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Resolvin D1 and Aspirin-Triggered Resolvin D1 Regulate Histamine-stimulated Conjunctival Goblet Cell Secretion

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Abstract

Resolution of inflammation is an active process mediated by pro-resolution lipid mediators. Since resolvin (Rv) D1 is produced in the cornea, pro-resolution mediators could be effective in regulating inflammatory responses to histamine in allergic conjunctivitis. Two key mediators of resolution are the D-series resolvins RvD1 or aspirin-triggered RvD1 (AT-RvD1). We used cultured conjunctival goblet cells to determine whether histamine actions can be terminated during allergic responses. We found cross-talk between two types of G protein-coupled receptors, as RvD1 interacts with its receptor GPR32 to block histamine-stimulated H_1 receptor increases in intracellular [Ca^{2+}] ($[Ca^{2+}]_i$) preventing H_1 receptor-mediated responses. In human and rat conjunctival goblet cells RvD1 and AT-RvD1 each block histamine-stimulated secretion by preventing its increase in $[Ca^{2+}]_i$ and activation of extracellular regulated protein kinase (ERK)1/2. We suggest that D-series resolvins regulate histamine responses in the eye and offer new treatment approaches for allergic conjunctivitis or other histamine-dependent pathologies.

INTRODUCTION

Inflammation plays a critical role in many widely occurring diseases and there now is a general consensus that failed endogenous resolution mechanisms can lead to uncontrolled and chronic inflammation[1, 2]. Uncontrolled inflammation is regarded as a critical component of the pathogenesis of two major diseases of the ocular surface, dry eye and allergic conjunctivitis, as well as dermatitis in the skin [3, 4]. Active resolution of the acute inflammatory response is orchestrated by a novel family of anti-inflammatory and pro-resolving mediators termed resolvins, which are a part of a wider genus of pro-resolving mediators [5, 6].

Histamine plays a central role in promoting allergic conjunctivitis [7] and is known to directly stimulate conjunctival goblet cell mucin secretion [8]. Martin et al recently demonstrated that RvD1 regulates histamine release by mast cell degranulation [9]. Herein, we addressed whether RvD1 could regulate the action of histamine on goblet cell secretion and

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Disclosure

CNS is an inventor on patents [resolvins] assigned to BWH and licensed to Resolvyx Pharmaceuticals. CNS is a scientific founder of Resolvyx Pharmaceuticals and owns equity in the company. CNS' interests were reviewed and are managed by the Brigham and Women's Hospital and Partners HealthCare in accordance with their conflict of interest policies.

characterized the histamine response by these cells. Recently, we found that resolvins of the D and E series (RvD1 and RvE1) regulate leukotriene-stimulated conjunctival goblet cell mucin secretion as can occur in the setting of allergic conjunctivitis[10]. Here we report that RvD1 interacts with its receptor GPR32 to block histamine-stimulated responses of conjunctival goblet cells that include increase in $[Ca^{2+}]_i$, activation of extracellular regulated kinase (ERK)1/2, and secretion of high molecular weight glycoproteins including MUC5AC mucin.

RESULTS

Histamine-stimulated increase in $[Ca^{2+}]_i$ in cultured human and rat conjunctival goblet cells was inhibited by resolvins

First, we determined if histamine alters $[Ca^{2+}]_i$ using the intracellular Ca^{2+} probe fura-2 in cultured human conjunctival goblet cells. Histamine increased $[Ca^{2+}]_i$ in a concentration-dependent manner with a maximum increase obtained at 10^{-5} M (Figure 1 A and B). We used RvD1 and its epimer AT-RvD1 that is produced in the presence of aspirin [5, 11]. In goblet cells exposure to either RvD1 or AT-RvD1 (10^{-10}-10^{-8} M) blocked histamine stimulated increases in $[Ca^{2+}]_i$ that were reduced by a maximum of 72.3 ± 24.2 and52.6 ± 23.%, respectively (Figure 1C and D). In rat goblet cells, histamine also increased $[Ca^{2+}]_i$ with maximum levels at 10^{-6} M a decreased concentration of histamine compared to that which was maximum for human cells (Figure 1E and F). At concentrations as low as 10^{-11} M, both RvD1 and AT-RvD1 significantly blocked the histamine stimulated increases in $[Ca^{2+}]_i$ (Figure 1G and H). A maximum inhibition of secretion of 82.3 ± 0.3, and 83.9 ± 6.4%. was obtained by RvD1 and AT-RvD1, respectively (Figure 1G and H).

