

## Microtubules facilitate the stimulated secretion of $\beta$ -hexosaminidase in lacrimal acinar cells

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### SUMMARY

Stimulation of lacrimal acini with secretagogues such as carbachol initiates movement and fusion of acinar secretory vesicles with the apical plasma membrane, resulting in release of protein into the nascent tear fluid. Using rabbit lacrimal acini reconstituted in vitro from isolated cells, we have investigated the organization of the apical cytoskeleton and its role in stimulated secretion. Confocal microscopy revealed a microtubule array emanating from the apical region of the acini; the apical region was also enriched in microfilaments and  $\gamma$ -tubulin. Cytokeratin-based intermediate filaments were apically concentrated, and also detected at the cell periphery. Neither confocal microscopy nor biochemical analysis revealed any reorganization of luminal microfilaments or microtubules which might accompany carbachol-stimulated release of secretory proteins. However, major

changes in the acinar microtubule array induced by taxol or nocodazole were correlated with inhibition of carbachol-dependent release of the secreted protein,  $\beta$ -hexosaminidase. Major changes in luminal microfilaments induced by jasplakinolide or cytochalasin D did not inhibit the carbachol-dependent release of  $\beta$ -hexosaminidase; rather, release of  $\beta$ -hexosaminidase from jasplakinolide- or cytochalasin D-treated carbachol-stimulated acini was markedly increased relative to the release from untreated stimulated acini. Our findings demonstrate that microtubules play a major role in stimulated lacrimal secretion, and suggest a contributory role for microfilaments.

Key words: Microtubule, Microfilament, Lacrimal gland, Stimulated secretion, Carbachol,  $\beta$ -Hexosaminidase

### INTRODUCTION

A major function of lacrimal acinar cells is the secretion of an isotonic NaCl- and protein-rich fluid in response to signals provided through parasympathetic innervation of the lacrimal gland. These secretory signals are mimicked in vitro by a number of agents including carbachol (Gierow et al., 1995a). Exposure of lacrimal acini to carbachol activates  $Ca^{2+}$ /calmodulin-protein kinase, protein kinase C and phospholipase D (Zoukhri et al., 1994; Zoukhri and Dartt, 1995; Hodges et al., 1994). The phosphorylation and consequent activation of specific protein targets of these second messenger pathways is believed to play a direct role in protein secretion (reviewed by Dartt, 1994). However, little is known about the identity of the target proteins which respond to cholinergic stimulation and changes in intracellular phosphorylation by facilitating the release of secretory fluid and proteins. Some possible candidates include the cytoskeleton and/or the microtubule (MT)-based motor proteins, kinesin and cytoplasmic dynein.

Over the past three decades, the involvement of MTs and microfilaments (MFs) in stimulated acinar secretion has been studied in pancreas, parotid gland, and lacrimal gland. Since MT-based vesicle transport facilitates stimulated secretion in

unpolarized cells including T-cells (Burchardt et al., 1993) and melanocytes (Rogers et al., 1997), a substantial precedent exists for the hypothesis that MTs may facilitate stimulated acinar secretion. In agreement with this model, stimulated release of secreted proteins is reduced by MT-targeted drugs in pancreas (Nevalainen, 1975; Pavelka and Ellinger, 1983) and lacrimal gland (Busson-Mabillot et al., 1982; Robin et al., 1995).

Other studies propose that apical MFs form a barrier restricting access of secretory vesicles to the apical membrane; stimulation of acini is thought to trigger a transient redistribution of apical MFs, allowing secretory vesicles to move past the MF barrier and fuse with the apical membrane (Lacy et al., 1973; Perrin et al., 1992; Jungerman et al., 1995). This model is supported by observations of MF fragmentation and/or changes in MF organization that accompany increased secretion (O'Konski and Pandolfi, 1990; Perrin et al., 1992; Jungerman et al., 1995).

Given the conflicting data regarding the participation of the cytoskeleton in acinar secretion, it is surprising that few studies have examined the role of MTs and MFs in parallel. Our goal for this study was to resolve the organization and function of the lacrimal acinar cytoskeleton. We found that the MT array was organized at the apical membrane, below the apical MFs surrounding the lumen. Colocalization of  $\gamma$ -tubulin with luminal

MFs revealed that MT minus-ends were apical. An elaborate array of cytokeratin intermediate filaments (IFs) was also detected at the apical region and around the cell periphery. No major changes in the cytoskeleton were associated with carbachol-induced stimulation of secretion. Studies using nocodazole and taxol to alter the MT array revealed that changes in acinar MTs were associated with reduced carbachol-dependent release of the secretory protein,  $\beta$ -hexosaminidase, and of bulk protein. Studies using jasplakinolide and cytochalasin D to alter luminal MFs revealed that the carbachol-dependent secretion of  $\beta$ -hexosaminidase was markedly increased in jasplakinolide- and cytochalasin D-treated acini relative to stimulated, untreated acini, probably due in part to the marked carbachol-independent release of  $\beta$ -hexosaminidase elicited by pretreatment with these actin-targeted agents. Our data suggest that MTs facilitate movement of membranes containing secretory protein to the apical plasma membrane but that MFs may also regulate the access of secretory vesicles to the apical plasma membrane.

## MATERIALS AND METHODS

### Reagents

Carbachol, nocodazole, rhodamine-phalloidin, cytochalasin D, FITC-conjugated goat anti-mouse and goat anti-rat secondary antibodies, mouse monoclonal anti-cytokeratin (K8.13), anti- $\alpha$  tubulin (B-5-1-2), anti- $\beta$  tubulin (TUB 2.1), anti-tyrosine tubulin (TUB-1A2), anti-acetylated tubulin (6-11B-1) and anti- $\gamma$ -tubulin primary antibodies were obtained from Sigma Chemical Co (St Louis, MO). The C4 mouse monoclonal antibody to actin was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Goat anti-rat and anti-mouse horseradish peroxidase-conjugated secondary antibodies were obtained from Amersham (Arlington Heights, IL). The rat monoclonal anti-tubulin antibody (YOL1/34) was obtained from Accurate Scientific (Westbury, NY). Matrigel was obtained from Collaborative Biochemicals (Bedford, MA). Taxol and jasplakinolide were purchased from LC Laboratories (Woburn MA) and Molecular Probes (Eugene, OR), respectively. All other chemicals were reagent grade and obtained from standard suppliers.

### Cell isolation and culture

All animal studies were done in accordance with the Guiding Principles for Use of Animals in Research. Female New Zealand white rabbits weighing between 1.8 and 2.2 kg were obtained from Irish Farms (Norco, CA). Lacrimal gland acinar cells were isolated as described previously (Gierow et al., 1996; Hamm-Alvarez et al., 1997) and cultured for 3 days. For confocal microscopy, acini were grown on 22 mm  $\times$  22 mm glass coverslips coated with Matrigel diluted 1:10 in Dulbecco's phosphate-buffered saline. For measurements of soluble and cytoskeletal actin and tubulin, acini were grown on 150 mm culture plates with or without diluted Matrigel. Since preliminary studies showed that the carbachol-stimulated release of  $\beta$ -hexosaminidase from acini cultured on dishes coated with diluted Matrigel versus no Matrigel was comparable, Matrigel-free 6-well or 24-well plates were utilized for measurements of  $\beta$ -hexosaminidase secretion. Under each of these culture conditions, the isolated cells aggregate into acinus-like structures around a central lumen; individual cells within these structures reveal distinct apical and basal-lateral domains (Gierow et al., 1996).

### Cell treatments

To stimulate secretion, reconstituted acini were incubated in medium with the muscarinic agonist, carbachol (10  $\mu$ M or 1 mM), at 37°C from 1-60 minutes. Changes in the MF array were elicited by cytochalasin D (Tannenbaum et al., 1977) or jasplakinolide (Bubb et al., 1994). Changes in the MT array were elicited by taxol (Schiff and Horwitz, 1981) or nocodazole (Lee et al., 1980). Experimental treatments included nocodazole (33  $\mu$ M from a 33 mM stock in

DMSO), taxol (10  $\mu$ M from a 4 mM stock in DMSO), jasplakinolide (0.5  $\mu$ M from a 0.5 mM stock in methanol) or cytochalasin D (5  $\mu$ M from a 5 mM stock in DMSO) for 60 minutes at 37°C either alone or prior to exposure to carbachol as indicated.

### Isolation and analysis of cytoskeletal and soluble tubulin and actin

After treatments, soluble and membrane proteins were separated from cytoskeletal proteins based on the procedure of Sontag et al. (1995). Acini were exposed to MT-stabilizing buffer (MTSB, 0.1 M Pipes, 1 mM EGTA, 1 mM MgSO<sub>4</sub>, 2 M glycerol, pH 6.75) for 10 minutes at room temperature. Then, additional MTSB containing 0.1% NP-40 or 0.1% Tx-100 was added to solubilize cytosol and membranes. This step was repeated as necessary, and samples containing cytosol and membrane fractions were pooled to obtain the 'soluble' pool. The remaining cellular material (cytoskeleton) was isolated in MTSB/1% NP-40.

