

# Effect of Histamine on Ca<sup>2+</sup>-Dependent Signaling Pathways in Rat Conjunctival Goblet Cells

Dayu Li, Richard B. Carozza, Marie A. Shatos, Robin R. Hodges, and Darlene A. Dartt

**PURPOSE.** The purpose of this study was to determine the Ca<sup>2+</sup>-dependent cellular signaling pathways used by histamine to stimulate conjunctival goblet cell secretion.

**METHODS.** Cultured rat goblet cells were grown in RPMI 1640. Goblet cell secretion of high molecular weight glycoconjugates was measured by an enzyme-linked lectin assay. Intracellular [Ca<sup>2+</sup>]<sub>i</sub> ([Ca<sup>2+</sup>]<sub>i</sub>) was measured by loading cultured cells with the Ca<sup>2+</sup> sensitive dye fura-2. The level of [Ca<sup>2+</sup>]<sub>i</sub> was measured using fluorescence microscopy. Extracellular regulated kinase (ERK) 2 was depleted using small interfering RNA (siRNA).

**RESULTS.** Histamine-stimulated conjunctival goblet cell secretion of high molecular weight glycoproteins was blocked by removal of extracellular Ca<sup>2+</sup> and depletion of ERK2 by siRNA. Histamine increase in [Ca<sup>2+</sup>]<sub>i</sub> was desensitized by repeated addition of agonist and blocked by a phospholipase C antagonist. Histamine at higher doses increased [Ca<sup>2+</sup>]<sub>i</sub> by stimulating influx of extracellular Ca<sup>2+</sup>, but at a lower dose released Ca<sup>2+</sup> from intracellular stores. Activation of each histamine receptor subtype (H<sub>1</sub>-H<sub>4</sub>) increased [Ca<sup>2+</sup>]<sub>i</sub> and histamine stimulation was blocked by antagonists of each receptor subtype. The H<sub>2</sub> receptor subtype increase in [Ca<sup>2+</sup>]<sub>i</sub> was cAMP dependent.

**CONCLUSIONS.** We conclude that histamine activates phospholipase C to release intracellular Ca<sup>2+</sup> that induces the influx of extracellular Ca<sup>2+</sup> and activates ERK1/2 to stimulate conjunctival goblet cell mucous secretion, and that activation of all four histamine receptor subtypes can increase [Ca<sup>2+</sup>]<sub>i</sub>. (*Invest Ophthalmol Vis Sci.* 2012;53:6928-6938) DOI:10.1167/iov.12-10163

Allergic conjunctivitis is a prevalent ocular surface disease affecting between 15% to 20% of Americans.<sup>1</sup> Symptoms of allergic conjunctivitis include chemosis, excess tearing, increase tear mucus, and itching. In some forms of allergic conjunctivitis excess tear mucus secretion predominates and is exacerbated by mucous fishing syndrome, in which the patient's attempts to clear away mucus causes additional mucus secretion.<sup>2</sup> There are no completely effective treatments for this type of allergic conjunctivitis and new approaches to treatment are warranted.

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Histamine, a major mediator of allergy, is secreted by mast cells recruited into the conjunctival stroma.<sup>3</sup> Although antihistamines, especially anti-H<sub>1</sub> and -H<sub>2</sub> antihistamines, are used to treat allergic conjunctivitis, the role of histamine in activating conjunctival goblet cells has only recently been explored. We recently published a study, in which we demonstrated that histamine directly stimulates secretion of high molecular weight glycoproteins from both human and rat conjunctival goblet cells in culture.<sup>4</sup> Surprisingly, all four histamine receptor subtypes (H<sub>1</sub>-H<sub>4</sub>) are present on goblet cells, and their activation by agonists stimulates secretion and inhibition by antagonists blocks histamine-induced goblet cell secretion.<sup>4</sup> Thus, conjunctival goblet cells are a direct target of histamine, suggesting that these cells play an integral role in the pathogenesis of allergic conjunctivitis.

The signaling pathways activated by histamine have not been investigated in goblet cells from any tissue including conjunctiva, lung, gastrointestinal tract, and nasal cavity. The typical signaling pathways activated by histamine receptor subtypes, however, are an increase in [Ca<sup>2+</sup>]<sub>i</sub> by H<sub>1</sub> and H<sub>4</sub> receptors, activation of adenylyl cyclase and production of cAMP by H<sub>2</sub> receptors, and a decrease in intracellular [Ca<sup>2+</sup>]<sub>i</sub> and cAMP by H<sub>3</sub> receptors.<sup>5</sup>

Known stimuli of conjunctival goblet cell secretion, including cholinergic agonists, vasoactive intestinal peptide (VIP), and cysteinyl leukotrienes, stimulate goblet cell secretion by increasing the [Ca<sup>2+</sup>]<sub>i</sub> and activating extracellular regulated kinase (ERK)1/2.<sup>6,7</sup> In the present study, we used rat conjunctival goblet cells in culture to determine if histamine increases [Ca<sup>2+</sup>]<sub>i</sub> and activates ERK1/2, if histamine-induced mucin secretion is dependent upon [Ca<sup>2+</sup>]<sub>i</sub> and activation of ERK1/2, and if so what cellular mechanisms histamine uses to increase [Ca<sup>2+</sup>]<sub>i</sub>, and which histamine receptor subtypes are used. We concluded that histamine activates phospholipase C to release intracellular Ca<sup>2+</sup> that induces the influx of extracellular Ca<sup>2+</sup> and activates ERK1/2 to stimulate conjunctival goblet cell mucous secretion. Interestingly, activation of all four histamine receptor subtypes increases [Ca<sup>2+</sup>]<sub>i</sub>, but the H<sub>2</sub> receptor uses cAMP and protein kinase A (PKA) to elevate [Ca<sup>2+</sup>]<sub>i</sub>.

## MATERIALS AND METHODS

### Materials

Histamine and the histamine receptor agonists 2-([3-trifluoromethyl]phenyl) histamine dimaleate (H<sub>1</sub>), amthamine dihydrobromide (H<sub>2</sub>), and 4-methylhistamine dihydrochloride (H<sub>4</sub>), the histamine receptor antagonists chlorpheniramine (H<sub>1</sub>), cimetidine (H<sub>2</sub>), and JNJ7777120 (H<sub>4</sub>) as well as histamine and thapsagargin were from Sigma (St. Louis, MO) while (R)α-methylhistamine dihydrochloride, the H<sub>3</sub> receptor agonist, conessine, the H<sub>3</sub> receptor antagonist, and U73122 were purchased from Tocris Bioscience (Ellisville, MO). VIP was from EMD Chemicals (Rockville, MA). H89 was purchased from R&D Systems (Minneapolis, MN). Amplex Red and fura2/AM were purchased from

Invitrogen (Grand Island, NY). The MUC5AC ELISA kit was purchased from Biotang Inc. (Waltham, MA).

## Animals

Male Sprague-Dawley rats weighing between 125 and 150 g were obtained from Taconic Farms (Germantown, NY). Rats were anesthetized with CO<sub>2</sub> for 1 minute followed by decapitation and the bulbar and fornical conjunctiva removed from both eyes. All experiments conformed to ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Schepens Eye Research Institute Animal Care and Use Committee.

## Cell Culture

Goblet cells from excised rat conjunctiva were grown in organ culture as described previously.<sup>8,9</sup> Pieces of minced tissue dissected from excised conjunctiva were placed in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 100 µg/mL penicillin-streptomycin. The tissue plug was removed after nodules of cells were observed. First passage goblet cells were used in all experiments. The identity of cultured cells was periodically confirmed by evaluating staining with antibody to cytokeratin 7 (that detects goblet cell bodies) and the lectin *Ulex europaeus* agglutinin (UEA)-1 or an antibody to MUC5AC (both detect goblet cell secretory products) to ensure that goblet cells predominated.

