, and on basement membrane matrix rafts for electron microscopy.<sup>23</sup> Under these conditions, the cells reconstitute acinus-like structures within 2 days and maintain histotypic morphology and responses to acute stimulation with CCh. This ex vivo model has the additional advantage that cellular metabolism during the initial 2 days in culture depletes endogenous immunoglobulins, permitting the use of antibodies made in rabbits for immunohistochemical and immunofluorescence studies. After the second day in culture, the cells were transferred to fresh media and incubated another 20 hours under control conditions, in the presence of 10 μM CCh, or in the presence of histamine, 5-HT, or forskolin at the concentrations indicated in the figures, before the cells were analyzed. Cells from one or two rabbits were used in each experiment.

**Reconstitution of Acinar Epithelial Monolayers**

A recently described reconstituted acinar epithelial monolayer model<sup>44</sup> was used to measure the vectorial secretion of β-hexosaminidase. Acinar cells freshly isolated from rabbits weighing approximately 4 kg each were suspended in medium (HepatoStim; BD Biosciences) and seeded onto microporous polyester Transwell inserts in 12-well plates. Transepithelial electrical resistance was measured; when the cells established confluent monolayers, as indicated by the appearance of a significant resistance, the medium was replaced by fresh media with and without CCh, histamine, and 5-HT. As with the reconstituted acinus-like structure models, the incubations continued another 20 hours before secretory function was assessed.

**Secretion of β-Hexosaminidase**

Fresh medium was added to wells containing reconstituted acini and to both the apical and the basal chambers of Transwells containing reconstituted epithelial monolayers, and incubations were continued for additional periods of 2 hours for the acini and 3 hours for the monolayers. Baseline samples were then taken from each well, and the cells were acutely stimulated with either CCh, at a final concentration of 100 μM, or with histamine, 5-HT, or forskolin at the various concentrations indicated in the figure legends. After 50 minutes of acute stimulation, supernatant media were collected, and the amounts of β-hexosaminidase activity secreted into the apical chamber and 5-HT, or forskolin at the various concentrations indicated in the figure legends. After 50 minutes of acute stimulation, supernatant media were collected, and the amounts of β-hexosaminidase activity secreted into the apical chamber and 5-HT, or forskolin at the various concentrations indicated in the figure legends. After 50 minutes of acute stimulation, supernatant media were collected, and the amounts of β-hexosaminidase activity secreted into the apical chamber and 5-HT, or forskolin at the various concentrations indicated in the figure legends.
Differences between the amounts of β-hexosaminidase secreted by control cells and cells that had been treated for 20 hours with 10 μM CCh, histamine, 5-HT, or forskolin were evaluated for significance with Student’s t-test.

Cytosolic Ca\(^{2+}\)

Coverslips were rinsed twice with extracellular solution and incubated at room temperature for 45 minutes in medium containing 4 μM fura-2 AM. The cells were then placed in fresh medium and kept at 37°C for 15 minutes. Coverslips were placed in a chamber containing extracellular solution, and a cluster of cells was chosen for observation under brightfield illumination. The cluster was acutely superfused with histamine, 5-HT, forskolin, or CCh delivered with a micropipette. Fluorescence excitation alternating between 350 nm and 380 nm was provided with a monochromator-based illumination system (Polychrome V; TILL Photonics, Eugene, OR) coupled to a microscope (Axiovert 100; Zeiss; Oberkochen, Germany). Emitted fluorescence was detected with a cascade camera (Cascade 512B; Photometrics, Tucson, AZ). Changes in cytosolic Ca\(^{2+}\) were evaluated based on changes in the ratio of the fluorescence emission intensities at the two excitation wavelengths.

Confocal Fluorescence Imaging of Actin Microfilaments and p150\(^{G\beta\delta}\)

Media were replaced with fresh control medium, medium containing 100 μM CCh, or medium containing test mediators at the concentrations indicated in the figure legends. Cells were then incubated at 37°C for 20 minutes. Media were aspirated, and cells were fixed and permabilized in 100% ethanol at −20°C for 10 minutes. After that, they were rinsed with PBS, rehydrated in PBS for at least 10 minutes, and placed in 1% BSA blocking solution. Then they were stained with primary antibody against p150\(^{G\beta\delta}\) for 45 minutes at 37°C, rinsed, and stained with goat anti-mouse FITC-conjugated secondary antibody and with rhodamine-conjugated phalloidin to label actin microfilaments. Cells were viewed on a confocal microscope in the Imaging Sub Core at the University of Southern California Research Center for Liver Diseases. Additional images were collected with a confocal microscope (LSM 510 META; Zeiss) and the manufacturer’s software package in the laboratory of Sarah F. Hamm-Alvarez (Department of Pharmacology and Pharmaceutical Sciences, University of Southern California School of Pharmacy).