Soluble and cytoskeletal pools were concentrated to equal volumes with a SpeedVac centrifuge and separated by 7.5% SDS-polyacrylamide gel electrophoresis before transfer to nitrocellulose filter paper. Blots were developed utilizing an enhanced chemiluminescence (ECL) detection kit (Amersham). Quantitation of tubulin and actin was by densitometry of western blots using a Bio-Rad GS-670 Imaging Densitometer. Scanned densitometry values fell within the linear range of the ECL system.

### Confocal fluorescence microscopy

After treatments, cells on coverslips were processed for MT and MF staining as described (Hamm-Alvarez et al., 1993; Sontag et al., 1995). Acini were rinsed with PBS, incubated with MTSB (2 minutes), rinsed again with PBS, incubated with MTSB + 0.1% Tx-100 (10 minutes), fixed with 0.1% glutaraldehyde/2% formaldehyde in PBS (15 minutes), quenched with 50 mM ammonium chloride, permeabilized (2 minutes) with 0.1% Triton X-100 in PBS and blocked with PBS/0.1% BSA. Samples were incubated (37°C, 30 minutes) with antibodies or affinity label. The MT array was visualized with the primary monoclonal  $\alpha$ -tubulin antibody, YOL1/34 (Kilmartin et al., 1982) and secondary FITC-conjugated goat anti-rat antibody. MT subpopulations were labeled with antibodies against acetylated (6-11B-1; LeDizet and Piperno, 1991) or tyrosinated tubulin (TUB-1A2; Kreis, 1987) and FITC-conjugated secondary goat anti-mouse antibody. MFs were detected by staining with rhodamine phalloidin or with a mouse monoclonal antibody to actin. Intermediate filaments were labeled with a monoclonal anti-cytokeratin antibody (K8.13) and FITC-conjugated secondary goat anti-mouse antibody. Slides were viewed using a Zeiss LSM-1 confocal laser scanning microscope equipped with filters for both FITC and RITC epifluorescence. Images were compiled using Adobe Photoshop 3.0 for Windows 95 (Adobe Systems Inc, Mountain View, CA).

Analysis of luminal MF integrity was done by confocal fluorescence microscopy of fixed acini labeled with rhodamine-phalloidin. Acini were scanned from bottom to top in approximately 1  $\mu$ m intervals. At each level, the integrity of each lumen was noted; compilation of these data at the end of the scan enabled the identification of intact or discontinuous luminal MFs (Table 1).

### Measurement of secreted $\beta$ -hexosaminidase activity

Following treatments, the medium bathing the cells was removed from the culture dishes and unattached cells were removed by centrifugation for 10 minutes at 600 rpm in a clinical centrifuge.  $\beta$ -Hexosaminidase in cell-free medium was determined using methyumbelliferyl- $\alpha$ -D-glucosaminide as substrate (Barrett and Heath, 1977) by utilizing a Perkin Elmer LS-5B Luminescence Spectrometer (excitation at 365 nm and emission at 460 nm).

### Measurement of secreted protein

Acini from 3 day cultures were collected by centrifugation in a clinical centrifuge, washed once with PBS, and resuspended in warm Ham's F-12 medium containing 10 mM Hepes, 2 mM butyrate, pH 7.6, and

supplemented with linoleic acid (0.3  $\mu\text{M}$ ), transferrin (5  $\mu\text{g/ml}$ ), insulin (5  $\mu\text{g/ml}$ ), sodium selenite (30 nM) and hydrocortisone (5 nM). Acini treated with and without nocodazole or taxol were exposed to either 1 mM carbachol (15 minutes) or 10  $\mu\text{M}$  carbachol (30 minutes) and the protein content of the medium from samples pre- and post-carbachol stimulation was measured.

### Protein synthesis experiments

The effects of taxol and nocodazole on protein synthesis were measured by incubating lacrimal gland acinar cells with  $^{35}\text{S}$ -Translabel (ICN, 6.6  $\mu\text{Ci/million cells}$ ) in sulfur-amino acid free medium with and without drugs for 60 minutes as previously described (Runnegar et al., 1997). The labeled cell pellet was extensively rinsed in PBS before suspension in 1 ml 5% TCA, centrifuged to pellet precipitated protein, rinsed in 5% TCA, and centrifuged again before dissolving in 100  $\mu\text{l}$  of 0.5 N NaOH for measurement of  $^{35}\text{S}$  and protein.

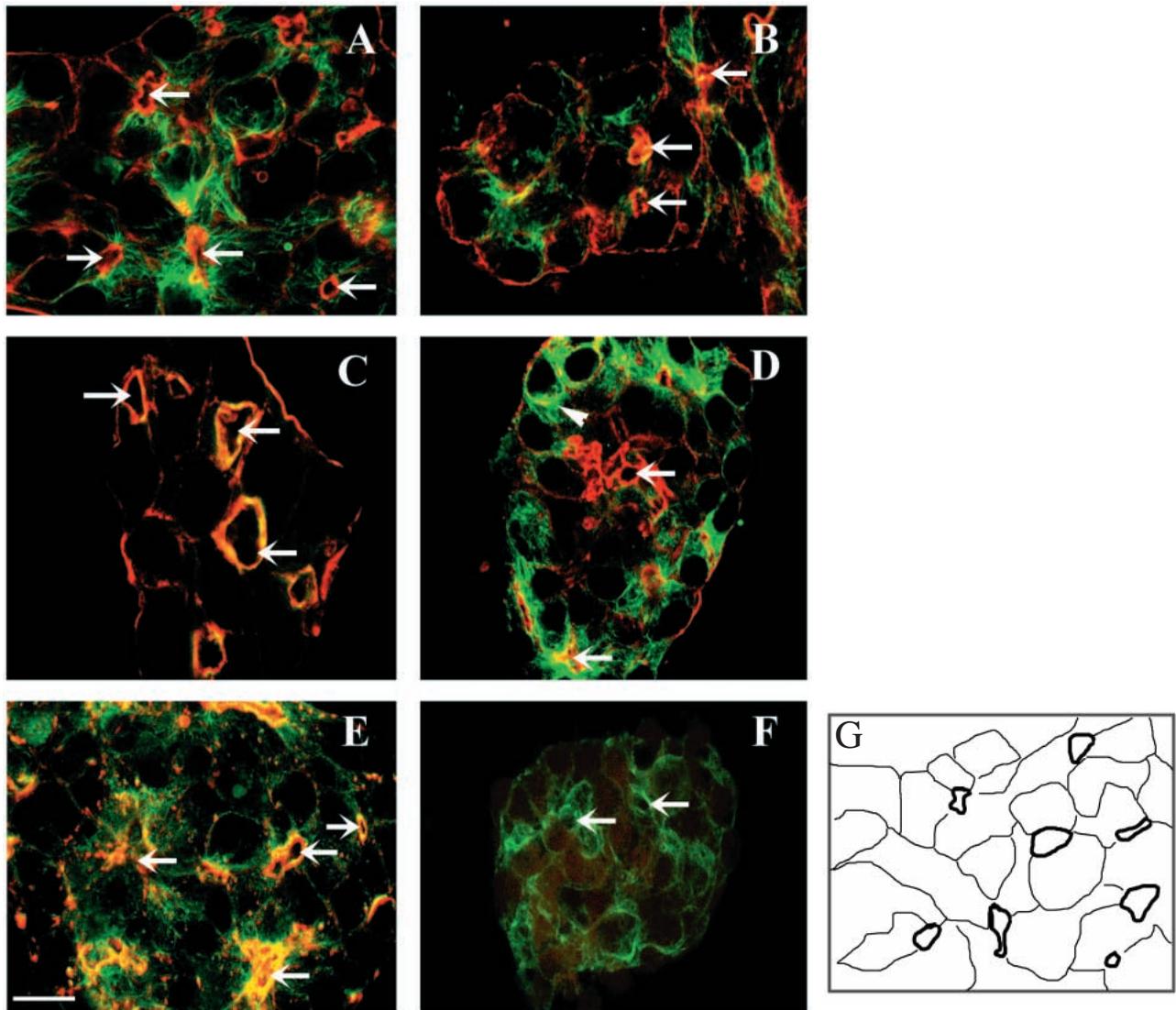
### Statistical analysis

Standard *t*-tests assuming equal variances were utilized for analysis of western blot densitometry. Paired *t*-tests (two-tailed) were utilized for comparison of  $\beta$ -hexosaminidase and protein secretion data values from different assays and for comparison of intact versus discontinuous luminal MFs in treated and control acini. A value of  $P \leq 0.05$  was considered statistically significant.