Resolvins block histamine-stimulated increase in $[Ca^{2+}]_i$ when added before but not after histamine

To examine the time-dependency of their effects, RvD1 and AT-RvD1 were each added simultaneously with histamine or 50 sec after histamine at the peak of $[Ca^{2+}]_i$ response (Figure 2A – D). When histamine and RvD1 or AT-RvD1 were added simultaneously, the peak $[Ca^{2+}]_i$ was significantly decreased compared to histamine alone (Figure 2A – D). In contrast, when RvD1 or AT-RvD1 were added at the peak of the histamine response, the $[Ca^{2+}]_i$ was not altered when the change in $[Ca^{2+}]_i$ (Figure 2A – D) was monitored or when the slope was calculated (data not shown). Both D-series resolvins when added before (Figure 1D and H) or together (Figure 2A – D) with histamine blocked the increase in $[Ca^{2+}]_i$ stimulated by histamine. However, these resolvins could not alter the histamine Ca^{2+} response if they were added within a minute after histamine had initiated the release of $Ca^{2+}{}_i$.

Resolvins and histamine receptors utilize the same intracellular Ca^{2+} pools

G protein-coupled receptors (GPCR) including histamine receptors release Ca^{2+} from intracellular capacitative Ca^{2+} stores located in the endoplasmic reticulum (ER) [12, 13]. Ca^{2+} release in turn activates Ca^{2+} influx from extracellular stores. The intracellular Ca^{2+} stores are refilled by Ca^{2+} ATPase pumping Ca^{2+} back into the ER. As thapsigargin depletes the intracellular Ca^{2+} store by blocking the Ca^{2+} ATPase, thapsigargin can be used to evaluate use of this store [14]. To determine if resolvins decrease $[Ca^{2+}]_i$ by preventing the release of this store, we measured the effect of resolvins on thapsigargin-induced $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+}. Histamine alone and thapsagargin alone increased $[Ca^{2+}]_i$ by 205.7 ± 48.7 and 146.8 ± 70.1 nM, respectively (Figure 2E). When RvD1 or AT-RvD1 was added before thapsigargin, the thapsigargin induced increase in $[Ca^{2+}]_i$ was not altered (Figure 2E). When the thapsigargin response was followed by addition of histamine, the histamine response was significantly decreased compared to histamine alone. When RvD1

or AT-RvD1 were followed by thapsagargin and then histamine, the histamine response was unchanged compared to the histamine response that followed the thapsagargin response. (Figure 2E). In support of this finding, we demonstrated earlier that after removal of extracellular Ca^{2+} a small increase in $[Ca^{2+}]_i$ remained after histamine 10^{-5} M stimulation.[15, 16] These results suggest that RvD1 and AT-RvD1 block the histamine-stimulated increase in $[Ca^{2+}]_i$ by either directly altering the histamine receptor or affecting a step before the release of intracellular Ca^{2+}.

Histamine stimulated increase in ERK 1/2 activity in cultured rat conjunctival goblet cells was inhibited by resolvins

We next investigated if histamine affects extracellular regulated kinase (ERK) 1/2 activity. Rat conjunctival goblet cells were stimulated with histamine and ERK1/2 activity measured by western blotting analysis. Histamine increased ERK1/2 activity in a concentration dependent manner with a maximum at 10^{-6} M (Figure 3A). Histamine also activated ERK1/2 in time-dependent manner with a maximum stimulation at 5 min (Figure 3B). Hence, at physiologic levels histamine activates ERK1/2 along with elevating $[Ca^{2+}]_i$.