As reported by Wang et al.,\(^{35}\) when acinar cells are acutely stimulated with 100 μM CCh, they mobilize the dynamin complex, marked by p150\(^{G\beta\delta}\), to the apical cytoplasm. Recruitment of p150\(^{G\beta\delta}\) elicited by acute stimulation with test mediators was compared to recruitment induced by acute stimulation with CCh. Two independent investigators scored multiple confocal images from each of three separate cell preparations. Recruitment equivalent to that induced by CCh was given a score of 2. Slight recruitment of p150\(^{G\beta\delta}\) was given a score of 1. Images showing no recruitment were scored as 0. Qian et al.\(^{13}\) found that chronic exposure to 10 μM CCh diminished the recruitment of p150\(^{G\beta\delta}\) induced by acute stimulation with 100 μM CCh. Confocal images of cells that had been chronically exposed to test mediators, then acutely stimulated with 100 μM CCh, were compared with images of control cells and cells that had been chronically stimulated with 10 μM CCh. Inhibition of CCh-induced p150\(^{G\beta\delta}\) recruitment similar to the inhibition caused by overnight stimulation with 10 μM CCh was given a score of −2. A slight inhibition of p150\(^{G\beta\delta}\) recruitment was given a score of −1. Images showing no decrease in recruitment were given a score of 0. Two independent investigators scored multiple confocal images from three separate cell preparations.

Electron Microscopy

Cells in basement membrane matrix (Matrigel; BD Biosciences) rafts were fixed in 3.0% glutaraldehyde in Sorensen phosphate buffer, pH 7.3, and transferred to microcentrifuge tubes. This and all subsequent fixation and dehydration steps were conducted in a microwave oven (3440; Pelco, Edmond, OK). After initial fixation, the samples were centrifuged at 2000 rpm for 3 to 5 minutes to form a pellet. The pellets were washed with buffer and fixed in 1% OsO\(_4\) in Sorensen phosphate buffer, pH 7.3. Pellets were dehydrated in graded chilled ethanol steps of 50% and 100%, each for 40 minutes at 37°C. Pellets were then processed in three changes of chilled acetone, followed by a 1:1 mixture of acetone and LR White resin for 15 minutes at 47°C. This was followed by two steps of 15 minutes each in pure LR White at 47°C. Pellets were then transferred to capped capsules (Beem; Ted Pella, Redding CA), covered with flexible film (Parafilm; Pechiney Plastic Packaging, Chicago, IL), and cured under water in the microwave for 10 minutes at 60°C, 10 minutes at 70°C, and 25 minutes at 80°C. Blocks were thin-sectioned by ultramicrotome (RMC-TMx; RMC, Tucson, AZ), collected on copper grids, stained with heavy metals, and photographed with a transmission electron microscope (1011; JEOL, Tokyo, Japan).

Results

β-Hexosaminidase Secretion

β-Hexosaminidase is directed to the lysosomes in all nucleated cells. Lacrimal acinar cells, however, like neutrophils, also traffic β-hexosaminidase to secretory vesicles, and they secrete it as an exocrine product in response to acute stimulation with cholinergic agonists.\(^{35}\)

Figure 1 summarizes the results of experiments testing the hypothesis that acute stimulation with histamine or 5-HT accelerates β-hexosaminidase secretion by reconstituted acinus-like structures. Histamine significantly stimulated secretion at concentrations of 1 μM, 10 μM, and 100 μM, but not 1 mM or 10 mM. 5-HT significantly stimulated secretion at a concentration of 10 μM; apparent effects at 1 μM, 100 μM, and 1 mM were not statistically significant; at 10 mM, 5-HT was cytotoxic for some preparations.