## RESULTS

### MT, MF and IF organization in lacrimal acini

The organization of the cytoskeleton in rabbit lacrimal acini was characterized by confocal fluorescence microscopy. As seen in Figs 1 and 2, the MF distribution in lacrimal acini is similar to



**Fig. 1.** MT and MF distributions in control and treated lacrimal acini. (A-E) Acini were exposed to rhodamine-phalloidin to label MFs (red) while a mouse monoclonal anti-actin antibody was utilized (F) for staining of MFs. The YOL1/34 antibody against  $\alpha$ -tubulin was utilized for MT labeling in all panels (green). (A) Control acini. (B) Acini exposed to 10  $\mu\text{M}$  carbachol for 30 minutes at 37°C. (C) Acini exposed to 33  $\mu\text{M}$  nocodazole. (D) Acini exposed to 10  $\mu\text{M}$  taxol. (E) Acini exposed to 5  $\mu\text{M}$  cytochalasin D. (F) Acini exposed to 0.5  $\mu\text{M}$  jasplakinolide. (C-F) Treatments were for 60 minutes at 37°C. Colocalization of MTs and actin is shown in yellow. Arrows reveal the locations of prominent lumens in each panel. Arrowhead shows MT bundling resulting from taxol treatment. Bar,  $\sim 10 \mu\text{m}$  (A,B,C,E);  $\sim 15 \mu\text{m}$  (D,F). (G) Schematic drawing of MF organization corresponding to A. Luminal/apical membranes are indicated by thick lines while basal-lateral membranes are indicated by thin lines.

that in other polarized epithelia. Fig. 1A (Control) shows that the MF array (red) in resting acini is concentrated at the apical/luminal region with a lower density of MFs distributed along the cell periphery. MF distribution and the position of the luminal regions can be more easily resolved by comparison of Fig. 1A to Fig. 1G, which schematically depicts the organization of the individual cells in Fig. 1A and delineates apical membranes (thick lines) and basal-lateral membranes (thin lines).

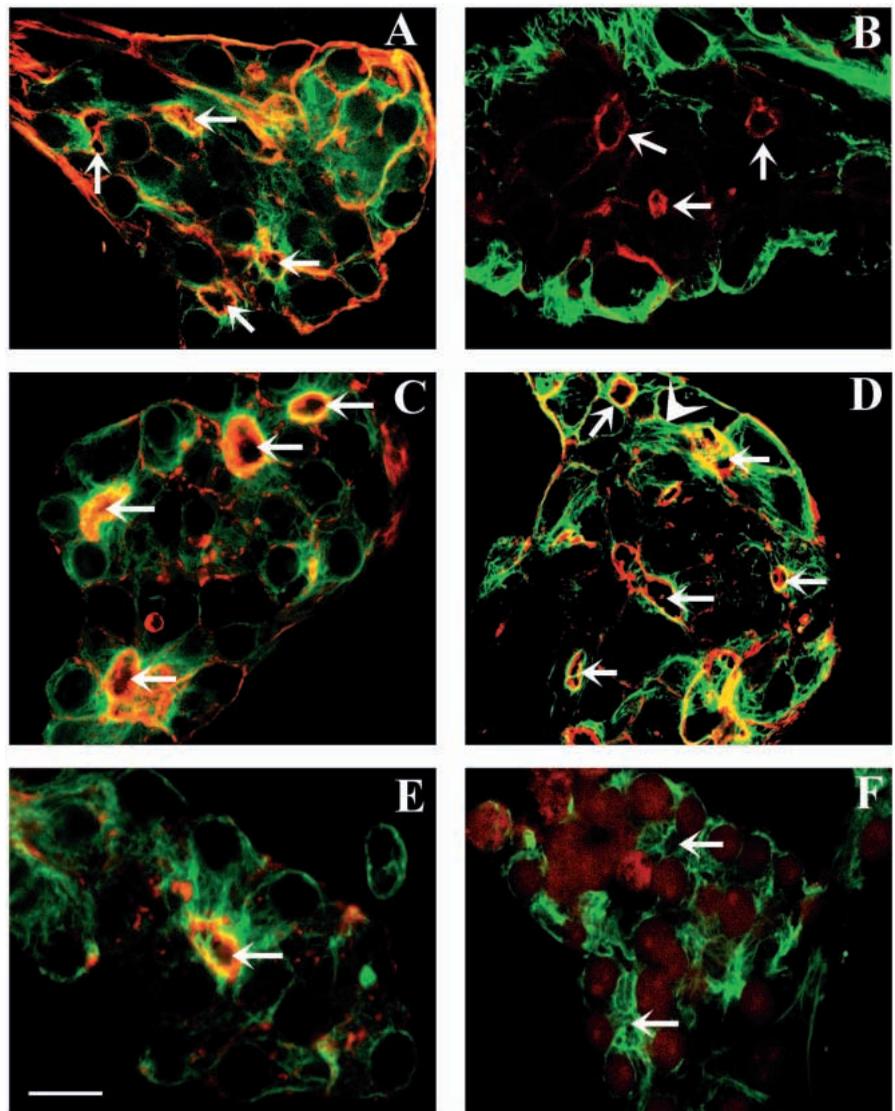
The MT array (green) appears to originate from an organizing center at the apical/luminal region and to radiate towards the basal-lateral surface (Fig. 1A). Although some MTs extended around the cell periphery to the basal-lateral surface (Fig. 1A, bottom right), most MTs were enriched at the apical pole of the acini. Two MT subpopulations were also examined in the lacrimal acini: stable MTs (marked by post-translational modifications including acetylation) and dynamic MTs (marked by the presence of a tyrosine residue at the carboxy terminus of the  $\alpha$ -tubulin)(reviewed by Gelfand and Bershadsky, 1991). Fig. 2A shows the distributions of stable MTs (green) and MFs (red) in resting acini; stable MTs in lacrimal acini are enriched at the apical regions. In contrast, the dynamic MTs (green) shown with the MF distribution

(red) in reconstituted lacrimal acini revealed an enrichment at the basal-lateral rather than apical regions (Fig. 2B).

Analysis of the cytokeratin IF distribution by confocal fluorescence microscopy revealed that reconstituted lacrimal acini have an extensive array of IFs which extend from the apical region and encompass the basal-lateral membrane, forming a 'basket-like' network of filaments around the cell periphery (Fig. 2C, IFs in green and MFs in red). IFs are densely packed around the luminal membrane, where they colocalize with MFs (yellow).

#### Treatment of lacrimal acini with jasplakinolide or cytochalasin D alters MF organization

To understand the factors involved in maintenance of this elaborate apical cytoskeletal array, we utilized jasplakinolide, a marine toxin, and cytochalasin D, a fungal metabolite, to alter F-actin organization. An anti-actin monoclonal antibody was used for detection of actin in Fig. 1F (red), since phalloidin and jasplakinolide have been shown to bind competitively to F-actin (Bubb et al., 1994). Compared to untreated acini (Fig. 1A) which showed an accumulation of MFs around the apical membrane and at the cell periphery, the MF organization in jasplakinolide-



**Fig. 2.** MT subpopulations and cytokeratin IFs in lacrimal acini. Acini in all panels were exposed to rhodamine-phalloidin to label MFs (red). (A-B) MTs and (C-F) cytokeratin IFs are shown in green. (A) Control acini labeled with a monoclonal antibody to acetylated tubulin. (B) Control acini labeled with a monoclonal antibody to tyrosinated  $\alpha$ -tubulin. A monoclonal antibody against cytokeratins (K8.13) was used to show the distribution of cytokeratin IFs in control acini (C), in acini exposed to 10  $\mu$ M taxol (D), in acini exposed to 5  $\mu$ M cytochalasin D (E) and in acini exposed to 0.5  $\mu$ M jasplakinolide (F). Treatments were for 60 minutes at 37°C. Colocalization of MTs and either MFs or IFs is shown in yellow. Arrows reveal the locations of prominent lumens in each panel. Arrowhead shows cytokeratin IF bundling resulting from taxol treatment. Bar, ~10  $\mu$ m.