## Secretion

Cultured goblet cells were seeded in 24-well plates and grown to confluence. Cells were serum starved for 2 hours before use, preincubated with antagonists for 30 minutes, and then stimulated with histamine for 2 hours in the presence of serum-free RPMI 1640, then supplemented with 0.5% bovine serum albumin (BSA). For small interfering (siRNA) experiments, goblet cells were incubated for 18 hours with 100 nM siRNA for ERK 2. The media was replaced and cells were allowed to grow for a total of 48 hours before use. Goblet cell secretion was measured using an enzyme-linked lectin assay (ELLA) with the lectin UEA-I. UEA-1 detects high molecular weight glycoconjugates including mucins produced by rat goblet cells. The media were collected and analyzed for the amount of lectin-detectable glycoconjugates, which quantifies the amount of goblet cell secretion. The standards and supernatant were spotted onto Nunc microplates (Thermo Scientific, Waltham, MA) and dried overnight at 60°C. The ELLA was performed according to a protocol from Pierce, Inc. (Rockford, IL) using UEA-I conjugated to horseradish peroxidase. The UEA-1 was then detected using Amplex Red (Invitrogen, Carlsbad, CA), which when oxidized by peroxidase in the presence of hydrogen peroxide, produces a highly fluorescent molecule. The fluorescence was quantified on a fluorescent ELISA reader (Synergy MX; Bio-Tek, Winooski, VT) with an excitation wavelength of 530 nm and an emission wavelength of 590 nm. The cells were removed and sonicated, and the cell homogenate analyzed for the total amount of protein by using the Bradford protein assay. High molecular weight glycoconjugate secretion was normalized to total protein in the homogenate. Bovine submaxillary mucin was used for the standard curve. High molecular weight glycoconjugate secretion was expressed as fold increase over basal that was set to 1. As a control we used a commercial rat MUC5AC ELISA kit to demonstrate that MUC5AC secretion is a component of high molecular weight glycoprotein secretion measured by ELLA (data not shown).

## Measurement of [Ca<sup>2+</sup>]<sub>i</sub>

Goblet cells were incubated for 1 hour at 37°C with Krebs-Ringer bicarbonate buffer with HEPES (KRB-HEPES) (119 mM NaCl, 4.8 mM KCl, 1.0 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 10 mM HEPES, and 5.5 mM glucose [pH 7.45]), plus 0.5% BSA

containing 0.5 µM fura-2/AM, 8 µM pluronic acid F127, and 250 µM sulfinpyrazone, followed by washing in KRB-HEPES containing sulfinpyrazone. Calcium measurements were made with a ratio imaging system (In Cyt Im2; Intracellular Imaging, Cincinnati, OH) using wavelengths of 340 and 380 nm and an emission wavelength of 505 nm. At least 10 cells were used for each condition and experiments were repeated in at least three separate animals. Antagonists of histamine (H) receptor subtypes H<sub>1</sub>-H<sub>4</sub> were dissolved in KRB-HEPES buffer and added 30 minutes before histamine. After addition of agonists data were collected in real time. Data are presented as the actual [Ca<sup>2+</sup>]<sub>i</sub> with time or as the change in peak [Ca<sup>2+</sup>]<sub>i</sub>. Change in peak [Ca<sup>2+</sup>]<sub>i</sub> was calculated by subtracting the average of the basal value (no added agonist) from the peak [Ca<sup>2+</sup>]<sub>i</sub>. Although data is not shown, the plateau [Ca<sup>2+</sup>]<sub>i</sub> was affected similarly to the peak [Ca<sup>2+</sup>]<sub>i</sub>.

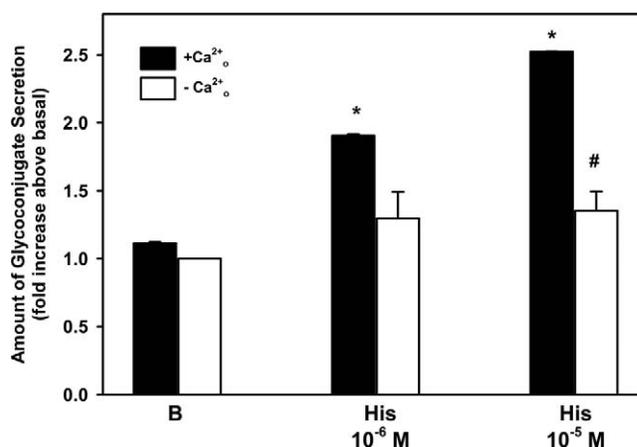
## Statistical Analysis

Results were expressed as the fold increase above basal or the percentage of inhibition of the net stimulation by agonist alone. Results are presented as mean ± SEM. Data were analyzed by Student's *t*-test. A *P* value less than 0.05 was considered statistically significant.

## RESULTS

### Effect of Extracellular Ca<sup>2+</sup> (Ca<sup>2+</sup><sub>o</sub>) Removal and Inhibition of Erk 2 on Histamine-Stimulated High Molecular Weight Glycoconjugate Secretion

As an increase in [Ca<sup>2+</sup>]<sub>i</sub> and in ERK1/2 activity is a major mechanism by which agonists stimulate conjunctival goblet cell secretion,<sup>4,10-13</sup> we investigated the extent to which histamine-stimulated secretion is dependent upon these signaling intermediates. We first determined the effect of extracellular (Ca<sup>2+</sup><sub>o</sub>) removal on histamine-stimulated goblet cell secretion, as we previously showed that histamine increases [Ca<sup>2+</sup>]<sub>i</sub> (data not shown). When Ca<sup>2+</sup><sub>o</sub> was removed, histamine-stimulated secretion was significantly blocked by 68% and 72% for histamine at 10<sup>-6</sup> and 10<sup>-5</sup> M, respectively (Fig. 1). Removal of Ca<sup>2+</sup><sub>o</sub> did not alter basal secretion.



**FIGURE 1.** Effect of removal of extracellular (Ca<sup>2+</sup><sub>o</sub>) on histamine-stimulated rat goblet cell high molecular weight glycoconjugate secretion. Cultured rat goblet cells were incubated in the presence (black bar) and absence (white bar) of extracellular Ca<sup>2+</sup> and then stimulated for 2 hours with no additions (B) or histamine (His) at 10<sup>-6</sup> and 10<sup>-5</sup> M. High molecular weight glycoconjugate secretion was measured by enzyme-linked lectin assay. Data are mean ± SEM of three independent experiments. \*Indicates statistical significance from no addition (B); #indicates statistical significance from histamine alone.

We then ascertained the extent that histamine-stimulated secretion is dependent upon activation of ERK1/2 by using siRNA for ERK2. We have shown that histamine activates ERK1/2 and, in addition, siRNA targeting ERK2, but not negative control, scrambled siRNA (small cytoplasmic RNA[scRNA]), decreased the amount of ERK 2.<sup>14</sup> When ERK2 was depleted with siRNA, histamine-stimulated secretion was significantly decreased by 68% and 78% for histamine at  $10^{-6}$  and  $10^{-5}$  M, respectively (Fig. 2). Incubation with scRNA did not alter the histamine-stimulated secretory response. In addition neither scRNA nor ERK2 siRNA altered basal secretion.

We conclude that in conjunctival goblet cells histamine-stimulated secretion is dependent upon both extracellular  $\text{Ca}^{2+}$  and activation of ERK 2.

### Effect of Multiple Applications of Histamine on $[\text{Ca}^{2+}]_i$

Histamine is a G protein-coupled receptor (GPCR) and one characteristic of GPCRs is desensitization. To explore whether activation of histamine receptors in conjunctival goblet cells desensitizes, histamine ( $10^{-5}$  M) was added sequentially three times at either 30 (Fig. 3A), 60 (Fig. 3B), or 120 (Fig. 3C) second intervals and  $[\text{Ca}^{2+}]_i$  measured. The second and third histamine responses were significantly decreased compared with the first, when histamine was added at 30, 60, or 120 second intervals, suggesting that histamine receptors in goblet cells desensitize and are GPCRs. Similar results were obtained if peak  $[\text{Ca}^{2+}]_i$  was expressed as a change compared with the basal  $[\text{Ca}^{2+}]_i$  before addition of histamine (data not shown) or the  $[\text{Ca}^{2+}]_i$  before each addition of histamine (Fig. 3A). Basal  $[\text{Ca}^{2+}]_i$  did not change over the course of the experiment (Fig. 3A).