To determine if D-series resolvins block histamine stimulation of ERK1/2 activity, we exposed rat goblet cells to resolvins for 30 min before stimulation with histamine at 10^{-5} M for 5 min. Both RvD1 and AT-RvD1 significantly inhibited histamine stimulated ERK1/2 activity with RvD1 inhibiting by a maximum of 94.0 ± 7.7 and AT-RvD1 by 81.9 ± 20.9% (Figure 3C).

Resolvins inhibit histamine-stimulated mucin secretion from cultured rat and human goblet cells

When goblet cell secretion was investigated, RvD1 or AT-RvD1 exposure completely blocked secretion stimulated by histamine (10^{-5} M) in human goblet cells and rat goblet cells at two concentrations of histamine (10^{-5} and 10^{-6} M, Figure 4A, B and C). RvD1 and AT-RvD1 blocked histamine-stimulated secretion a maximum of 78.1 ± 9.0 and 90.3 ± 18.6%, respectively in human goblet cells and 76.9 ± 15.1 and 83.0 ± 9.3% respectively in rat goblet cells. RvD1 and AT-RvD1 blocked histamine-stimulated increase in $[Ca^{2+}]_i$, ERK1/2 activity, and high molecular weight glycoconjugate including MUC5AC secretion in both human and rat conjunctival goblet cells.

Resolvins increase mucin secretion in cultured human and rat conjunctival goblet cells, $[Ca^{2+}]_i$, and ERK1/2 in rat conjunctival goblet cells

RvD1 activates its receptor GPR32 [11], which is present in human and rat conjunctival goblet cells [10], and also can activate the lipoxin A_4 receptor (ALX/FPR2) [17]. We tested whether RvD1 and AT-RvD1 themselves alter secretion, $[Ca^{2+}]_i$, and ERK1/2 in conjunctival goblet cells. RvD1 and AT-RvD1 each increased goblet cell secretion in both human (Figure 5A) and rat cells (Figure 5C). A maximum action was recorded at 10^{-9} M RvD1 and AT-RvD1 in human cells and 10^{-9} M RvD1 and 10^{-10} M AT-RvD1 in rat cells. The secretory effect of the D-series resolvins was significantly less than that stimulated by histamine at 10^{-5} M which was 2.2 ± 0.2 fold above basal (not shown). RvD1 and AT-RvD1 caused a maximum stimulation at 1 h in both human and rat cells, which was shorter than histamine, which caused its maximum response at 2 h of stimulation [16] (Figure 5B and D).

RvD1 and AT-RvD1 increased the peak $[Ca^{2+}]_i$ in a concentration-dependent manner (Figure 6A and B) with a maximum increase obtained with 10^{-9} M RvD1 and 10^{-8} M AT-RvD1. In the same experiments histamine increased $[Ca^{2+}]_i$ to a higher level elevating it by 220.0 ± 49.0 nM. To determine the cellular Ca^{2+} pool used by RvD1 and AT-RvD1, extracellular Ca^{2+} was omitted before stimulation with the resolvins. In the absence of

extracellular Ca^{2+}, RvD1 stimulated increase in $[Ca^{2+}]_i$ was partially decreased, but the increase stimulated by AT-RvD1 was completely blocked (Figure 6C). The role of the intracellular capacitative Ca^{2+} pool was investigated using thapsigargin in the absence and presence of extracellular Ca^{2+}. Independent of the presence of extracellular Ca^{2+}, when thapsigargin was added first, the increase in $[Ca^{2+}]_i$ by the subsequent addition of RvD1 was not altered (Figure 6D). When RvD1 was added first, the thapsigargin-induced Ca^{2+} response was also not altered. Similar results were obtained when AT-RvD1 was used (Figure 6E). Thus neither RvD1 nor AT-RvD1 appear to use the IP_3 sensitive capacitative Ca^{2+} pool.