**Table 1. Cytochalasin D but not carbachol generates discontinuities in luminal MFs**

Treatments	% Total lumens remaining intact
Control	71.85±0.19 (90/3)
Carbachol	72.90±0.15 (89/3)
Cytochalasin D	29.02±0.11* (114/3)

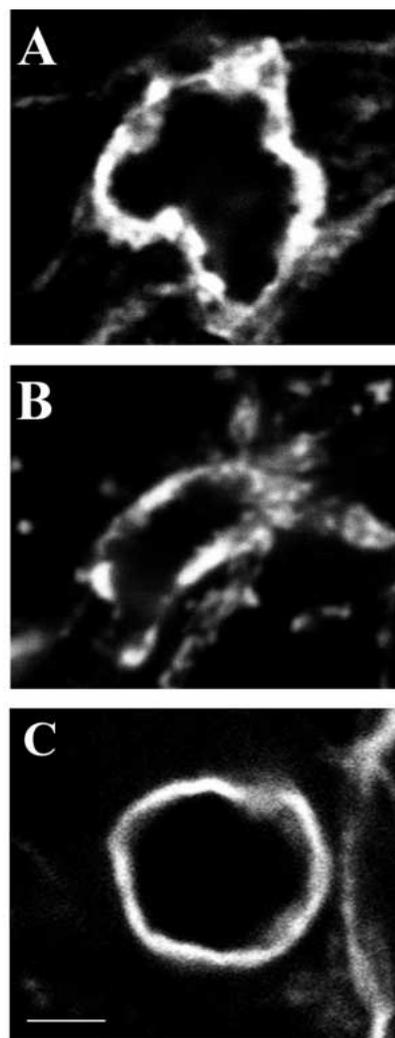
Lacrimal acini were treated with 10  $\mu$ M carbachol (37°C, 5 minutes) or 5  $\mu$ M cytochalasin D (37°C, 60 minutes) and processed for confocal microscopy using rhodamine-phalloidin to detect MFs. Scanning of acini for analysis of lumens was done from the bottom to the top of each in approximately 1  $\mu$ m intervals, facilitating the identification of intact or discontinuous luminal MFs. *n* = no. of samples/no. of cell preparations. Values shown are mean  $\pm$  s.e.m. and \* indicates significant at  $P \leq 0.05$ .

treated cells was completely disrupted, as evidenced by the diffuse staining distributed throughout the cells and the complete lack of luminal MFs (Fig. 1F). Rhodamine-phalloidin labeling of MFs (red) in jasplakinolide-treated acini also yielded a diffuse labeling pattern with no evidence of luminal MFs (Fig. 2F). Analysis of rhodamine-phalloidin labeled acini by phase contrast microscopy revealed that most of the rhodamine-phalloidin staining was nuclear (data not shown), counter to the diffuse cytoplasmic staining seen with anti-actin antibody. This lack of luminal MFs in jasplakinolide-treated cells revealed by both labeling methods did not impact on the MT array (Fig. 1F, green), which retained an apparent apical organization that resembled that seen in untreated acini (Fig. 1A). The cytokeratin IF network also remained intact following jasplakinolide treatment (Fig. 2F, green).

As shown in Fig. 1E, cytochalasin D treatment also disrupted MFs (red), as evidenced by the patches of actin staining found throughout the cell and at the cell periphery. In contrast to the jasplakinolide-treated acini, the cytochalasin D-treated cells maintained an apparent dense network of luminal MFs. To determine whether cytochalasin D elicited a major reorganization of apical MFs, we used confocal microscopy to examine the luminal regions of control and cytochalasin-D treated lacrimal acini at higher magnifications. As shown in Fig. 3A, the lumen of control acini revealed a continuous and thick ring of F-actin. In contrast, acini exposed to cytochalasin D (Fig. 3B) displayed large discontinuities in the luminal MFs encircling the apical surface of the acini. As shown in Table 1, control acini occasionally exhibited this pattern of discontinuity in the luminal MFs, but cytochalasin D-treated acini showed a significant ( $P \leq 0.05$ ) increase in luminal MF breakage and discontinuity. Similar to the findings with jasplakinolide, disarrangement of the MF network by cytochalasin D did not alter the MT (Fig. 1E, green) or IF (Fig. 2E, green) arrays.

### Taxol alters the MT and IF arrays in lacrimal acini

The changes in the MT array following exposure to nocodazole and taxol were assessed. Nocodazole caused loss of MT staining (green) but no additional effects on MFs (Fig. 1C, red) or cytokeratin IFs (data not shown). The nocodazole-induced loss of MT polymer seen by confocal microscopy was also confirmed biochemically (Table 2). Despite the loss of the majority of acinar MTs, a few nocodazole-resistant MTs remained in these lacrimal acini (Fig. 1C). Exposure of acini to taxol resulted in an apparent increase in MTs and some apical MT bundling (Fig. 1D, green); the increase in MT



**Fig. 3.** Cytochalasin D induces luminal gaps in the MF array. (A) Control acini. (B) Acini exposed to 5  $\mu$ M cytochalasin D for 60 minutes at 37°C. (C) Acini exposed to 10  $\mu$ M carbachol for 5 minutes at 37°C. Rhodamine phalloidin was used to label MFs in these preparations. Bar,  $\sim$ 5  $\mu$ m.

polymer was also confirmed biochemically (Table 2). We also noted an apparent accumulation of cytokeratin IFs in the taxol-treated acini (Fig. 2D, green).

To better resolve the effects of taxol on the MT and IF arrays, we examined their distributions in the luminal regions of control and taxol-treated acini at higher magnification. Relative to the MT array in control acini (Fig. 4A), prominent bundles of MTs in the apical region of the acini were induced by taxol treatment (Fig. 4B). Relative to control acini (Fig. 4C), taxol treatment also caused an obvious bundling of cytokeratin IFs that paralleled the MT bundling (Fig. 4D).

### $\gamma$ -Tubulin is localized to the apical membrane in lacrimal acini

$\gamma$ -Tubulin is associated with MT minus ends and appears to play an important role in MT nucleation (Joshi et al., 1992). Other studies have localized  $\gamma$ -tubulin beneath the apical membrane of polarized epithelial cells, consistent with MT minus-end organization at the apical surface of epithelia (Meads and

**Table 2. Measurement of cytoskeletal tubulin and actin after inhibitor treatments**

Treatment	% Total $\alpha$ -tubulin pool	% Total actin pool
Control	63.16 $\pm$ 4.6 (5/5)	69.2 $\pm$ 3.9 (5/4)
Taxol	87.36 $\pm$ 4.8 *(5/5)	–
Nocodazole	48.69 $\pm$ 1.8 *(4/4)	–
Jasplakinolide	–	75.9 $\pm$ 10.9 (5/4)
Cytochalasin D	–	69.8 $\pm$ 7.6 (4/4)

Acini were treated (60 minutes, 37°C) with nocodazole (33  $\mu$ M), taxol (10  $\mu$ M), jasplakinolide (0.5  $\mu$ M) or cytochalasin D (5  $\mu$ M). *n* = no. of samples/no. of cell preparations; \* indicates significant ( $P \leq 0.05$ ) relative to control.

Schroer, 1995). To investigate the orientation of the MT array in lacrimal acini, we utilized confocal fluorescence microscopy to probe the distribution of  $\gamma$ -tubulin in lacrimal acini (Fig. 5A) relative to the distribution of luminal MFs in the same sample (Fig. 5B).  $\gamma$ -Tubulin staining is present exclusively at the apical membrane of lacrimal acini, suggesting that MT minus-ends in lacrimal acini are organized at the apical region.

#### Secretion of $\beta$ -hexosaminidase is maximal at 10 $\mu$ M carbachol

$\beta$ -Hexosaminidase is enriched in secretory membranes (Hamm-Alvarez et al., 1997) and secreted in response to carbachol (Gierow and Mircheff, 1998). In order to determine optimal treatment conditions for further studies on stimulated secretion, the time course of release of  $\beta$ -hexosaminidase from lacrimal acini was measured following exposure to 10  $\mu$ M or 1 mM carbachol (Fig. 6). At all time points,  $\beta$ -hexosaminidase secretion was greater at 10  $\mu$ M carbachol than at 1 mM carbachol. This observation is consistent with other measurements of the optimal carbachol dose required for maximal stimulation in lacrimal acini (Gierow et al., 1995a).