### Effect of Inhibition of Phospholipase C with U73122 on Histamine-Stimulated Increase in $[\text{Ca}^{2+}]_i$

We next determined if, like other GPCRs, histamine in rat conjunctival goblet cells activates phospholipase C. Histamine at  $10^{-6}$  M significantly increased  $[\text{Ca}^{2+}]_i$  by  $170 \pm 35.9$  nM

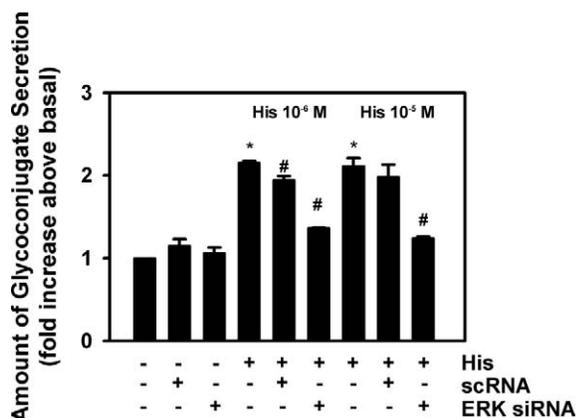


FIGURE 2. Effect of depletion of ERK2 on histamine-stimulated rat goblet cell high molecular weight glycoconjugate secretion. Cultured rat goblet cells were preincubated for 48 hours with no additions, siRNA for ERK2 (ERK siRNA), or siRNA for a scrambled sequence (scRNA), and then stimulated with histamine (His) at  $10^{-6}$  and  $10^{-5}$  M for 2 hours. High molecular weight glycoconjugate secretion was measured by enzyme-linked lectin assay. Data are mean  $\pm$  SEM of three independent experiments. \*Indicates statistical significance from no addition; #indicates statistical significance from histamine alone.

(Figs. 4A, 4B). When goblet cells were pre-incubated for 30 minutes with the phospholipase C inhibitor U73122 ( $10^{-6}$ – $10^{-8}$  M), the histamine-stimulated goblet cell increase in  $[\text{Ca}^{2+}]_i$  was significantly decreased. A maximum decrease of 78% was obtained (Fig. 4C). Thus, histamine interacts with GPCRs and activates phospholipase C to increase the  $[\text{Ca}^{2+}]_i$ .

### Effect of Removal of $\text{Ca}^{2+}_o$ on Histamine-Stimulated Increase in $[\text{Ca}^{2+}]_i$

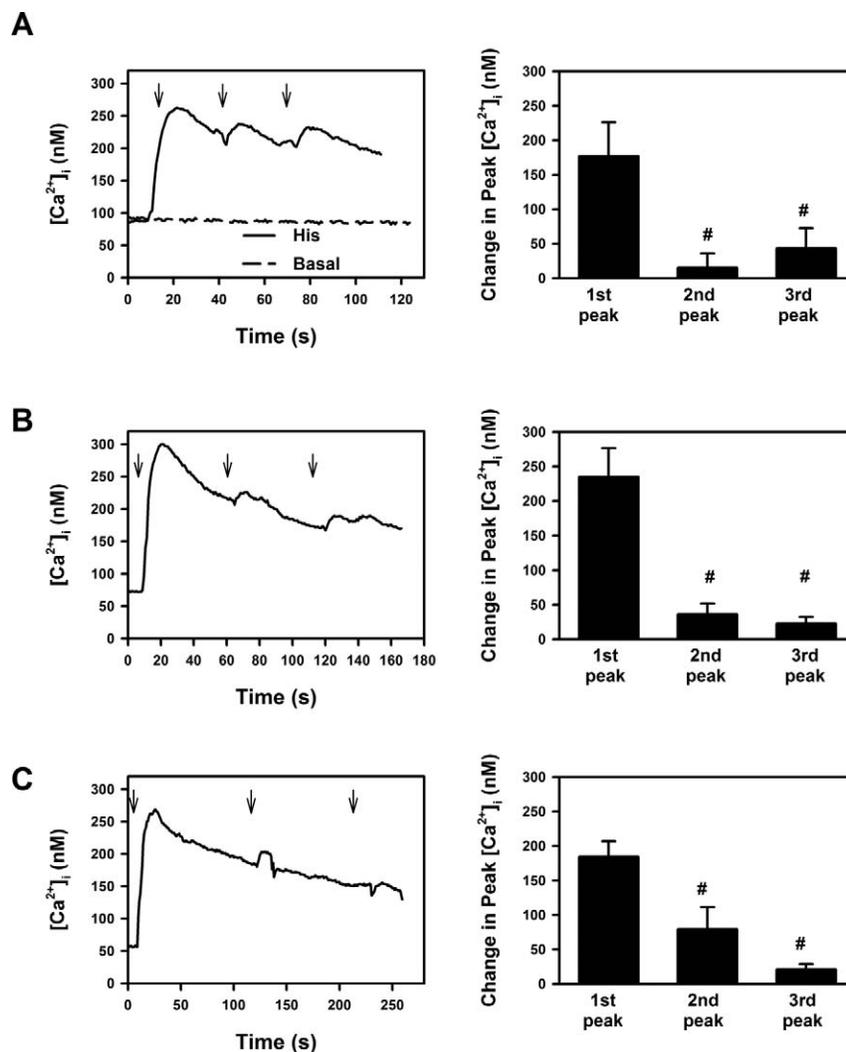
We next explored the cellular  $\text{Ca}^{2+}$  pools used by histamine to elevate  $[\text{Ca}^{2+}]_i$ . To determine the role of  $\text{Ca}^{2+}_o$ , we removed  $\text{Ca}^{2+}_o$  before stimulation with histamine. In the absence of  $\text{Ca}^{2+}_o$  the histamine ( $10^{-6}$  and  $10^{-5}$  M) stimulated increase in  $[\text{Ca}^{2+}]_i$  was almost completely inhibited (Figs. 5A–C). At the lowest concentration of histamine ( $10^{-7}$  M) removal of  $\text{Ca}^{2+}_o$  did not change the level of  $[\text{Ca}^{2+}]_i$  caused by histamine (Fig. 5C), suggesting that at higher concentrations the histamine response was almost completely dependent on  $\text{Ca}^{2+}_o$ , but was not similarly dependent at the lower histamine concentration. Removal of  $\text{Ca}^{2+}_o$  blocks both the histamine-stimulated increase in  $[\text{Ca}^{2+}]_i$  (Fig. 5) and secretion (Fig. 1) at histamine concentrations of  $10^{-5}$  and  $10^{-6}$  M and links the two functions.

We then determined if the lower concentration of histamine released intracellular  $\text{Ca}^{2+}$  stores. As a control, goblet cells were stimulated with histamine at  $10^{-7}$  M in the presence of  $\text{Ca}^{2+}_o$  which increased  $[\text{Ca}^{2+}]_i$  by  $70.2 \pm 16.1$  nM (Figs. 6A, 6B). In the next set of experiments, goblet cells were first stimulated with thapsigargin ( $10^{-6}$  and  $10^{-5}$  M) in the absence of  $\text{Ca}^{2+}_o$ , which blocks the  $\text{Ca}^{2+}$ -ATPase, a  $\text{Ca}^{2+}$  pump, of intracellular  $\text{Ca}^{2+}$  stores. This maneuver empties intracellular  $\text{Ca}^{2+}$  stores by preventing the re-uptake of  $\text{Ca}^{2+}$  after it passively leaks out. After the intracellular  $\text{Ca}^{2+}$  stores were depleted with thapsigargin, cells were stimulated by histamine. Compared with the histamine ( $10^{-7}$  M) stimulated increase in  $[\text{Ca}^{2+}]_i$  response in the absence of thapsigargin (Fig. 6A left panel, 6B) the histamine response in the presence of thapsigargin ( $10^{-6}$  and  $10^{-5}$  M) was completely blocked (Fig. 6A right panel, 6B). We suggest that histamine at low concentrations releases intracellular  $\text{Ca}^{2+}$  stores that stimulate influx of extracellular  $\text{Ca}^{2+}$ , but at higher concentrations the influx of extracellular  $\text{Ca}^{2+}$  overwhelms the release of intracellular  $\text{Ca}^{2+}$ .