Figure 1 also summarizes the effects of chronic exposure to histamine or 5-HT on the secretion of β-hexosaminidase induced by acute stimulation with 100 μM CCh. Chronic exposure to the highest histamine concentration tested, 10 mM, significantly reduced the response to CCh, but no significant effects were detected after chronic exposure to lower histamine concentrations, including those that acutely stimulated β-hexosaminidase secretion. Chronic exposure of reconstituted acinus-like structures to 5-HT at concentrations between 1 μM and 1 mM had no significant effect on secretion induced by acute stimulation with 100 μM CCh.

Although the M3/ChR, H1 histamine receptor, and 5-HT2 serotonin receptors signal primarily through G\(_{\alpha}\) and G\(_{\alpha,i}\), the histamine- and the 5-HT receptor families also include subtypes that signal through G\(_{\alpha}\). Given that one of the prominent actions of G\(_{\alpha}\) is to activate adenyl cyclase, studies were conducted to test the hypotheses that forskolin, which activates adenyl cyclase independently of G\(_{\alpha}\), acutely stimulates secretion and, after chronic exposure, impairs the response to acute CCh stimulation. Acute stimulation with forskolin significantly accelerated β-hexosaminidase secretion by acinus-like structures only at the highest concentration tested, 100 μM. Chronic exposure to forskolin at the concentrations tested had no significant effect on CCh-induced secretion.

Elevation of Cytosolic Ca\(^{2+}\)

Experiments were conducted to test the hypotheses that acute stimulation with histamine or 5-HT would elevate cytosolic Ca\(_{\text{i}}\) and that chronic exposure to either mediator would diminish the Ca\(_{\text{i}}\) elevation induced by acute stimulation with CCh. No significant elevation of Ca\(_{\text{i}}\) was observed after acute stimulation with histamine, 5-HT, or forskolin (data not shown). As summarized in Figure 2, chronic exposure to histamine at a con-
centration of 1 mM caused a 33% decrease in the peak Ca<sub>i</sub> elevation induced by acute stimulation with 100 μM CCh, but it caused only a negligible change in the total area under the curve. Chronic exposure to histamine at 10 mM decreased the peak Ca<sub>i</sub> elevation by 43% and the area under the curve by 53%. Chronic exposure to 5-HT and forskolin failed to significantly impair secretion. Number of separate cell preparations analyzed is indicated by n. Significant differences from the amount of CCh-induced secretion by control cells are indicated by stars.

**Actin Microfilament Organization and p150<sub>Glued</sub> Recruitment**

Meshworks of actin microfilaments underlie the apical- and basal-lateral plasma membranes of epithelial cells. Dynamic interconversion between the monomeric and polymerized forms of actin appear to be involved in the cycle of events underlying secretory vesicle exocytosis triggered by acute stimulation with CCh: docking and exocytotic fusion of secretory vesicles at the apical plasma membrane, expulsion of secretory products, and endocytotic retrieval of secretory vesicle membrane constituents for recycling through the endosomes, Golgi complex, and trans-Golgi network.29,32,36,37 Actin-mediated processes triggered by CCh are coordinated with microtubule-based phenomena: recruitment of the minus end-directed microtubule-based molecular motor, cytoplasmic dynein, into the basal-lateral plasma membrane; and chronic exposure to 5-HT or forskolin would impair the cells’ ability to recruit dynactin complexes, marked by p150<sub>Glued</sub>, to the immediate subapical region in response to acute stimulation with 100 μM CCh. Representative confocal fluorescence images are presented in Figures 3 and 4, and the findings are summarized graphically in Figure 5.

In control cells, illustrated in Figure 3, actin microfilament meshworks were prominently localized as intense fluorescent bands just beneath the luminal membranes and as continuous, but less intense, bands beneath the basal-lateral membranes; appreciable cytoplasmic staining was not present. In contrast, p150<sub>Glued</sub> staining was distributed throughout the cytoplasm. Acute stimulation with histamine often decreased the intensity of the subapical actin band slightly without causing discontinuities, and it caused much of the cytoplasmic p150<sub>Glued</sub> to redistribute to the immediate subapical region. Acute stimulation with histamine at 100 μM CCh or forskolin significantly increased the cytoplasmic p150<sub>Glued</sub> staining. Number of separate cell preparations analyzed is indicated by n. Significant differences from the amount of CCh-induced secretion by control cells are indicated by stars.