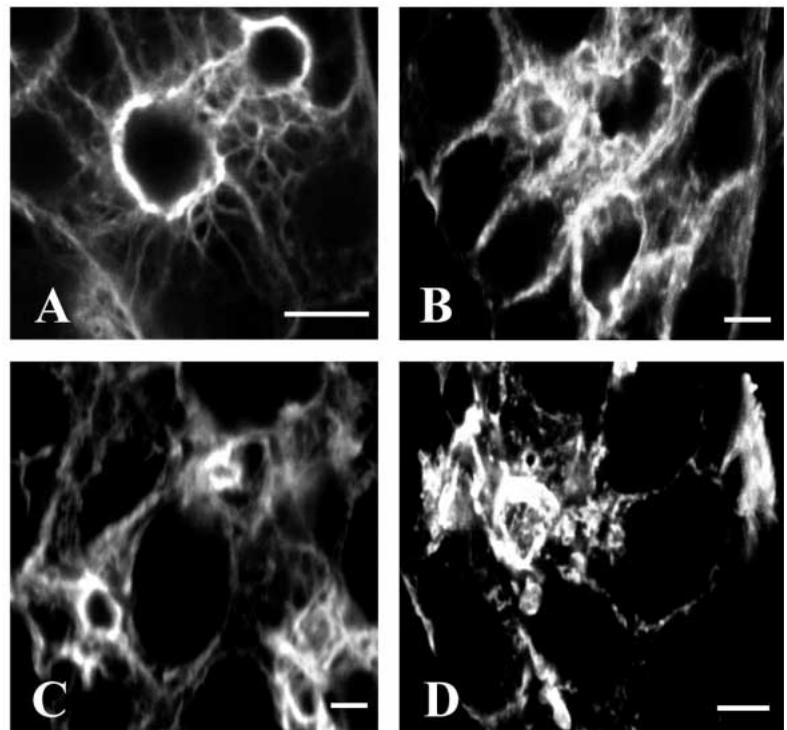
Exposure to 10  $\mu$ M carbachol caused a steady release of  $\beta$ -hexosaminidase that was maintained throughout the time course (1-60 minutes). Exposure to 1 mM carbachol elicited a biphasic release of  $\beta$ -hexosaminidase secretion, with a rapid phase complete within 10 minutes and a slower phase which continued from 10 to 60 minutes.

#### Stimulation with carbachol does not alter the MF or MT arrays

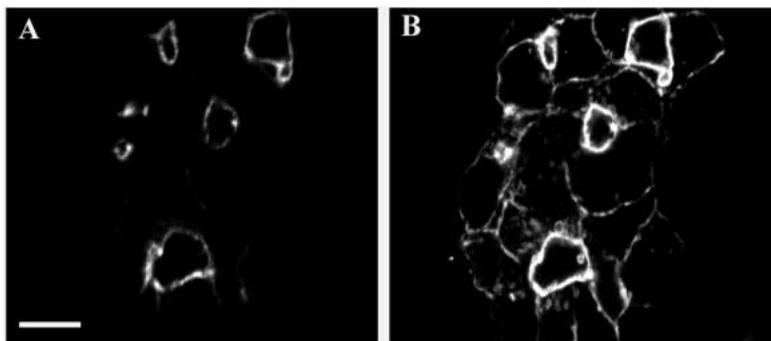
The data in Fig. 6 suggested that acini would be in an active secretory phase following exposure to 10  $\mu$ M carbachol for at least 30 minutes. To analyze possible changes in MF and MT organization that might accompany carbachol-stimulated secretion, acini were treated with 10  $\mu$ M carbachol for up to 30 minutes prior to processing for confocal fluorescence microscopy. As shown in Fig. 1B, stimulation of acinar secretion with 10  $\mu$ M carbachol for 30 minutes did not elicit detectable changes in either MT (green) or MF (red) distribution or density. To ensure that we were not missing the MF disassembly hypothesized by the MF barrier model of acinar secretion, we examined luminal MFs from carbachol-treated (10  $\mu$ M, 5 minutes) acini at higher magnification for evidence of possible MF loss or discontinuity at the luminal surface (Fig. 3C). No major changes in MF organization were seen; additionally, quantitative analysis revealed no evidence for the development of discontinuities in luminal MFs in carbachol-treated acini (10  $\mu$ M, 5 minutes) relative to controls (Table 1). As well, stimulation of acini with 1 mM carbachol from 1-30 minutes did not cause detectable changes in MFs or MTs (data not shown).

#### Analysis of cytoskeletal tubulin and actin from resting and carbachol-stimulated acini

Although confocal microscopy revealed no major cytoskeletal changes in MFs or MTs following stimulation, we wanted to confirm these findings through biochemical analysis. Fractions



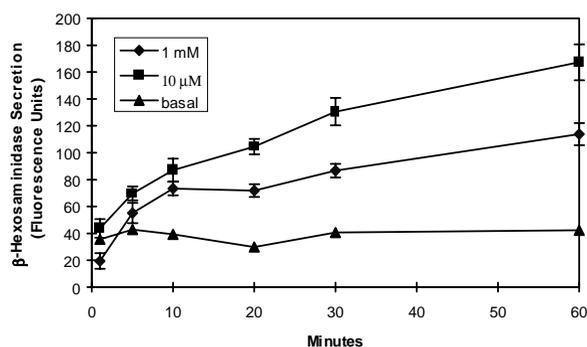
**Fig. 4.** Taxol causes bundling of MTs and IFs. (A) MTs in control acini. (B) MTs in acini exposed to 10  $\mu$ M taxol. (C) Cytokeratin IFs in control acini. (D) Cytokeratin IFs in acini exposed to 10  $\mu$ M taxol. Taxol treatments were for 60 minutes at 37°C. The YOL1/34 antibody against  $\alpha$ -tubulin was used for MT labeling and the K8.13 monoclonal antibody was used for IF labeling. Bar,  $\sim$ 5  $\mu$ m.



**Fig. 5.**  $\gamma$ -Tubulin staining is localized to the luminal region of acini. (A)  $\gamma$ -Tubulin distribution and (B) MF distribution in the same field. MFs were labeled with rhodamine phalloidin and  $\gamma$ -tubulin with a monoclonal antibody as indicated in Materials and Methods. Bar,  $\sim 10 \mu\text{m}$ .

enriched in soluble and cytoskeletal proteins were isolated from resting and carbachol-stimulated acini, and cytoskeletal tubulin and actin contents were measured. Evidence of cytoskeletal disassembly following carbachol treatment would be decreased recovery of the actin or tubulin in the cytoskeletal pool. As shown in Fig. 7, stimulation of acini with either  $10 \mu\text{M}$  or  $1 \text{mM}$  carbachol caused no significant changes in the amount of actin or tubulin associated with the cytoskeletal pool, consistent with the confocal microscopy data presented in Fig. 1B, Fig. 3C, and Table 1.

The major changes in MT morphology observed after nocodazole (Fig. 1C) and taxol (Fig. 1D) treatment by confocal microscopy were directly correlated with decreased and increased recoveries of cytoskeletal tubulin, respectively (Table 2). The major changes in MF morphology seen after cytochalasin D (Fig. 1E) or jasplakinolide (Fig. 1F) were not associated with any significant changes in cytoskeletal actin (Table 2). Confocal microscopy of jasplakinolide-treated acini labeled with a monoclonal anti-actin antibody (Fig. 1F) revealed diffuse staining rather than the absence of staining, suggesting that the actin in jasplakinolide-treated acini could be present as very short MFs throughout the cytoplasm. This speculation is consistent with the biochemical quantitation and also with observations that jasplakinolide induces MF polymerization (Bubb et al., 1994). The finding that cytochalasin D treatment did not reduce the cytoskeletal actin content may be explained

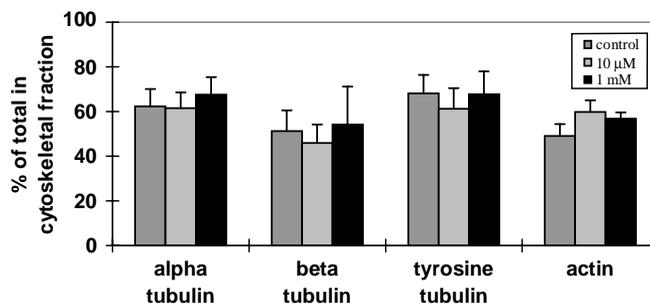


**Fig. 6.** Time course for release of  $\beta$ -hexosaminidase in resting and stimulated acini.  $\beta$ -Hexosaminidase activity released into the culture medium of resting acini ( $\blacktriangle$ ) and acini stimulated with  $10 \mu\text{M}$  carbachol ( $\blacksquare$ ) or  $1 \text{mM}$  carbachol ( $\circ$ ) was measured at the indicated times. For carbachol stimulation,  $n=23$  to 35 samples per timepoint from 3-5 cell preparations and values are mean  $\pm$  s.e.m. For resting acini,  $n=12$  samples per timepoint from 2 cell preparations and results are expressed as an average from the mean of the two experiments.

by previous reports that cytochalasin D treatment does not cause net loss of MFs (Morris and Tannenbaum, 1980) and can even induce random nucleation of MFs in the cytoplasm (Friederich et al., 1993). These findings showing that jasplakinolide and cytochalasin D do not significantly reduce cytoskeletal actin are consistent with some previous reports. However, the failure of these agents to demonstrate a loss in polymeric actin leaves us without a positive control that would confirm the ability of our analysis system to detect a carbachol-induced loss in F-actin levels. Since changes in cytoskeletal tubulin are biochemically detectable in our system, our data demonstrate definitively that MT loss is not associated with carbachol-stimulated secretion.