### Effect of Specific Histamine Receptor Subtype Agonists on $[\text{Ca}^{2+}]_i$

We previously found that all four histamine receptor subtypes,  $\text{H}_1$ – $\text{H}_4$ , were present in human and rat conjunctival goblet cells and agonists for each receptor subtype stimulated rat goblet cell secretion.<sup>4</sup> In the present experiments on rat conjunctival goblet cells in culture, agonists of each histamine receptor subtype significantly increased intracellular  $[\text{Ca}^{2+}]_i$  in a concentration-dependent manner (Figs. 7A–D).

As  $\text{H}_2$  receptors have been shown to increase cAMP, we determined if the rise in  $\text{Ca}^{2+}$  stimulated by the  $\text{H}_2$  receptor agonist amthamine was inhibited by H89, a specific inhibitor of cAMP-dependent protein kinase, known as protein kinase A (PKA). Rat goblet cells were pre-incubated for 30 minutes with H89 at  $10^{-6}$  and  $10^{-5}$  M. Amthamine ( $10^{-5}$  M) or the positive control VIP ( $10^{-7}$  M) was then added and  $[\text{Ca}^{2+}]_i$  measured. H89 at both concentrations significantly inhibited amthamine-stimulated increase in  $[\text{Ca}^{2+}]_i$  (Fig. 7E). VIP-stimulated increase in  $[\text{Ca}^{2+}]_i$  was also significantly inhibited by H89 at both concentrations (Fig. 7E).



**FIGURE 3.** Effect of repeated additions of histamine on intracellular  $[Ca^{2+}]_i$  ( $[Ca^{2+}]_i$ ) in rat conjunctival goblet cells. Cultured rat goblet cells were loaded with the  $Ca^{2+}$  indicator dye fura 2. Histamine ( $10^{-5}$  M) was then added at 30 (A), 60 (B), or 120 seconds (C) intervals (indicated by the arrows) and the  $[Ca^{2+}]_i$  measured. Traces shown in left panels are mean of five individual experiments. Peak  $[Ca^{2+}]_i$  was calculated from  $[Ca^{2+}]_i$  prior to histamine addition and shown as mean  $\pm$  SEM in right panels. #Indicates statistical significance from first histamine addition.

### Effect of Specific Histamine Receptor Subtype Antagonists on Histamine-Stimulated Increase in $[Ca^{2+}]_i$

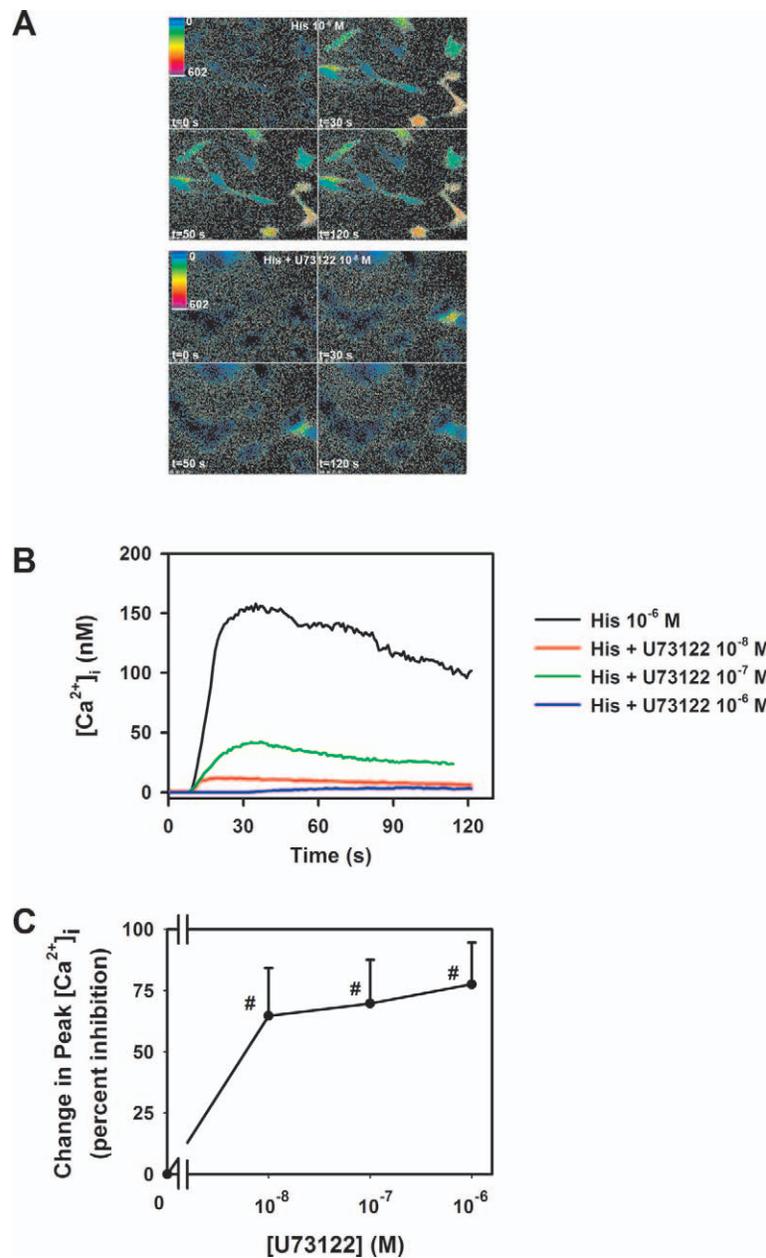
Similarly to the agonists, antagonists of all four histamine receptor subtypes significantly blocked the histamine-stimulated increase in  $[Ca^{2+}]_i$  in a concentration-dependent manner (Figs. 8A–D). A maximum inhibition of  $86.8\% \pm 4.8\%$  was obtained at  $10^{-5}$  M chlorpheniramine the  $H_1$  antagonist, of  $83.3\% \pm 3.3\%$  by  $10^{-6}$  M of cimetidine the  $H_2$  antagonist, of  $97.3\% \pm 0.4\%$  by  $10^{-5}$  M of conessine the  $H_3$  antagonist, and of  $83.1\% \pm 6.3\%$  by  $10^{-6}$  M of JNJ777120 the  $H_4$  antagonist. As the antagonists could have non-specific effects we determined the effect of each histamine receptor subtype antagonist on its respective agonist. The  $H_1$  antagonist chlorpheniramine at  $10^{-5}$  M inhibited the  $Ca^{2+}_i$  response to the  $H_1$  agonist histamine dimaleate ( $10^{-6}$  M) by  $52.9\% \pm 10.7\%$  (Fig. 8E). The  $H_2$  antagonist cimetidine at  $10^{-6}$  M inhibited the  $Ca^{2+}_i$  response to the  $H_2$  agonist amthamine ( $10^{-5}$  M) by  $19.3\% \pm 8.5\%$  (Fig. 8E). The  $H_4$  antagonist JNJ777120 at  $10^{-6}$  M inhibited the  $Ca^{2+}_i$  response to the  $H_4$  agonist 4-methylhistamine ( $10^{-4}$  M) by  $39.2\% \pm 16.5\%$  (Fig. 8E).

Since the effect of the  $H_3$  agonist increased substantially between  $10^{-6}$  and  $10^{-5}$  M, we tested the effect of the  $H_3$  antagonist conessine on  $\alpha$ -methylhistamine at  $10^{-7}$ – $10^{-5}$  M. The  $H_3$  antagonist conessine at  $10^{-6}$  M inhibited the  $Ca^{2+}_i$  response to the  $H_3$  agonist by a maximum  $55.3\% \pm 19.1\%$  at  $10^{-7}$  M  $\alpha$ -methylhistamine (Fig. 8F). The effect of the  $H_3$  antagonist did not differ with the concentration of  $H_3$  agonist used.

Although subtype receptor antagonists were not as effective on subtype receptor antagonists as on histamine, these data taken together suggest that activation of each of the four histamine receptor subtypes is a potent and effective stimulus of intracellular  $Ca^{2+}$ . In conjunctival goblet cells histamine could activate all four histamine receptor subtypes to increase intracellular  $Ca^{2+}$ .

### DISCUSSION

In the present study, we found that an increase in intracellular  $Ca^{2+}$  and activation of ERK1/2 is a major mechanism by which histamine stimulates conjunctival goblet cell secretion of MUC5AC and other high molecular weight glycoproteins.