**FIGURE 1.** Left: secretion of β-hexosaminidase after acute agonist stimulation with histamine, 5-HT, or forskolin. β-Hexosaminidase secreted into the supernatant culture media was measured with a standard assay for catalytic activity against a model substrate. Acute stimulation with histamine at 1 μM to 10 μM appeared to be as effective as CCh at stimulating β-hexosaminidase release, but secretion decreased as the histamine concentration increased. 5-HT also stimulated secretion at lower, but not higher, concentrations. In contrast, forskolin stimulated secretion only at the highest concentration tested. Number of separate cell preparations analyzed is indicated by n. Significant differences (P < 0.05) from the amount of secretion in the absence of acute CCh stimulation are indicated by stars. Right: influence of chronic exposure to histamine or 5-HT on β-hexosaminidase secretion induced by acute stimulation with CCh (100 μM). Chronic stimulation by histamine at 10 mM appeared to be as effective as chronic treatment with CCh at decreasing the cell’s ability to secrete β-hexosaminidase. Chronic exposure to 5-HT and forskolin did not significantly impair secretion. Number of separate cell preparations analyzed is indicated by n. Significant differences from the amount of CCh-induced secretion by control cells are indicated by stars.
to the immediate subapical region. Acute stimulation with 5-HT between 1 μM and 100 μM also recruited p150\textsuperscript{Glued} without altering the subapical actin meshwork. However, at 1 mM, 5-HT moderately enlarged some lumina and expanded the subapical actin bands. Acute stimulation with forskolin caused appreciable p150\textsuperscript{Glued} recruitment at all concentrations tested. Between 10 μM and 1 mM, it also caused dose-dependent enlargement of lumina and expansion of subapical actin bands without decreasing the actin staining intensity (arrowhead). Thus, histamine and 5-HT acutely induced p150\textsuperscript{Glued} recruitment throughout the range of lower concentrations that did not significantly increase β-hexosaminidase secretion, forskolin acutely induced p150\textsuperscript{Glued} recruitment at concentrations that failed to increase β-hexosaminidase secretion, and histamine failed to induce p150\textsuperscript{Glued} recruitment at 1 μM, the concentration at which it maximally increased β-hexosaminidase secretion.

Chronic exposure to CCh, illustrated in Figure 4, disrupted the actin staining pattern in many cells. The subapical actin bands became markedly less intense, whereas the intensity of actin bands beneath the basal-lateral membranes increased to the extent that it became difficult to discern cellular polarity and to distinguish actin-bound lumina from actin-bound vacuoles. Subsequent acute stimulation with CCh for 20 minutes further altered actin filament organization, and it failed to induce p150\textsuperscript{Glued} recruitment. Chronic exposure to histamine caused a slight enlargement of the lumina of some acini, and, in contrast to CCh, increased the thickness of the actin band in the subapical region without altering the thinner actin band subjacent to the lateral and basal plasma membranes. The actin band remained intact during subsequent acute stimulation with 100 μM CCh. Chronic exposure to 5-HT also enlarged acinar lumina and thickened the subapical actin bands; when cells had been chronically exposed to the highest 5-HT concentration tested, 1 mM, subsequent acute stimulation with 100 μM CCh caused lumina to enlarge further and caused the apparent formation of vacuoles. Chronic exposure to forskolin also caused thickening of the subapical actin bands and the formation of actin-bound vacuoles. In contrast to cells that had been exposed to histamine or 5-HT, and some cells that had been chronically exposed to forskolin responded to subsequent acute stimulation with 100 μM CCh with disorganization of the subapical actin bands as well as enlargement of the lumina and formation of large actin-bound vacuoles.

**Ultrastructure**

Recent studies have shown that chronic exposure of acinar cells to elevated levels of the protein hormone prolactin induces a cellular physiological transformation characterized by decreased expression of the apparatus for exocrine protein secretion and induction of a novel apparatus for paracrine protein secretion.\textsuperscript{38,39} Those findings suggested the hypothesis that chronic exposure to histamine at concentrations between 1 μM and 1 mM, or to 5-HT at all concentrations tested, would cause CCh-induced β-hexosaminidase secretion to be redirected from the apical- to the basal-lateral plasma membrane without altering the total amount secreted. The plausibility of this hypothesis was evaluated in an ultrastructural survey; representative images are presented in Figure 6.