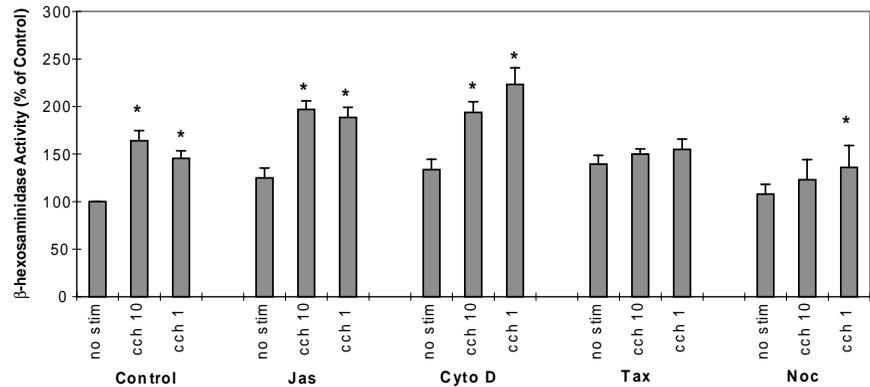
#### MF-targeted agents may enhance the release of $\beta$ -hexosaminidase

To further correlate changes in apical MFs with stimulated secretion, the effects of jasplakinolide and cytochalasin D on the release of  $\beta$ -hexosaminidase were measured (Fig. 8). Neither jasplakinolide nor cytochalasin D blocked the release of  $\beta$ -hexosaminidase elicited by  $10 \mu\text{M}$  or  $1 \text{mM}$  carbachol, as evidenced by the observation of significant ( $P \leq 0.05$ ) carbachol-stimulated release of  $\beta$ -hexosaminidase in acini with and without these treatments. The statistics shown in Fig. 8 reflect comparisons between  $\beta$ -hexosaminidase release from carbachol-stimulated/treated samples versus the pretreated samples alone. However, the release of  $\beta$ -hexosaminidase from treated (jasplakinolide or cytochalasin D), stimulated acini was also significantly increased ( $P \leq 0.05$ ) relative to untreated, stimulated acini at both  $10 \mu\text{M}$  and  $1 \text{mM}$  carbachol.



**Fig. 7.** The cytoskeletal composition of tubulin and actin is not altered by carbachol. Lacrimal acini were stimulated with  $10 \mu\text{M}$  or  $1 \text{mM}$  carbachol for 30 minutes. The percentage of total  $\alpha$  (alpha)-,  $\beta$  (beta)- and tyrosinated tubulin and actin recovered in soluble and cytoskeletal fractions was determined by densitometry of samples analyzed by western blots. The percent of total tubulin and actin in the cytoskeletal pools of control and treated acini are shown here  $\pm$  s.e.m.  $n=4$ -12 samples from 2-3 cell preparations for each treatment.

**Fig. 8.** MT-targeted agents impede carbachol-dependent secretion of  $\beta$ -hexosaminidase in lacrimal acini. Control and treated acini were stimulated (30 minutes, 37°C) with either 10  $\mu$ M or 1 mM carbachol before assay of  $\beta$ -hexosaminidase release into the culture medium. Each of the following treatments was for 60 minutes at 37°C prior to carbachol stimulation: taxol (Tax, 10  $\mu$ M,  $n=108/10$ , where  $n$  = no. of samples/no. of cell preparations), nocodazole (Noc, 33  $\mu$ M,  $n=48/4$ ), jasplakinolide (Jas, 0.5  $\mu$ M,  $n=96/8$ ), and cytochalasin D (Cyto D, 5  $\mu$ M,  $n=84/7$ ). \*Release of  $\beta$ -hexosaminidase is significant ( $P\leq 0.05$ ) relative to the unstimulated controls.  $n$  ranged from 16/4 to 143/23 in matched unstimulated controls.



The role of carbachol in this increased response of jasplakinolide and cytochalasin D-treated acini to carbachol was difficult to assess, since a marked carbachol-independent release of  $\beta$ -hexosaminidase was also seen after pretreatment with jasplakinolide or cytochalasin D (Fig. 8). This carbachol-independent increase was not due to the methanol vehicle used for jasplakinolide or the DMSO vehicle used for cytochalasin D (data not shown). Although it represented release of 20-30% more  $\beta$ -hexosaminidase, this carbachol-independent increase was also not significant ( $P\leq 0.05$ ) relative to untreated acini. However, this carbachol-independent increase during pretreatment with jasplakinolide or cytochalasin D certainly contributes to the larger secretory response to carbachol in jasplakinolide- and cytochalasin D-treated acini relative to untreated, stimulated acini. In any case, these data suggest that the lack of luminal MFs in carbachol-stimulated cells leads to significant increases in  $\beta$ -hexosaminidase secretion.

#### Treatment with MT-targeted agents reduces the carbachol-dependent secretion of $\beta$ -hexosaminidase

To further correlate effects on cellular MTs with stimulated secretion, the effects of taxol and nocodazole on the release of  $\beta$ -hexosaminidase were measured. As shown in Fig. 8, the significant ( $P\leq 0.05$ ) release of  $\beta$ -hexosaminidase normally elicited by either 10  $\mu$ M or 1 mM carbachol was prevented by taxol. As well, nocodazole treatment prevented the significant ( $P\leq 0.05$ ) release of  $\beta$ -hexosaminidase at 10  $\mu$ M carbachol. Under these same conditions (10  $\mu$ M taxol or 33  $\mu$ M nocodazole, 60 minutes, 37°C), no major inhibition of protein synthesis occurred during a 60 minute pulse:  $^{35}\text{S}$ /protein for

nocodazole-treated acini was  $91\pm 16\%$  of control while  $^{35}\text{S}$ /protein for taxol-treated acini was  $96\pm 4\%$  of control ( $n=4-6$  samples from 1-2 cell preparations).

Some carbachol-independent release of  $\beta$ -hexosaminidase also occurred during pretreatment with taxol, which was not attributable to its DMSO vehicle (data not shown). Although a similar effect was observed with jasplakinolide and cytochalasin D pretreatment, it is likely that this carbachol-independent effect of taxol occurs through a separate mechanism.

We assessed whether taxol and nocodazole also elicited an inhibition of general protein release. As shown in Table 3, both taxol and nocodazole reduced the carbachol-stimulated release of protein at both 10  $\mu$ M and 1 mM carbachol. Intriguingly, we also found that 1 mM carbachol elicited a slightly greater release of bulk protein than 10  $\mu$ M carbachol, counter to the opposite trend measured for  $\beta$ -hexosaminidase release (Fig. 6). As well, nocodazole reduced the release of protein at 1 mM carbachol to a greater extent than at 10  $\mu$ M carbachol, counter to its effects on  $\beta$ -hexosaminidase release.

#### DISCUSSION

This work defines the organization of the cytoskeleton in lacrimal acini and probes its participation in stimulated secretion. Confocal images delineate a MT array emanating from an apical/luminal region that is also enriched in MFs and  $\gamma$ -tubulin. Confocal microscopy and biochemical analysis of acini stimulated with carbachol showed no major organizational changes in MTs or MFs under conditions where acini were actively secreting  $\beta$ -hexosaminidase. However, major changes in the MT array caused by taxol and nocodazole were correlated with reductions in carbachol-stimulated secretion of  $\beta$ -hexosaminidase and protein, suggesting that carbachol-stimulated secretion has MT-dependent components. Major changes in luminal MFs elicited by jasplakinolide and cytochalasin D were not associated with reductions in the carbachol-stimulated secretion of  $\beta$ -hexosaminidase, rather, both actin-targeted drugs caused a trend toward increased release of  $\beta$ -hexosaminidase with and without carbachol.

We utilized the carbachol-stimulated release of  $\beta$ -hexosaminidase as our model for stimulated protein secretion. Previous work with rabbits has shown that total amounts of  $\beta$ -hexosaminidase in secreted lacrimal gland fluid increase in direct proportion to the secreted fluid volume when rabbits are stimulated with pilocarpine (Gierow et al., 1995b; Gierow and Mircheff, 1998). Moreover, reconstituted lacrimal acini release

**Table 3. Taxol and nocodazole reduce bulk protein secretion in lacrimal acini**

Treatments	Protein release/1 mM CCH % of control	Protein release/10 $\mu$ M CCH % of control
Taxol	73 $\pm$ 8*	77 $\pm$ 3*
Nocodazole	67 $\pm$ 4*	82 $\pm$ 4

Samples were incubated with taxol (10  $\mu$ M) or nocodazole (33  $\mu$ M) for 60 minutes at 37°C prior to addition of 1 mM carbachol (CCH) for 15 minutes or 10  $\mu$ M carbachol for 30 minutes. Data are expressed as a percentage of the protein release obtained in carbachol-stimulated cells alone  $\pm$  s.e.m.; 1 mM carbachol stimulated protein release by  $180\pm 6\%$  of control while 10  $\mu$ M carbachol stimulated protein release by  $168\pm 6\%$  of control. \*Significant ( $P\leq 0.05$ ) difference relative to carbachol-stimulated cells.  $n = 3-4$  separate preparations.