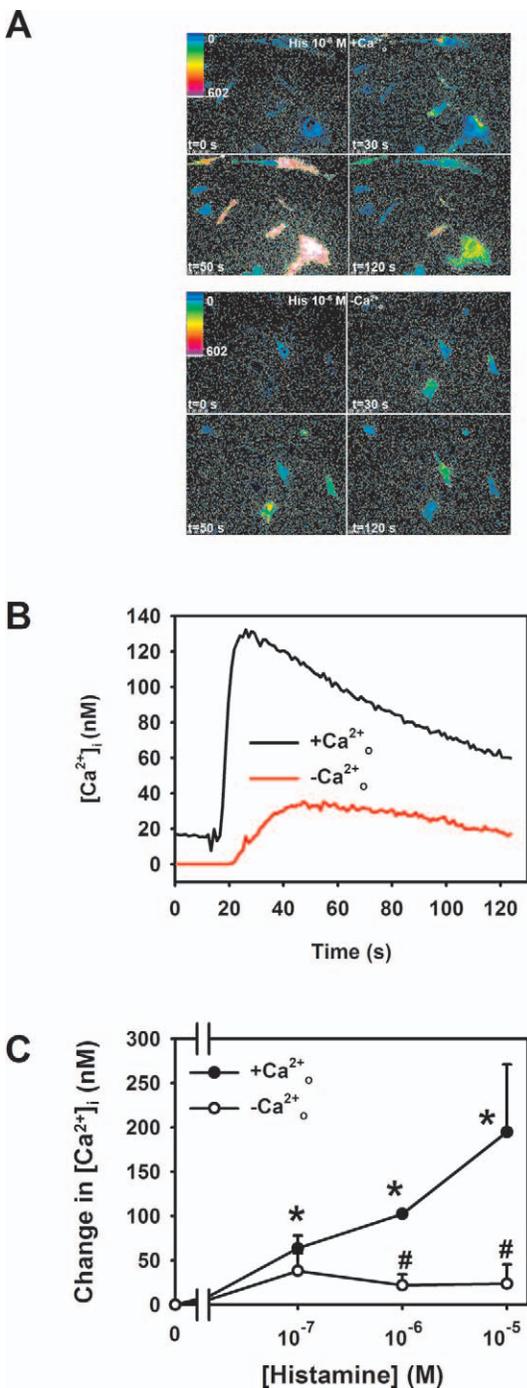
**FIGURE 4.** Effect of a phospholipase C inhibitor on intracellular  $[Ca^{2+}]_i$  stimulated by histamine in rat conjunctival goblet cells. Cultured rat goblet cells were loaded with the  $Ca^{2+}$  indicator dye fura 2 and pre-incubated for 30 minutes with the phospholipase C inhibitor U73122. The  $[Ca^{2+}]_i$  was visualized in single goblet cells by pseudocolor as indicated in (A). The *top four panels* indicate the  $[Ca^{2+}]_i$  response to histamine (His,  $10^{-6}$  M) at 0, 30, 50, and 120 seconds. The *bottom four panels* indicate the  $[Ca^{2+}]_i$  response to histamine ( $10^{-6}$  M) at 0, 30, 50, and 120 seconds after pre-incubation with U73122 ( $10^{-8}$  M). Traces shown in (B) are mean of four individual experiments. The percent inhibition of peak  $[Ca^{2+}]_i$  was calculated and shown as mean  $\pm$  SEM in (C). #Indicates statistical significance from histamine alone.

Furthermore, we demonstrated that histamine functions as a typical GPCR as it desensitizes, activates phospholipase C, releases  $Ca^{2+}$  from intracellular stores, and stimulates influx of extracellular  $Ca^{2+}$ . Histamine also activates ERK1/2. These effects of histamine are in common with all stimuli of conjunctival goblet cell secretion studied to date.<sup>7,11</sup> We also discovered that histamine activates all four histamine receptor subtypes to increase the  $[Ca^{2+}]_i$ .

Histamine also stimulates secretion from goblet cells located in other tissues including nasal epithelium, intestine, colon, and airways.<sup>15-20</sup> However, there is contradictory evidence in colon, airway, and pancreatic duct goblet cells whether histamine increases  $[Ca^{2+}]_i$ .<sup>21-23</sup> Unlike the conjunc-

tival goblet cells, the cellular mechanisms used by histamine to increase  $[Ca^{2+}]_i$  in the goblet cells from these nonconjunctival tissues has yet to be published.

The role  $[Ca^{2+}]_i$  in the histamine-stimulated response has been investigated in nongoblet cells.<sup>24</sup> In human conjunctival epithelial cells, which are likely to be stratified squamous cells, histamine activates phospholipase C and increases  $[Ca^{2+}]_i$ .<sup>25</sup> In another cell type, astrocytoma cells, histamine increases  $[Ca^{2+}]_i$ .<sup>26</sup> However, the increase in  $[Ca^{2+}]_i$  in most tissues, depends upon the histamine receptor subtype. All four histamine receptor subtypes are present in conjunctival goblet cells<sup>4</sup> and activation of each subtype increases  $[Ca^{2+}]_i$  (present study) and stimulates secretion (although H<sub>2</sub> and H<sub>3</sub> receptors