In control acini, typified by Figure 6A, secretory vesicles were frequently evident in the apical cytoplasm; vesicle contents were generally flocculent and homogeneous in electron density, though the densities varied even within a single cell. Some vacuoles were present, and occasional large vacuoles appeared to be located near the apical surface. Vacuoles differed from secretory vesicles by their more heterogeneous contents, including dense particles or clusters of small vesicles (Fig. 6A). After chronic exposure to 10 μM CCh (Fig. 6B), secretory vesicles appeared to be relatively more abundant, and much of the cytoplasm appeared to be occupied by numerous vesicles that had undergone homotypic fusion. Frequently the multiple-fused vesicles appeared to communicate exocytotically with the acinar lumina; however, subsequent...
analyses of vectorial secretion by reconstituted acinar epithelial monolayers indicated that chronic exposure to 10 μM CCh decreased, rather than increased, the baseline rate of secretion. Large secretory vesicles were increasingly abundant in samples that had been chronically exposed to 1 μM histamine (Fig. 6C) and were abundant to an even greater extent in samples that had been chronically exposed to 10 mM histamine (Fig. 6D). Some cells that had been chronically stimulated with 1 μM histamine exhibited clusters of microvilli anomalously located at the basal-lateral membrane. In some cells that had been chronically stimulated with 10 mM histamine, unusually dense meshworks of actin microfilaments were interposed between the apical plasma membrane and the secretory vesicles and vacuoles in the apical cytoplasm. Apical actin filaments and adjacent junctional complexes helped distinguish the lumina in these cultures. Extensive vacuole formation and large secretory vesicles also were evident in cells that had been chronically exposed to 10 μM 5-HT (Fig. 6E) or to 10 μM forskolin (Fig. 6F). Exocytosis of vesicles to the lumina was rare in these cells. However, some secretory vesicles were located immediately subjacent to the basal surfaces of cells that had been chronically exposed to histamine or 5-HT.

**Figure 3.** Influence of acute stimulation on microfilament organization and recruitment of p150Glued. Reconstituted acinus-like structures were grown on basement membrane matrix-coated coverslips for 3 days and were treated for 20 minutes with CCh, histamine, 5-HT, or forskolin at the indicated concentrations. Cells were then fixed with ethanol and blocked with a 1% BSA solution in PBS. Cells were stained with mouse antibody to p150Gluced, goat anti–mouse FITC-conjugated secondary antibody (green), and rhodamine-conjugated phalloidin, which labels to actin microfilaments (red). Arrows indicate subapical actin bands. Arrowheads indicate expanded lumina delineated by subapical actin bands.

**Vectorial β-Hexosaminidase Secretion**

The appearance of secretory vesicles near the basal-lateral surfaces of cells that had been chronically exposed to histamine or 5-HT appeared consistent with the hypothesis that such cells might release some secretory material basally, rather than apically, if they were acutely stimulated with 100 μM CCh. The recently described reconstituted lacrimal acinar epithelial monolayer model was used test this hypothesis. As illustrated in Figure 7, acute stimulation with 100 μM CCh caused a roughly 11-fold increase in the rate at which monolayers secreted β-hexosaminidase to their apical medium, but it had no significant effect on the rate of secretion to the basal medium. Chronic exposure to histamine at 1 μM or 1 mM did not significantly alter secretion to the apical medium in the absence or presence of acute stimulation with 100 μM CCh. However, chronic exposure to histamine at 10 mM, a concentration that significantly decreased CCh-induced secretion in the ex vivo model used in Figure 1, prevented the monolayers from establishing confluence and maintaining significant trans-
epithelial electrical resistances essential for vectorial transport (data not shown). Chronic exposure to 5-HT at a concentration of 10 μM did not affect β-hexosaminidase secretion, but 1 mM 5-HT decreased secretion to the apical medium by 50% (P < 0.05).

CCh-induced secretion to the basal media was highly variable, and no significant effects of chronic exposure to histamine or 5-HT were evident. Notably, however, in the absence of acute stimulation with 100 μM CCh, control monolayers and monolayers that had been chronically exposed to 10 μM CCh, 10 μM 5-HT, or 1 mM 5-HT secreted β-hexosaminidase to their basal media at roughly twice the rate they secreted it to their apical media.