$\beta$ -hexosaminidase into the culture medium in response to cholinergic stimulation (Gierow et al., 1995b; Gierow and Mircheff, 1998). As well, our previous work with analytical subcellular fractionation methods has shown that membrane-associated kinesin codistributed with an apparent post-Golgi secretory compartment containing  $\beta$ -hexosaminidase in resting acini (Hamm-Alvarez et al., 1997). Exposure of acini to carbachol resulted in corresponding parallel redistributions of membrane-associated kinesin and  $\beta$ -hexosaminidase that appeared to reflect a return of material to the Golgi complex from the secretory pathway via the apical plasma membrane, as reported previously by Herzog and Farquhar (1976). Although we cannot eliminate the possibility that some  $\beta$ -hexosaminidase may exit the cell through basal-lateral pathways during stimulation, these data support an exodus through the apical secretory pathway.

Inhibition of stimulated secretion of  $\beta$ -hexosaminidase by nocodazole is consistent with a role for MT-dependent vesicle movement, since removal of MT tracks would inhibit the movement of vesicles along MTs to their cellular destination. Because the removal of the majority of the MTs was correlated with marked reductions in  $\beta$ -hexosaminidase and protein release after carbachol stimulation, a logical conclusion is that these MTs supported the carbachol-stimulated movement of vesicles containing  $\beta$ -hexosaminidase to the apical membrane. Careful examination of Fig. 1C shows that a few drug-resistant MTs persist even after exposure to 33  $\mu$ M nocodazole. Previous work has shown that some MTs are drug-resistant, particularly those enriched in deetyrosinated or acetylated  $\alpha$ -tubulin (Baas and Black, 1990). The respective abilities of stable (drug-resistant) and dynamic (drug-sensitive) MTs to support MT-based vesicle transport are unknown, but this small number of resistant MTs could be responsible for sustaining remaining levels of stimulated secretion in nocodazole-treated acini.

Our finding that taxol inhibits stimulated secretion in lacrimal acini is consistent with other studies showing that taxol inhibits MT-dependent vesicle transport in cultured cells (Hamm-Alvarez et al., 1993). Since taxol-induced stabilization of MTs does not inhibit vesicle movement *in vitro* (Hamm-Alvarez et al., 1993), it is conceivable that suppression of normal MT dynamics by taxol can alter the binding of MT-associated proteins to the MT, thus impeding vesicle movement. It is also possible that the MT bundling observed by confocal microscopy (Fig. 4B) impedes vesicle movement along the MT through steric constraints.

The observation of a trend toward carbachol-independent release of  $\beta$ -hexosaminidase in taxol-treated acini was somewhat puzzling (Fig. 8). It is likely that this effect is independent of the trend toward carbachol-independent release of  $\beta$ -hexosaminidase in jasplakinolide- and cytochalasin D-treated acini, which is discussed below. Our finding that taxol-induced MT stabilization caused concomitant bundling of cytokeratin-based IFs reveals an interaction between cytokeratin IFs and MTs, similar to reports in cultured cells (Gurland and Gundersen, 1995). Taxol could conceivably increase the accessibility of the apical membrane to membrane vesicles via effects on cytokeratin IFs: Fig. 4 reveals that the densely packed IF array is bundled by taxol, introducing gaps which might enhance carbachol-independent access of secretory proteins to the lumen. Alternatively, taxol could act through a MT-independent pathway to elicit this carbachol-independent release of  $\beta$ -hexosaminidase.

Although both taxol and nocodazole reduced stimulated secretion of  $\beta$ -hexosaminidase, the reductions were more pronounced at 10  $\mu$ M than at 1 mM carbachol (Fig. 8). In fact, nocodazole did not detectably impede release of  $\beta$ -hexosaminidase at 1 mM carbachol. Intriguingly, this effect was not observed for bulk protein release (Table 3), where both taxol and nocodazole showed more inhibition of protein secretion at 1 mM carbachol rather than at 10  $\mu$ M carbachol. These small differences in sensitivity of carbachol-stimulated secretion of  $\beta$ -hexosaminidase and bulk protein to taxol and nocodazole suggest that their release might occur through different pathways, although both putative pathways show evidence for MT-dependence.

Confocal microscopy and biochemical analysis of resting and carbachol-stimulated acini revealed no evidence for major changes in apical MF organization that might accompany stimulated secretion. The failure to detect possible changes in MF organization in carbachol-treated acini by high resolution confocal microscopy or biochemical analysis suggested that secretion is not facilitated by a gross rearrangement of apical MFs; however, we cannot rule out more subtle carbachol-induced changes in MFs that may participate in regulated secretion. In fact, our data suggest that MFs can regulate access to the apical plasma membrane in lacrimal acini. Carbachol-stimulated release of  $\beta$ -hexosaminidase in acini lacking luminal MFs (jasplakinolide- or cytochalasin D-treated) was significantly increased relative to stimulated, untreated acini, suggesting that removal of luminal MFs can further enhance stimulated secretion.

We propose that MTs normally facilitate stimulated secretion of  $\beta$ -hexosaminidase in lacrimal acini by supporting the movement of secretory vesicles containing  $\beta$ -hexosaminidase to the apical membrane. The observation that nocodazole and taxol reduce stimulated secretion is consistent with this model. We also suggest that changes in luminal MFs can modulate stimulated secretion in lacrimal acini by regulating the access of secretory vesicles containing  $\beta$ -hexosaminidase to the apical membrane. In jasplakinolide- or cytochalasin D-treated acini, the trend toward increased carbachol-independent release of  $\beta$ -hexosaminidase may represent the enhanced ability of secretory vesicles positioned close to the apical membrane to fuse with the apical membrane in the absence of luminal MFs. The increased carbachol-dependent release of  $\beta$ -hexosaminidase in acini exposed to jasplakinolide or cytochalasin D may represent the enhanced ability of secretory vesicles transported along MTs to the apical membrane at an accelerated rate by carbachol to fuse with the apical membrane in the absence of MFs. However, the extent that carbachol actually promotes MF reorganization in order to enhance release of  $\beta$ -hexosaminidase is still unclear. The observation that removal of luminal MFs generates an additive secretory response over and above the response to carbachol alone suggests that luminal MFs are normally present in carbachol-treated acini, as do our observations that luminal MFs remain intact following carbachol stimulation (Fig. 3C, Table 1).

Previous studies have suggested major roles for MTs (Busson-Mabillot et al., 1982; Robin et al., 1995) or MFs (O'Konski and Pandol, 1990; Jungerman et al., 1995; Perrin et al., 1992) in stimulated exocrine secretion. Our work supports a role for MTs in the carbachol-dependent secretion of  $\beta$ -hexosaminidase and protein. Our work also suggests that elimination of luminal MFs by actin-targeted drugs can

enhance secretion of  $\beta$ -hexosaminidase in lacrimal acini, but that this mechanism is not necessarily utilized by carbachol. Together, these studies suggest that stimulated acinar secretion may proceed via MT and/or MF-dependent pathways. Although we have focused on carbachol, which utilizes  $\text{Ca}^{2+}$ -dependent signal transduction pathways (Dartt, 1994; Zoukhri et al., 1997), lacrimal secretion can also be stimulated via cAMP-dependent pathways (Meneray et al., 1997). Conceivably, the nature of the cargo and/or the secretagogue may determine the choice of a particular pathway. Further studies will reveal whether particular secretory proteins or signaling pathways rely to different extents on MF- versus MT-mediated secretion. The relationships between trafficking mechanism, cargo, and signal transduction must be systematically investigated at the cellular level before the choice of a predominantly MT-based or MF-based mechanism can be fully understood.