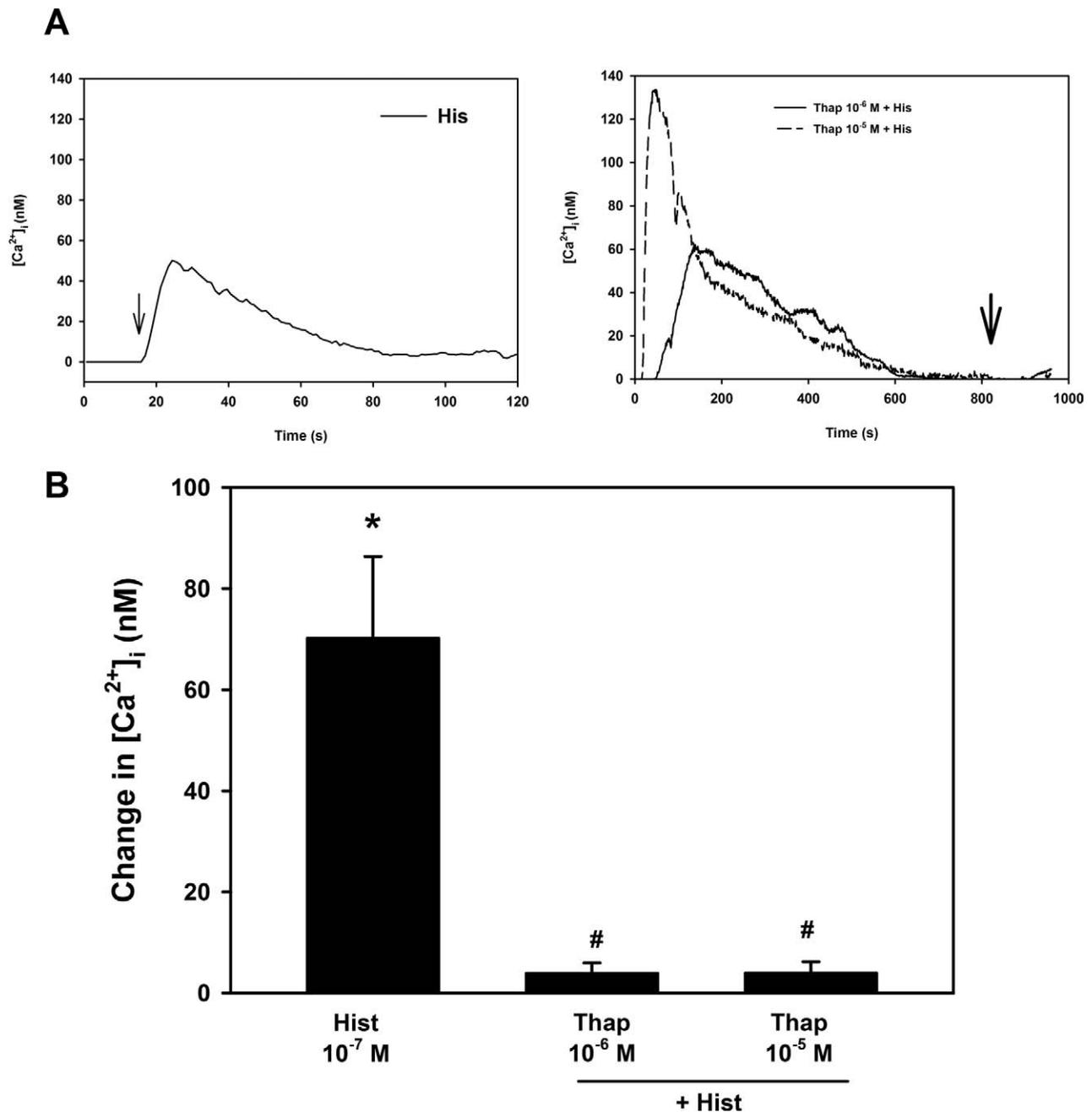


**FIGURE 5.** Effect of removal of extracellular  $Ca^{2+}$  on histamine-stimulated increase in intracellular  $[Ca^{2+}]_i$  in rat conjunctival goblet cells. Cultured rat goblet cells were loaded with the  $Ca^{2+}$  indicator dye fura 2 and incubated in the presence and absence of extracellular  $Ca^{2+}$  ( $Ca^{2+}_o$ ). Cells were then stimulated with histamine ( $10^{-7}$ ,  $10^{-6}$ , and  $10^{-5}$  M). The  $[Ca^{2+}]_i$  was indicated in single goblet cells by pseudocolor as indicated in (A). The top four panels indicate the  $[Ca^{2+}]_i$  response to histamine ( $10^{-6}$  M) at 0, 30, 50, and 120 seconds in the presence of extracellular  $Ca^{2+}$ . The bottom four panels indicate the  $[Ca^{2+}]_i$  response to histamine ( $10^{-6}$  M) at 0, 30, 50, and 120 seconds in the absence of extracellular  $Ca^{2+}$ . Traces shown in (B) represent the  $[Ca^{2+}]_i$  induced by histamine ( $10^{-6}$  M) in the presence and absence of extracellular  $Ca^{2+}$  and are the mean of three individual experiments. Peak  $[Ca^{2+}]_i$  was calculated and shown as mean  $\pm$  SEM in (C). \*Indicates statistical significance from no addition (0); #indicates statistical significance from histamine in the presence of extracellular  $Ca^{2+}$ .

are more effective than  $H_1$  and  $H_4$ ).<sup>4</sup> Furthermore, inhibition of histamine activation of each receptor subtype by antagonists decreases  $[Ca^{2+}]_i$  (present study) and blocks secretion (except for  $H_4$ ).<sup>4</sup> These results were unexpected as not all histamine receptor subtypes are known to increase  $[Ca^{2+}]_i$ . When histamine receptor subtypes were studied in conjunctival epithelial cells, unlike the conjunctival goblet cells of the present study, histamine stimulation of  $[Ca^{2+}]_i$  did not desensitize, and was blocked by  $H_1$ , but not by  $H_2$  or  $H_3$  antagonists.<sup>25</sup>  $H_4$  antagonists were not available at the time of these experiments. We suggest that both conjunctival cell types (stratified squamous and goblet cell) respond to histamine, but the subtypes of histamine receptors that are active differ. In other cell types activation of histamine  $H_1$  increased  $[Ca^{2+}]_i$  in salivary gland cells, cytotrophoblasts, and purkinje cell neurons, microvascular endothelial cells, and endothelial cells.<sup>27-31</sup> When  $H_4$  receptors were induced in mast cells,  $[Ca^{2+}]_i$  increased.<sup>32</sup> In contrast in cardiac sympathetic nerve endings, activation of  $H_3$  receptors decreased  $[Ca^{2+}]_i$  and  $H_1$  and  $H_4$  receptors increased  $[Ca^{2+}]_i$ .<sup>33</sup> Thus, in most tissues  $H_1$  and  $H_4$  receptors increase  $[Ca^{2+}]_i$ ,  $H_3$  receptors decrease  $[Ca^{2+}]_i$ , and  $H_2$  receptors have not been tested as they are expected to activate adenylate cyclase. In the current study, we showed that in conjunctival goblet cells activation of all four histamine receptor subtypes increases  $[Ca^{2+}]_i$ . Activation of  $H_1$  and  $H_4$  receptors in conjunctival goblet cells is in agreement with the results in other cell types. In contrast, however, activation of  $H_2$  receptors has not been shown to alter  $Ca^{2+}$  and activation of  $H_3$  receptors usually decreases  $[Ca^{2+}]_i$ . In fact, we found herein that the  $H_2$  agonist uses cAMP and PKA to increase in  $[Ca^{2+}]_i$  as the amthamine-stimulated  $Ca^{2+}$  response was blocked by the PKA inhibitor H89. Activation of  $H_3$  receptors usually decreases  $[Ca^{2+}]_i$ , but perhaps when co-expressed with  $H_1$  and  $H_4$  subtypes, as in conjunctival goblet cells, the  $H_3$  receptor subtype is either not linked to an inhibitory pathway or the inhibitory pathway is altered. Conjunctival goblet cells are, thus, unusual in possessing all four histamine receptor subtypes<sup>4</sup> and by all four subtypes having the same effect on  $[Ca^{2+}]_i$  as shown in the present study.

Shown in Figure 9 is a schematic of the possible mechanisms the histamine subtype receptors could use to increase  $[Ca^{2+}]_i$  and activate ERK1/2 to stimulate conjunctival goblet cell secretion. We propose that  $H_1$  receptors are linked to the G protein (G)  $G_{\alpha q}$  that activates phospholipase C to release  $Ca^{2+}$  from intracellular stores and stimulate  $Ca^{2+}$  influx. The increase in  $[Ca^{2+}]_i$  either by itself or by activating ERK1/2 stimulates goblet cell secretion.  $H_2$  receptors, in contrast, are couple to  $G_{\alpha s}$  that activates adenylate cyclase. Activation of this cyclase produces cAMP from adenosine triphosphate (ATP) that activates PKA. PKA perhaps by interacting with the  $IP_3$  receptor releases intracellular  $Ca^{2+}$  and then stimulates  $Ca^{2+}$  influx. The increase in  $[Ca^{2+}]_i$  either by itself or by activating ERK1/2 stimulates goblet cell secretion. We propose that  $H_3$  and  $H_4$  receptors are each coupled to  $G_{\alpha i/o}$ . By some mechanism, perhaps phospholipase C, these receptors increase  $[Ca^{2+}]_i$  that either by itself or by activating ERK1/2 stimulates goblet cell secretion.

When histamine was used as an agonist, each of the four histamine receptor subtype antagonists blocked the increase in  $[Ca^{2+}]_i$  by 83% to 97%. In contrast, when specific subtype agonists, and their respective antagonists, were used inhibition of  $[Ca^{2+}]_i$  was less. We suggest that the antagonists were more effective on histamine itself for two reasons. First, activation of all four histamine receptor subtypes in conjunctival goblet cells converge on increasing  $[Ca^{2+}]_i$ . Second, the selected histamine receptor subtype agonists and antagonists are not completely subtype specific. Thus, a non-specific effect of an