**DISCUSSION**

This study has demonstrated that histamine and 5-HT acutely stimulate β-hexosaminidase secretion by rabbit lacrimal gland acinar cells but that dose-response relationships for both GPCR agonists are complex, with the secretion decreasing as the agonist concentrations increased. Histamine and 5-HT previously have been identified as secretagogues for acinar cells of rat, mouse, and pig lacrimal glands, but biphasic dose-response relationships have not been reported in these tissues. This phenomenon, referred to as supramaximal stimulation, has been studied extensively in rat and rabbit pancreas and ex vivo pancreatic acinar cell models; in those models, it is associated with activation of muscarinic acetylcholine receptors or of cholecystokinin receptors, which also transduce their signals primarily through Gαq and Gα11.[42]

This study has also demonstrated that chronic exposure to histamine or 5-HT induces cytopathologic changes and exocrine dysfunction and that the relationship between the two phenomena is surprisingly complex. The observed cytopathologic changes included the formation of multiple-fused secretory vesicles, which sometimes aberrantly localized in the basal cytoplasm, and of large vacuoles with heterogeneous contents; in some cases, microvilli formed at the basal-lateral membranes, and the actin microfilament meshwork underlying the apical membranes thickened. Strikingly, histamine and 5-HT induced cytopathologic changes even at concentrations that did not significantly impair β-hexosaminidase secretion, and chronic exposure to forskolin induced similar cytopathologic changes without significantly impairing secretion at any concentration tested.
Chronic exposure to histamine and 5-HT altered two CCh-activated functions thought to be involved in the secretory process: elevation of Ca\(^{2+}\) and recruitment of p150\(^{Glued}\). Nonstimulated cells were scored with a numerical value of 0. Robust recruitment of p150\(^{Glued}\), similar to that induced by acute stimulation with 100 \(\mu\)M CCh, was scored a value of 2. Smaller amounts of recruitment were scored a value of 1. Acute stimulation with increasing concentrations of histamine induced increasing amounts of p150\(^{Glued}\) recruitment. 5-HT induced recruitment with no evident dose-response relationship.

Several findings suggest that the impairment of p150\(^{Glued}\) recruitment might not have been directly related to impairment of \(\beta\)-hexosaminidase secretion. p150\(^{Glued}\) recruitment was impaired by chronic forskolin, which, as noted, did not significantly impair \(\beta\)-hexosaminidase secretion, also did not alter CCh-induced Ca\(^{2+}\) elevation. In contrast, chronic histamine and 5-HT decreased CCh-induced Ca\(^{2+}\) elevation at the high concentrations that significantly impaired \(\beta\)-hexosaminidase secretion. Chronic histamine and 5-HT decreased Ca\(^{2+}\) elevation to smaller extents at concentrations of 1 mM and 100 \(\mu\)M, respectively.

Several findings suggest that the impairment of p150\(^{Glued}\) recruitment might not have been directly related to impairment of \(\beta\)-hexosaminidase secretion. p150\(^{Glued}\) recruitment was impaired by chronic forskolin, which, as noted, did not impair secretion, and it was induced by acute stimulation with histamine, 5-HT, and forskolin at concentrations that did not elicit significant increases of secretion. Although impairment of p150\(^{Glued}\) recruitment cannot be identified as one of the molecular mechanisms underlying exocrine dysfunction, it is possible that alterations of p150\(^{Glued}\) activation and recruitment, and the dissociation of p150\(^{Glued}\) recruitment from \(\beta\)-hexosaminidase secretion, might have contributed to the cytopathologic changes that occurred during chronic exposure to histamine, 5-HT, and forskolin.

Chronic exposure to histamine at the highest concentration tested, 10 mM, caused two changes that might be regarded as exocrine dysfunction: impairment of CCh-induced \(\beta\)-hexosaminidase secretion comparable to that caused by chronic exposure to 10 \(\mu\)M CCh (Fig. 1) and impairment of the cells’ ability to form continuous monolayers capable of maintaining a significant transepithelial electrical resistance (data not shown). Chronic exposure to 5-HT at the highest noncytotoxic concentration tested, 1 mM, caused significant impairment of CCh-induced secretion at the apical membranes of reconstituted epithelial monolayers. It may be noted that such an effect was not evident in the reconstituted acinus-like structures used for the experiments presented in Figure 1. It is possible that this discrepancy reflected inherent technical limitations of the reconstituted acinus-like structures as an ex vivo model. One limitation is essentially topological, stemming from the fact that though individual cells were well polarized, the system was essentially symmetrical because secretion to the apical medium could not be distinguished from secretion to the basal medium. The experiments with the reconstituted epithelial