As RvD1 and AT-RVD1 can also activate the ALX/FPR2 receptor[11], we determined the role that the ALX receptor plays in RvD1 and AT-RvD1 stimulated $[Ca^{2+}]_i$ response in rat conjunctival goblet cells. In cells in which the ALX/FPR2 receptor was knocked down with siRNA, the $[Ca^{2+}]_i$ over time in response to RvD1 (Figure 6F), AT-RvD1 (Figure 6G), or, as a control lipoxin A_4 (data not shown) was abolished. Peak $[Ca^{2+}]_i$ response was decreased significantly decreased with all three compounds (Figure 6H). The $[Ca^{2+}]_i$ response was unchanged when cells were incubated with either a scrambled siRNA or the transfection reagent alone (Figure 6F–H). In rat goblet cells, RvD1 and AT-RvD1 each activate the ALX/FPR2 receptor.

When ERK1/2 activity was studied, both RvD1 and AT-RvD1 stimulated ERK1/2 activity in a concentration- and time-dependent manner. A maximum increase in ERK1/2 activity was not reached even at 10^{-8} M RvD1 or AT-RvD1 (Figure 7A and B). A maximum increase in time was detected at 20 min of resolvin stimulation. The resolvins were slower at activating ERK1/2 than histamine whose maximum stimulation was obtained at 5 min (Figure 7C and D). Both RvD1 and AT-RvD1 themselves caused goblet cell secretion, elevation in $[Ca^{2+}]_i$, and increase in ERK1/2 activity.

H1 histamine and DRV1-GPR32 receptors are present and co-localize in cultured human conjunctival goblet cells

As all four histamine receptors (H_1–H_4) are activated by histamine in conjunctival goblet cells [8], we focused on the H_1 receptor to determine if RvD1 uses its receptor GPR32 to alter the action of histamine. Using fluorescence microscopy, we examined the location of the histamine H_1 receptors and the DRV1-GPR32 receptor in human goblet cells. Both receptors displayed a punctate pattern of localization (Figure 8A and B). They were detected in the same cells and their immunofluorescence location overlapped (Figure 8C).

DRV1-GPR32 using protein kinase C and GRK 2, blocks the histamine-stimulated increase in $[Ca^{2+}]_i$

To determine if RvD1 used its receptor GPR32 to block histamine stimulated $[Ca^{2+}]_i$, we transfected CHO cells with DRV1-GPR32 alone, the H_1 histamine receptor alone, both receptors, or mock transfection. Cells were stimulated with histamine, RvD1, or RvD1 followed by histamine. When both the H_1 and GPR32 receptors were transfected, histamine increased $[Ca^{2+}]_i$, (Figure 9A) and RvD1 blocked histamine stimulation. RvD1 did not alter $[Ca^{2+}]_i$. When only the H_1 receptor was transfected, histamine increased $[Ca^{2+}]_i$ and prior exposure to RvD1 did not alter this response (Figure 9B). When only the GPR32 receptor was transfected, no combination of histamine or RvD1 increased the $[Ca^{2+}]_i$ (Figure 9C). In mock transfected cells neither histamine nor RvD1 increased $[Ca^{2+}]_i$ and the RvD1 treatment did not alter the histamine response (Figure 9D). Together these results suggest that RvD1 uses its receptor DRV1-GPR32 to block the effect of histamine on $[Ca^{2+}]_i$. As a control, cells were also stimulated with RvE1 or RvE1 followed by histamine in CHO cells transfected with both H1 receptor and DRV1-GPR32. RvE1 alone had no effect on $[Ca^{2+}]_i$