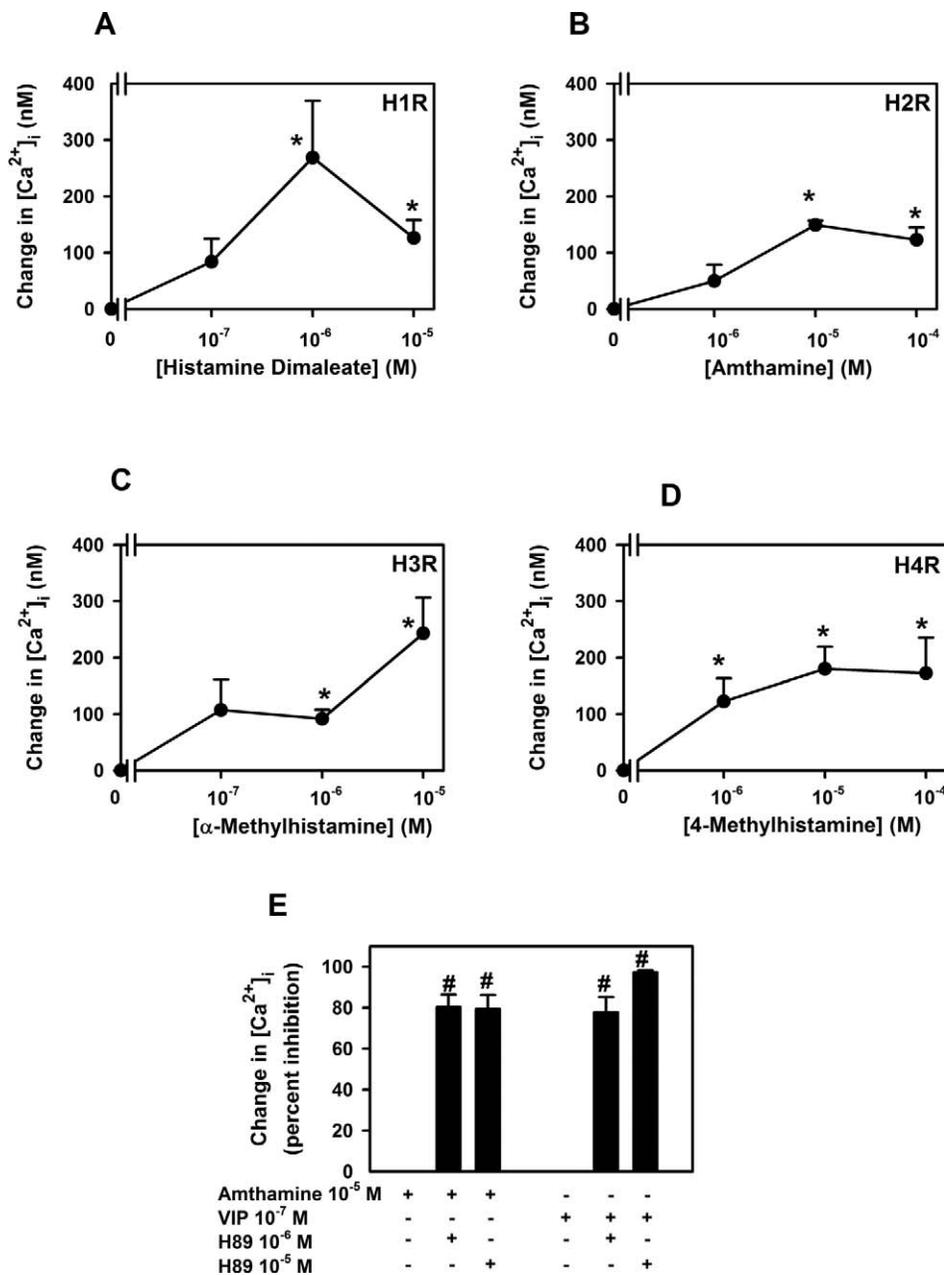
**FIGURE 6.** Effect of pre-incubation with thapsigargin on histamine-stimulated increase in intracellular [Ca<sup>2+</sup>] ([Ca<sup>2+</sup>]<sub>i</sub>) in rat conjunctival goblet cells. Cultured rat goblet cells were loaded with the Ca<sup>2+</sup> indicator dye fura 2 and either stimulated with histamine (His) alone in the presence of Ca<sup>2+</sup><sub>o</sub> or preincubated with thapsigargin and then stimulated with histamine indicated by the *arrow* in the absence of Ca<sup>2+</sup><sub>o</sub>. The [Ca<sup>2+</sup>]<sub>i</sub> was measured. Traces shown in (A) are the mean of three individual experiments. Peak [Ca<sup>2+</sup>]<sub>i</sub> was calculated and shown as mean ± SEM in (B). \*Indicates statistical significance from no addition (0); #indicates statistical significance from histamine alone.

antagonist on a second histamine receptor subtype would cause an additional inhibition of the [Ca<sup>2+</sup>]<sub>i</sub> response to histamine.

Histamine is also known to activate ERK1/2. In vascular smooth muscle cells histamine increases ERK1/2, an effect dependent upon [Ca<sup>2+</sup>]<sub>i</sub>.<sup>34</sup> Histamine also activates ERK1/2 in nerves.<sup>35</sup> Activation of several histamine receptor subtypes activates ERK1/2. In splenocytes, activation of all four histamine receptors increased ERK1/2 activity.<sup>36</sup> H<sub>1</sub> activation increased ERK1/2 phosphorylation in adrenal chromaffin cells as did activation of H<sub>2</sub> in HEK293 cells, and gastric epithelial cells stimulated ERK1/2 activity.<sup>37,38</sup> Finally, stimulation of H<sub>3</sub>

receptors decreases ERK1/2 phosphorylation in cholangiocytes.<sup>39</sup> Although the effect of the different histamine receptor subtypes on ERK1/2 was not tested in conjunctival goblet cells, we would predict that activation of each of the four histamine receptor subtypes would increase ERK1/2 activity as an increase in [Ca<sup>2+</sup>]<sub>i</sub> stimulates ERK1/2 activity.

The evidence that all four histamine receptors function in conjunctival goblet cells is robust as all four receptors are present by PCR, Western blotting analysis, and immunofluorescence microscopy<sup>4</sup>; agonist activation of all four receptors stimulates secretion (although H<sub>2</sub> and H<sub>3</sub> agonists are more effective than H<sub>1</sub> and H<sub>4</sub> agonists) and antagonists of all four