**FIGURE 5.** Left: summary of influences of acute stimulation with CCh, histamine, 5-HT, or forskolin on p150\(^{Glued}\) recruitment. Images were scored on a 3-point scoring system to categorize the recruitment of p150\(^{Glued}\). Nonstimulated cells were scored with a numerical value of 0. Robust recruitment of p150\(^{Glued}\), similar to that induced by acute stimulation with 100 \(\mu\)M CCh, was scored a value of 2. Smaller amounts of recruitment were scored a value of 1. Acute stimulation with increasing concentrations of histamine induced increasing amounts of p150\(^{Glued}\) recruitment. 5-HT induced recruitment with no evident dose-response relationship. Right: summary of influences of chronic exposure to CCh, histamine, 5-HT, or forskolin on recruitment of p150\(^{Glued}\) induced by acute stimulation with 100 \(\mu\)M CCh. Inhibition of recruitment similar that induced by chronic exposure to 10 \(\mu\)M CCh was given a score of −2. Slight inhibition of p150\(^{Glued}\) recruitment was given a score of −1. Images showing no decrease in recruitment were given a score of zero. Images from three separate cell preparations were examined by two independent observers.
The monolayer model produced the unexpected finding that resting acinar cells—cells that had not been acutely stimulated with a secretagogue—secreted β-hexosaminidase at their basolateral membranes twice as rapidly as at their apical membranes. In retrospect, this finding should not have been surprising because β-hexosaminidase is a catabolic lysosomal enzyme and a secretory product in lacrimal acinar cells. Many lysosomal hydrolases traffic from the cells’ biosynthetic apparatus to their lysosomal-autophagic apparatus by way of the early endosome and the late endosome, and evidence began appearing sometime ago that lacrimal acinar cells maintained an unusually vigorous traffic of transport vesicles between their early endosomes and basolateral plasma membranes. Because the resting rate of secretion at the basolateral membrane was so high, the CCh-induced component of secretion at the apical medium represented between 75% and 80% of the total amount secreted to the medium (Fig. 1), whereas it represented roughly 95% of the amount secreted to the apical medium (Fig. 7). Not only was CCh-induced secretion at the apical membrane a smaller fraction of the total when secretion was measured in the symmetrical system, but estimates of its magnitude were influenced by variability in the resting rates of secretion at both membranes. A second potential technical limitation of the reconstituted-acinus like model was because it was more physiological in that this model may be more susceptible to mechanical stress associated with the changes of incubation media associated with the assay procedure itself. Thus, the impairment of CCh-induced β-hexosaminidase secretion by chronic exposure to 5-HT (Fig. 7) seems more likely to be the physiologically relevant finding.

Serotonergic fibers are present in the rat lacrimal gland, and some innervate mast cells. 5-HT activates acinar cell protein secretion directly and indirectly by inducing mast cells to release histamine, which also activates protein secretion. Therefore, mast cells appear to play a role in the normal rat lacrimal gland, analogous to the role they play in the gastric glands, where they release histamine in response to stimulation.