and RvE1 followed by histamine did not have an effect on histamine-stimulated $[Ca^{2+}]_i$ (Figure 9E). Results using transfected CHO cells suggested that RvD1 and AT-RvD1 blocked the histamine-stimulated response at a step before the release of intracellular Ca^{2+} and thus a potential mechanism of action for RvD1 is to counterregulate the histamine receptor. Examination of the phosphorylation sites of the H_1 receptor using Scan Site (http://scansite.mit.edu/motifscan_id.phtml) and the kinases that phosphorylate the H_1 receptor using Phospho.ELM (http://phospho.elm.eu.org/pELMBlastSearch.html) indicates potential PKC and βARK1 (also known as GRK2) phosphorylation sites. CHO cells transfected with the human H_1 and GPR32 receptors were stimulated with histamine; RvD1 followed by histamine; or the PKC inhibitor calphostin C (Figure 10A) or the βARK1inhibitory peptide (Figure 10B) followed either by histamine or RvD1 and then histamine. RvD1 blocked the histamine-induced Ca^{2+} response. Calphostin C and βARK1 both reversed the inhibitory effect of RvD1 on the histamine $Ca^{2+}{}_i$ response. Neither calphostin C nor βARK1 alone blocked the histamine receptor response.

In rat goblet cells, both the PKC inhibitor Ro-317549 (Figure 10C) and βARK1 inhibitory peptide (Figure 10D) blocked the RvD1 inhibition of histamine dimaleate, a specific agonist for the H1 histamine receptor.

These data indicate that RvD1 uses PKC and βARK1 to counterregulate the H_1 receptor.

Resolvins inhibit histamine-stimulated increase in $[Ca^{2+}]_i$ to the same extent as H1 receptor inhibitor

The inhibitory actions of both RvD1 and AT-RvD1 on the histamine-induced increase in $[Ca^{2+}]_i$ in cultured conjunctival goblet cells was compared with that of the H_1 antagonist chlorpheniramine (Figure 11A). Maximally effective concentrations of the three inhibitors each blocked the histamine-stimulated increase in $[Ca^{2+}]_i$ to essentially the same extent (Figure 11B).

DISCUSSION

Our results demonstrate that histamine uses its receptors to increase $[Ca^{2+}]_i$, activate ERK1/2, and stimulate secretion. RvD1 blocks the effects of histamine by interacting with its receptor GPR32 to counterregulate the histamine receptor to prevent the release of intracellular Ca^{2+} and the activation of ERK1/2 thus inhibiting secretion. RvD1 must be added before histamine in order to prevent the release of the intracellular Ca^{2+} store. AT-RvD1 has similar effects in conjunctival goblet cells as RvD1. Previous studies have demonstrated that RvD1 and AT-RvD1 block inflammatory processes in a variety of tissues, but the cellular mechanism of this inhibition has not been demonstrated. Here we show that these resolvins prevent the increase in Ca^{2+} and activation of ERK1/2 used by histamine and its H_1 receptor subtype to induce goblet cell secretion by activating PKC and βARK1 to counterregulate the histamine receptor. RvD1 is known to interact with its receptor DRV1-GPR32. Here, we show that there is cross-talk between human GPR32 and the H_1 receptor. Use of GPR32 is required for the inhibitory actions of RvD1 on the signaling pathway activated by another GPCR the H_1 receptor activated by histamine. In rat goblet cells, use of the ALX/FPR2 receptor by RvD1 also activates PKC and βARK1 to counter regulate the H1 receptor.

As Ca^{2+} is major mechanism by which histamine evokes it's response, the actions of the D-series resolvins RvD1 and AT-RvD1 on blocking the action of histamine could prevent allergic or inflammatory responses in any tissue in which histamine receptors are located. These tissues include the lung, skin, immune cells, smooth muscle, blood vessels, glands, and nerves.