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## REFERENCES

- Baas, P. W. and Black, M. M. (1990). Individual microtubules in the axon consist of domains that differ in both composition and stability. *J. Cell Biol.* **111**, 495-509.
- Barrett, A. J. and Heath, M. F. (1977). Lysosomes. *Laboratory Handbook* (ed. J. T. Dingle), pp. 118-120. Elsevier/North Holland Biomedical Press, Amsterdam.
- Bubb, M. R., Senderowicz, A. M., Sausville, E. A., Duncan, K. L. and Korn, E. D. (1994). Jaspilakinolide, a cytotoxic natural product, induces actin polymerization and competitively inhibits the binding of phalloidin to F-actin. *J. Biol. Chem.* **269**, 14869-14871.
- Burcharde, J. K., McIlvain, J. M., Sheetz, M. P. and Argon, Y. (1993). Lytic granules from cytotoxic T cells exhibit kinesin-dependent motility on microtubules in vitro. *J. Cell Sci.* **104**, 151-162.
- Busson-Mabillot, S., Chambaut-Guerin, A. M., Obracht, L., Muller, P., and Rossignol, B. (1982). Microtubules and protein secretion in rat lacrimal glands: localization of short-term effects of colchicine on the secretory process. *J. Cell Biol.* **95**, 105-117.
- Dartt, D. A. (1994). Signal transduction and activation of the lacrimal gland. In *Principles and Practice of Ophthalmology* (ed. D. M. Albert and F. A. Jakobiec), pp. 458-465. Saunders, Philadelphia, 2nd Edition.
- Friederich, E., Kreis, T. E. and Louvard, D. (1993). Villin-induced growth of microvilli is reversibly inhibited by cytochalasin D. *J. Cell Sci.* **105**, 765-775.
- Gelfand, V. I. and Bershadsky, A. D. (1991). Microtubule dynamics: mechanism, regulation, and function. *Annu. Rev. Cell Biol.* **7**, 93-116.
- Gierow, J. P., Lambert, R. W. and Mircheff, A. K. (1995a). Fluid phase endocytosis by isolated rabbit lacrimal gland acinar cells. *Exp. Eye Res.* **60**, 511-525.
- Gierow, J. P., Bekmezian, A., Rafisolyman, S., Norian, J. M., Azzarolo, A. M., Platler, B. W., Huang, Z. M., Warren, D. W. and Mircheff, A. K. (1995b). Secretion of  $\beta$ -hexosaminidase by rabbit lacrimal gland and reconstituted acini. *Invest. Ophthalmol. Vis. Sci.* **36**, S990.
- Gierow, J. P., Yang, T., Bekmezian, A., Liu, N., Norian, J. M., Kim, S. A., Rafisolyman, S., Wood, R. L. and Mircheff, A. K. (1996). Na,K-ATPase in lacrimal gland acinar cell endosomal system. Correcting a case of mistaken identity. *Am. J. Physiol.* **271**, C1685-C1698.
- Gierow, J. P. and Mircheff, A. K. (1998). Role of protein kinases in regulation of apical secretion and basal-lateral membrane recycling traffic in reconstituted rabbit lacrimal gland acini. In *The Lacrimal Gland, Tear Film, and Dry Eye Syndromes II* (ed. D. A. Sullivan). Plenum, New York (in press).
- Gurland, G. and Gundersen, G. G. (1995). Stable, deetyrosinated microtubules function to localize vimentin intermediate filaments in fibroblasts. *J. Cell Biol.* **131**, 1275-1290.
- Hamm-Alvarez, S. F., Kim, P. Y. and Sheetz, M. P. (1993). Regulation of microtubule-dependent vesicle transport in CV-1 cells and extracts. *J. Cell Sci.* **106**, 955-966.
- Hamm-Alvarez, S. F., da Costa, S. R., Yang, T., Wei, X., Gierow, J. P. and Mircheff, A. K. (1997). Cholinergic stimulation of lacrimal acinar cells promotes redistribution of membrane-associated kinesin and the secretory protein  $\beta$ -hexosaminidase, and activation of soluble kinesin. *Exp. Eye Res.* **64**, 141-156.
- Herzog, V. and Farquhar, M. G. (1976). Luminal membrane retrieved after exocytosis reaches most Golgi cisternae in secretory cells. *Proc. Nat. Acad. Sci. USA* **74**, 5073-5077.
- Hodges, R. R., Dicker, D. M. and Dartt, D. A. (1994). Role of protein kinase C in alpha 1-adrenergic and cholinergic agonist stimulated protein secretion. *Advan. Exp. Med. Biol.* **350**, 147-150.
- Joshi, H. C., Palacios, M. J., McNamara, L. and Cleveland, D. W. (1992). Gamma-tubulin is a centrosomal protein required for cell-cycle-dependent microtubule nucleation. *Nature* **356**, 80-83.
- Jungerman, J., Lerch, M. M., Weidenbach, H., Lutz, M. P., Kruger, B. and Adler, G. (1995). Disassembly of rat pancreatic acinar cell cytoskeleton during supramaximal secretagogue stimulation. *Am. J. Physiol.* **268**, G328-G338.
- Kilmartin, J. V., Wright, B. and Milstein, C. (1982). Rat monoclonal anti-tubulin antibodies derived by using a new nonsecreting rat cell line. *J. Cell Biol.* **93**, 576-582.
- Kreis, T. E. (1987). Microtubules containing deetyrosinated tubulin are less dynamic. *EMBO J.* **6**, 2597-2606.
- Lacy, P. E., Klein, N. J. and Fink, C. J. (1973). Effect of cytochalasin B on the biphasic release of insulin in perfused rat islets. *Endocrinology* **92**, 1458-1468.
- LeDizet, M. and Piperno, G. (1991). Detection of acetylated alpha-tubulin by specific antibodies. *Meth. Enzymol.* **196**, 264-274.
- Lee, J. C., Field, D. J. and Lee, L. L. Y. (1980). Effects of nocodazole on structures of calf brain tubulin. *Biochemistry* **19**, 6209-6215.
- Meads, T. and Schroer, T. A. (1995). Polarity and nucleation of microtubules in polarized epithelial cells. *Cell Motil. Cytoskel.* **32**, 273-288.
- Meneray, M. A., Fields, T. Y. and Bennett, D. J. (1997). Gs and Gq/11 couple vasoactive intestinal peptide and cholinergic stimulation to lacrimal secretion. *Invest. Ophthalmol. Vis. Sci.* **38**, 1261-1270.
- Morris, A. and Tannenbaum, J. (1980). Cytochalasin D does not produce net depolymerization of actin filaments in HEP-2 cells. *Nature* **287**, 637-639.
- Nevalainen, T. J. (1975). Inhibition of pancreatic exocrine secretion by vinblastine. *Res. Exp. Med.* **165**, 163-168.
- O'Konski, M. S. and Pandol, S. J. (1990). Effects of caerulein on the apical cytoskeleton of the pancreatic acinar cell. *J. Clin. Invest.* **86**, 1649-1657.
- Pavelka, M. and Ellinger, A. (1983). Effect of colchicine on the Golgi complex of rat pancreatic acinar cells. *J. Cell Biol.* **97**, 737-748.
- Perrin, D., Möller, K., Hanke, K. and Söling, H. D. (1992). cAMP and  $\text{Ca}^{2+}$ -mediated secretion in parotid acinar cells is associated with reversible changes in the organization of the cytoskeleton. *J. Cell Biol.* **116**, 127-134.
- Robin, P., Rossignol, B. and Raymond, M.N. (1995). Effect of microtubule network disturbance by nocodazole and docetaxel (Taxotere) on protein secretion in rat extraorbital lacrimal and parotid glands. *Eur. J. Cell Biol.* **67**, 227-237.
- Rogers, S. L., Tint, I. S., Fanapour, P. C. and Gelfand, V. I. (1997). Regulated bidirectional motility of melanophore pigment granules along microtubules in vitro. *Proc. Nat. Acad. Sci. USA* **94**, 3720-3725.
- Runnegar, M., Wei, X., Berndt, N. and Hamm-Alvarez, S. F. (1997). Transferrin receptor recycling in hepatocytes is regulated by protein phosphatase 2A, possibly through effects on microtubule-dependent transport. *Hepatology* **26**, 176-185.
- Schiff, P. B. and Horwitz, S. B. (1981). Taxol assembles tubulin in the absence of exogenous guanosine 5'-triphosphate or microtubule-associated proteins. *Biochemistry* **20**, 3247-3252.
- Sontag, E., Nungakdi-Craig, V., Bloom, G. S. and Mumby, M. C. (1995). A novel pool of protein phosphatase 2A is associated with microtubules and is regulated during the cell cycle. *J. Cell Biol.* **128**, 1131-1144.
- Tannenbaum, J., Tannenbaum, S. and Godman, G. (1977). Binding and subcellular localization of titrated cytochalasin D. *Exp. Cell Res.* **91**, 47-56.
- Zoukhri, D., Hodges, R. R., Dicker, D. M. and Dartt, D. A. (1994). Role of protein kinase C in cholinergic stimulation of lacrimal gland protein secretion. *FEBS Lett.* **351**, 67-72.
- Zoukhri, D. and Dartt, D. A. (1995). Cholinergic activation of phospholipase D in lacrimal gland acini is independent of protein kinase C and calcium. *Am. J. Physiol.* **268**, C713-20.
- Zoukhri, D., Hodges, R. R., Sergheraert, C., Toker, A. and Dartt, D. A. (1997). Lacrimal gland PKC isoforms are differentially involved in agonist-induced protein secretion. *Am. J. Physiol.* **272**, C263-269.