**Figure 6.** Influences of chronic exposure to CCh, histamine, 5-HT, or forskolin on acinar cell ultrastructure. Acinus-like structures were reconstituted in basement membrane matrix rafts, which were treated as indicated and processed. (A) Control. Secretory vesicles (arrows) are evident near the apical surface in this raft. Vacuoles near the apical surface often make it difficult to determine whether they are in continuity with the lumen or are within the cytosol. (B) Chronic CCh (10 μM). Low-magnification view of a cluster of small rafts. Secretory vesicles are abundant in these rafts, and exocytosis is dramatic (arrows), as seen at high magnification. (C) Chronic histamine (1 μM). Low-magnification view of a cluster of small rafts. Secretory vesicles are abundant in these rafts, and exocytosis is dramatic (arrows), as seen at high magnification. (D) Chronic 5-HT (10 mM). Low-magnification view of a cluster of small rafts. Secretory vesicles are abundant in these rafts, and exocytosis is dramatic (arrows), as seen at high magnification. (E) Chronic 5-HT (10 mM). Low-magnification view of a cluster of small rafts. Secretory vesicles are abundant in these rafts, and exocytosis is dramatic (arrows), as seen at high magnification. (F) Chronic forskolin (10 μM). Secretory vesicles are abundant in these rafts, and exocytosis is dramatic (arrows), as seen at high magnification.
Although mast cells have been demonstrated in the canine lacrimal gland and are present in human salivary glands, it is not clear that one may extrapolate the paradigm for mast cell mediation of neural control of exocrine function to the lacrimal glands of humans or rabbit models because, to the authors' knowledge, their presence cells in human and rabbit lacrimal glands has not been documented. However, serotonergic fibers have been shown to innervate the lacrimal glands of the pig, camel, and monkey, as well as the rat. Therefore, neuronal release of 5-HT may be involved in the acute regulation of lacrimal secretion in a broad range of species.

Mast cells become highly abundant in the lacrimal glands of aging mice and rats and it has been reasonable to infer that they mediate age-related lacrimal gland cytopathology in rodents. The present findings support this inference by demonstrating that chronic exposure to histamine or 5-HT causes cytopathological changes.

The authors are not aware of evidence that mast cells play roles analogous to their role in aging rodent lacrimal glands in autoimmune-mediated or age-related lacrimal gland pathophysiology in humans or rabbit models. However, in addition to serotonergic fibers, the lacrimal glands of several species have been shown to be innervated by fibers containing vasoactive intestinal peptide, leu-enkephalin, substance P,53-56 and neuropeptide Y.57 Like the biogenic amines, these neuropeptides activate G protein-coupled receptors. Although vasoactive intestinal peptide, substance P, and neuropeptide Y are secretagogues, enkephalins acutely inhibit protein secretion.53 Therefore, the present findings suggest the hypothesis that dysregulated aminergic and peptidergic neurotransmission might cause exocrine dysfunction and cytopathology by altering the same mechanisms that the mast cell mediators alter.

In addition to secreting cytokines, activated lymphocytes, antigen-presenting cells, and leukocytes also secrete biogenic amines, prostaglandins, leukotrienes, and chemokines, all of which interact with G protein-coupled receptors. Moreover, certain of the cytokines that activated immune cells secrete induce parenchymal cells to express biogenic amines, prostaglandins, leukotrienes, and chemokines, thereby initiating intracellular, autocrine, and paracrine feedback interactions that maintain inflammatory states. Thus, the actions of histamine and 5-HT demonstrated in this study may be emblematic of the actions of a wide range of inflammatory mediators and of neuropeptides and neurotransmitters.

If diverse mediators induce cytopathology and, at higher concentrations, exocrine dysfunction, then the multiple mediators associated with an ongoing immunopathophysiological process are likely to exert combinatorial effects. This principle suggests an explanation for the range of exocrine dysfunction encountered in clinical practice and population studies. The profound exocrine quiescence that manifests clinically as decreased production of ocular surface fluid and symptomatic dry eye disease may be caused by different mediators or combinations of mediators in patients with Sjögren's syndrome, graft-versus-host disease, sarcoidosis, Wegener granulomatosis, and the diffuse infiltrative lymphocytosis syndrome. Similarly, varying degrees of exocrine dysfunction falling short of profound quiescence may be caused by different mediator spectra in patients with Mikulicz' disease, premature ovarian failure, and the common histopathologic syndrome associated with aging.

The present findings may have implications for the pathogenesis of inflammatory lacrimal gland disease as well as for its pathophysiology. Lacrimal acinar cells secrete lysosomal enzymes to their stromal space constitutively. In the absence of other perturbations, the induction of lysosomal hydrolase secretion by inflammatory mediators during response to infection or trauma, or by neurotransmitters and neuropeptides elicited during environmental stress, may not be a provocation significant enough to account for the development of chronic inflammatory autoimmune disease.

Acknowledgments
The authors thank Francie Yarber, Michelle Aloni, and Michael Pidgeon for technical assistance; and Chuanqing Ding, Sarah F. Hams-Alvarez, Melvin D. Trousdale, and Dwight W. Warren for advice during the study and in the preparation of the manuscript.

References