The actions of the D-series resolvins on goblet cell functions are complex as a short-term incubation with the resolvins results in significant activation of ERK 1/2. This activity is short lived however, as 30 min after addition of the resolvins the ERK 1/2 activity has returned to basal levels. In this study, goblet cells were exposed to D-series resolvins for 30 min prior to addition of histamine. Thus resolvins counterregulate the histamine receptors to prevent activation of ERK 1/2.

E-series and D-series resolvins are effective in blocking different types of inflammatory responses in the ocular surface. The E-series resolvin RvE1 can reverse inflammation in the cornea by blocking corneal hemangiogenesis activated in a suture or pellet implanted into the mouse cornea [18]. RvE1 induces corneal epithelial cell migration and attenuates herpes simplex induced ocular inflammation, as well as corneal epithelial barrier disruption and goblet cell loss in a murine model of dry eye [19, 2021]. RvE1 also stimulates tear production and decreases inflammation in a mouse model of dry eye [22] and in humans in phase 1 and 2 clinical trial reduces the signs and symptoms of dry eye syndrome (ClinicalTrials.gov Identifier NCT00799552). The D-series resolvin RvD1 blocks corneal hemangiogenesis [18] and previously we demonstrated that RvD1 blocks cholinergic agonist and leukotriene stimulation of conjunctival goblet cell secretion. The present results demonstrate that RvD1 blocks histamine responses and provides a novel function for resolvins in termination of allergic inflammation.

Our findings demonstrate a new use for resolvins as endogenous mediators that could be used to prevent the actions of histamine to treat allergic responses including allergic conjunctivitis in both the mild and severe forms. In the eye, nose, and lung, steroids are the mainstay of regulating inflammation, but their use carries the major side effect of ocular hypertension and glaucoma [23, 24]. Resolvins are not immunosuppressant, unlike steroids or other anti-inflammatory agents currently used in the eye. Resolvin D1 control of histamine stimulated responses could represent a novel approach with local anti-inflammatory and pro-resolution mediators to treat allergic conjunctivitis and allergies in other susceptible tissues such as lung and skin.

METHODS

Animals

Male Sprague-Dawley rats (Taconic Farms, Germantown, NY) weighing between 125 and 150 g were anesthetized with CO_2 for 1 min, decapitated, and the bulbar and forniceal conjunctival membranes removed from both eyes. All experiments were approved by the Schepens Eye Research Institute Animal Care and Use Committee.

Human Material

Human conjunctival tissue was obtained from Heartland Lions Eye Bank (Kansas City, MO). Tissue was placed in Optisol media within 6 hours of death.

Cell Culture

Goblet cells from rat and human conjunctiva were grown in organ culture as described previously [25, 26]. The tissue plug was removed after nodules of cells were observed. First passage goblet cells were used in all experiments. Cultured cells were periodically checked by evaluating staining with antibody to cytokeratin 7 (detects goblet cell bodies) and the lectin Ulex europaeus agglutinin (UEA)-1 (detects goblet cell secretory product) to ensure that goblet cells predominated.

Measurement of $[Ca^{2+}]_i$

Goblet cells were incubated for 1 h at 37 °C with Krebs-Ringer bicarbonate buffer with HEPES (KRB-HEPES) (119 mM NaCl, 4.8 mM KCl, 1.0 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM HEPES, and 5.5 mM glucose (pH 7.45) plus 0.5% BSA containing 0.5 μM fura-2/AM (Invitrogen, Grand Island, NY), 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 selected in each experimental condition, and experiments were repeated in at least 3 separate animals. RvD1 or AT-RvD1 (Cayman Chemical, Ann Arbor, MI) were added 30 min before histamine (Sigma Aldrich, St. Louis, MO). Calphostin C, Ro-317549, β-adrenergic receptor kinase 1 (βARK1) inhibitor, and H89 (EMD Millipore, Billerica, MA) were added 15 min prior to addition of RvD1. After addition of agonists data were collected in real time. Data are presented as the actual $[Ca^{2+}]_i$ with time or as the change in peak $[Ca^{2+}]_i$. Change in peak $[Ca^{2+}]_i$ was calculated by subtracting the average of the basal value (no added agonist) from the peak $[Ca^{2+}]_i$. Although data is not shown, the plateau $[Ca^{2+}]_i$ was affected similarly to the peak $[Ca^{2+}]_i$.