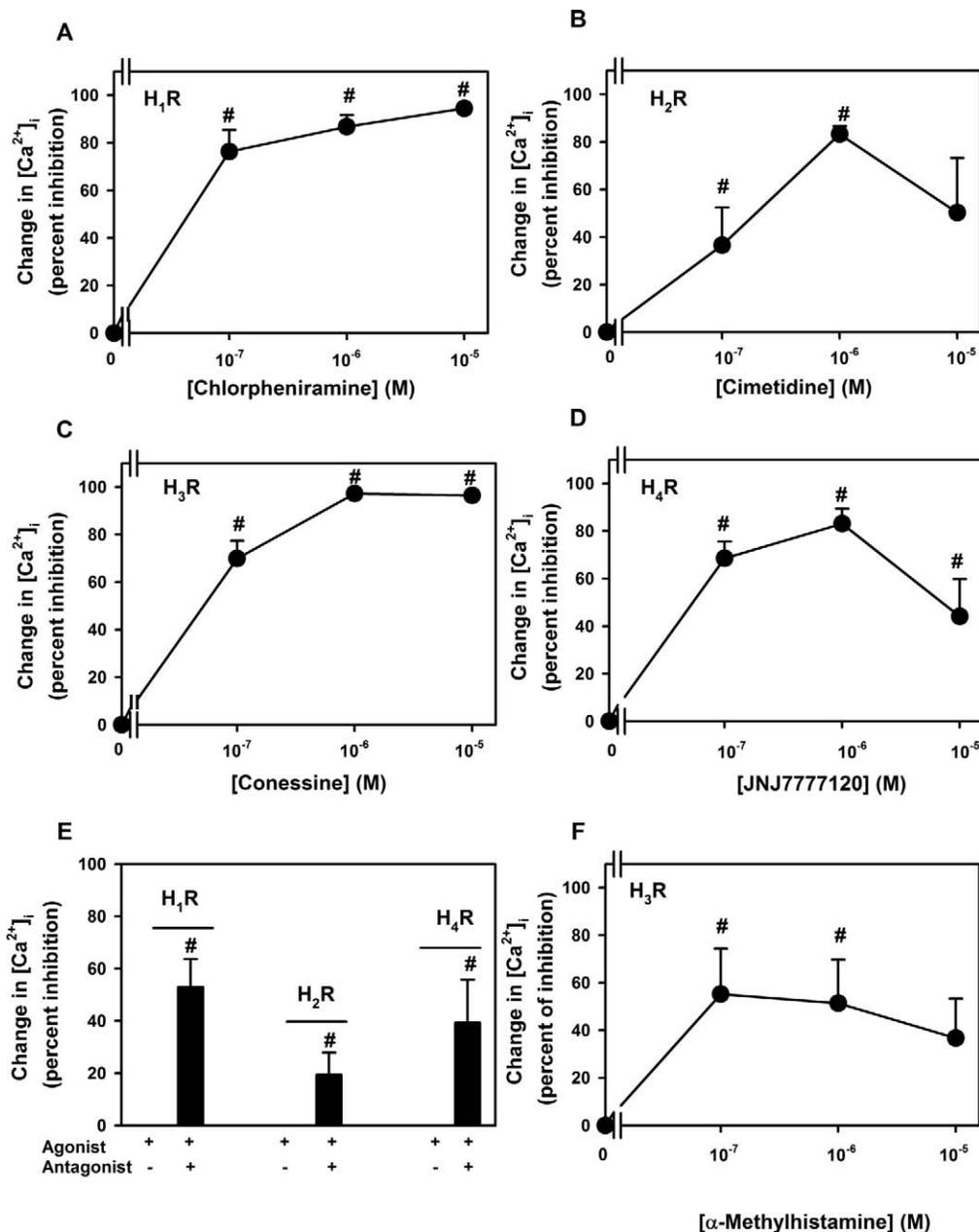


**FIGURE 7.** Effect of histamine receptor subtype agonists on intracellular [Ca<sup>2+</sup>]<sub>i</sub> ([Ca<sup>2+</sup>]<sub>i</sub>) in rat conjunctival goblet cells. Cultured rat goblet cells were loaded with the Ca<sup>2+</sup> indicator dye fura 2 and either stimulated with an H<sub>1</sub> agonist histamine dimaleate (A), an H<sub>2</sub> agonist amthamine (B), an H<sub>3</sub> agonist α-methylhistamine (C), or an H<sub>4</sub> agonist 4-methylhistamine (D). Peak [Ca<sup>2+</sup>]<sub>i</sub> was calculated and shown as mean ± SEM from three independent experiments (A–D). Cultured rat goblet cells were also pre-incubated with H89 (10<sup>-6</sup> and 10<sup>-5</sup> M) for 30 minutes and stimulated with either amthamine (10<sup>-5</sup> M) or VIP (10<sup>-7</sup> M) (E). Mean ± SEM of the percent inhibition shown in (E) is from four independent experiments. \*Indicates statistical significance from no addition (0). #Indicates statistical significance from amthamine alone.

receptors except H<sub>4</sub> block histamine-stimulated secretion<sup>4</sup>; and agonist activation of all four receptor subtypes increases [Ca<sup>2+</sup>]<sub>i</sub>, and antagonists of all four receptor subtypes blocks histamine-induced elevation of [Ca<sup>2+</sup>]<sub>i</sub>, as shown in the present study. Involvement of multiple types of histamine receptors in one type of allergic conjunctivitis was shown in vernal keratoconjunctivitis. Leonardi et al.<sup>40</sup> found that H<sub>1</sub>, H<sub>2</sub>, and H<sub>4</sub>, but not H<sub>3</sub>, receptors were overexpressed in the conjunctiva from patients with vernal keratoconjunctivitis than controls. In addition Leonardi et al.<sup>40</sup> found that cytokines present in allergic conjunctivitis can upregulate H<sub>2</sub>, but not H<sub>4</sub> receptors in conjunctival cells. Thus, the role of the histamine

receptor subtypes may change in the setting of allergic conjunctivitis. Multiple histamine receptor subtypes appear to play a role in at least one type of allergic conjunctivitis.

Since goblet cell mucous secretion functions to protect the surface of the eye and, indeed, the entire eye from a variety of challenges from the external environment, it is not surprising that there are multiple redundant pathways for goblet cell secretion. Our findings with histamine suggest that goblet cell mucous secretion is an important component of the innate immune system that has layers of protective mechanisms designed to keep the eye safe from the environment. In diseases such as allergic conjunctivitis, stimulation of mucous



**FIGURE 8.** Effect of histamine receptor subtype antagonists on intracellular  $[Ca^{2+}]_i$  ( $[Ca^{2+}]_i$ ) stimulated by histamine in rat conjunctival goblet cells. Cultured rat goblet cells were loaded with the  $Ca^{2+}$  indicator dye fura 2 and pre-incubated with an  $H_1$  antagonist chlorpheniramine (A), an  $H_2$  antagonist cimetidine (B), an  $H_3$  antagonist conessine (C), and an  $H_4$  antagonist JNJ7777120 (D), and then stimulated with histamine ( $10^{-6}$  M). Peak  $[Ca^{2+}]_i$  was calculated and shown as mean  $\pm$  SEM of the percent inhibition. Cultured goblet cells were loaded with the  $Ca^{2+}$  indicator dye fura 2 and preincubated with an  $H_1$  antagonist chlorpheniramine ( $10^{-5}$  M) prior to stimulation with an  $H_1$  agonist histamine dimaleate ( $10^{-6}$  M) (E), an  $H_2$  antagonist cimetidine ( $10^{-6}$  M) prior to stimulation with an  $H_2$  agonist amthamine ( $10^{-5}$  M) (E), an  $H_4$  antagonist JNJ7777120 ( $10^{-6}$  M) prior to stimulation with an  $H_4$  agonist 4-methylhistamine ( $10^{-4}$  M) (E), and an  $H_3$  antagonist conessine ( $10^{-5}$  M) prior to stimulation with an  $H_3$  agonist  $\alpha$ -methylhistamine ( $10^{-7}$ ,  $10^{-6}$ , and  $10^{-5}$  M) (F). Peak  $[Ca^{2+}]_i$  was calculated and shown as mean  $\pm$  SEM of the percent inhibition. #Indicates statistical significance from agonist alone.

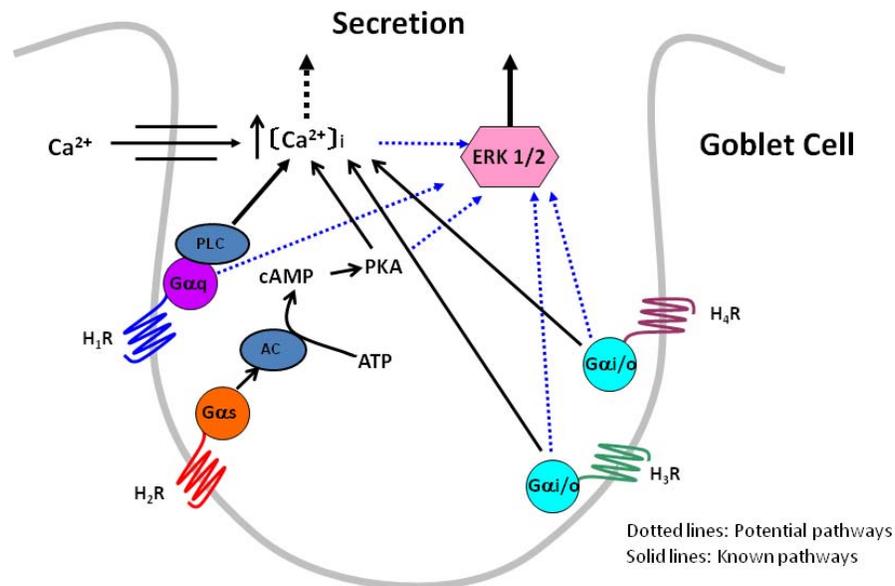
secretion by histamine, and other mediators of the allergic response becomes uncontrolled or excessive. Thus, excess mucous secretion can be a component of the pathology of allergic conjunctivitis. In this case development of antagonists of multiple histamine receptor subtypes as treatments of ocular allergy would be beneficial to normalize conjunctival goblet cell secretion.

We conclude that histamine activates phospholipase C to release intracellular  $Ca^{2+}$  that induces the influx of extracellular  $Ca^{2+}$  and activates ERK1/2 to stimulate conjunctival

goblet cell mucous secretion. Interestingly, activation of all four histamine receptor subtypes increases  $[Ca^{2+}]_i$ , but the  $H_2$  receptor uses cAMP and PKA for this effect.

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**FIGURE 9.** Proposed mechanism of the role of histamine receptor subtypes in goblet cell signaling and secretion. H<sub>1</sub>R to H<sub>4</sub>R, histamine receptor subtypes 1 to 4; Gαq, G protein of the αq subtype; Gαs, G protein of the αs subtype; Gαi/o, G protein of the αi/o subtype; PLC, phospholipase C; AC, adenylate cyclase; PKA, protein kinase A; ERK 1/2, extracellular regulated kinase 1/2.

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