Synthetic resolvins at 10 μg/μl (dissolved in ethanol as purchased from the manufacturer) were stored at −80 °C with minimal exposure to light. Immediately prior to use, the resolvins were diluted in KRB-HEPES buffer to the desired concentrations and added to the cells. The cells were then incubated at 37 °C in the dark. Daily working stock dilutions were discarded following each experiment. Concentrations were confirmed and the resolvin structures validated using LC-MS-MS and were consistent with reported characteristics [27].

Western blotting

Cultured goblet cells were incubated with increasing concentrations of histamine, RvD1, or AT-RvD1. Cells were also incubated with histamine 10^{-5} M for 0–10 min or RvD1 (10^{-9} M) or AT-RvD1 (10^{-9} M) for 0–30 min. In additional experiments, cells were preincubated with increasing concentrations of RvD1 or AT-RvD1 for 30 min prior to addition of histamine (10^{-5} M). Cells were lysed in RIPA buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, and 1 mM EDTA) in the presence of a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The homogenate was centrifuged at 2000g for 30 min at 4°C. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and processed for western blotting. Primary antibodies used were phosphorylated (active) ERK 1/2 or total ERK 2 (Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:1000. Immunoreactive bands were visualized by the enhanced chemiluminescence method. The films were analyzed with Image J software (http://rsbweb.nih.gov/ij/). ERK activation was expressed as fold increase over basal that was set to 1.

Secretion

Cultured goblet cells were serum starved for 2 h before use, preincubated with RvD1 or AT-RvD1 for 30 min, and then stimulated with histamine in serum-free RPMI 1640 supplemented with 0.5% BSA for 0–4 h. 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 as described earlier [10]. Glycoconjugate secretion was expressed as fold increase over basal that was set to 1.

Immunofluorescence Microscopy

First passage cells were grown on glass cover slips and fixed in methanol. Both anti-GPR32 (Gene Tex, Irvine, CA) and anti-H_1 receptor antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used at 1:100 dilution overnight at 4 °C. Secondary antibodies were conjugated to either Cy2 or Cy 3 (Jackson ImmunoResearch Laboratories, West Grove, PA) and were used at a dilution of 1:150 for 1.5 h at room temperature. Negative control experiments included incubation with the isotype control antibody. The cells were viewed by fluorescence microscopy (Eclipse E80i; Nikon, Tokyo, Japan) and micrographs were taken with a digital camera (Spot; Diagnostic Instruments, Inc, Sterling Heights, MI).

Transfection of Chinese Hamster Ovary (CHO) Cells

CHO cells (1×10^6 cells) were transfected with mock (pcDNA3), human H1 (NM_000861.2; OriGene, Rockville, MD), or human GPR32 receptors (O75388; [11]) using FuGENE transfection reagent (Promega, Madison, WI) following manufacturer's instruction. At 48 hours post transfection, cells were harvested for calcium mobilization experiments.

Knockdown of ALX Receptor

Goblet cells incubated with 100 μM siRNA against a scrambled sequence or ALX receptor for 72 h (ThermoScientific, Waltham, MA) in Accell delivery media according to manufacturer's instructions (ThermoScientific, Waltham, MA). The siRNA was a pool of 4 molecules whose sequences were: 1) CCAUCAGGUUCGUUAUUGG 2) CCUGCAGACAUUGAGAUAA 3) GUUUAAUACUCGUUACGGA and 4) GUACAAACACUUGUGAAA.

Statistical analysis

Results were expressed as the fold-increase above basal. Results are presented as mean ± SEM. Data were analyzed by Student's *t*-test. $P<0.05$ was considered statistically significant.

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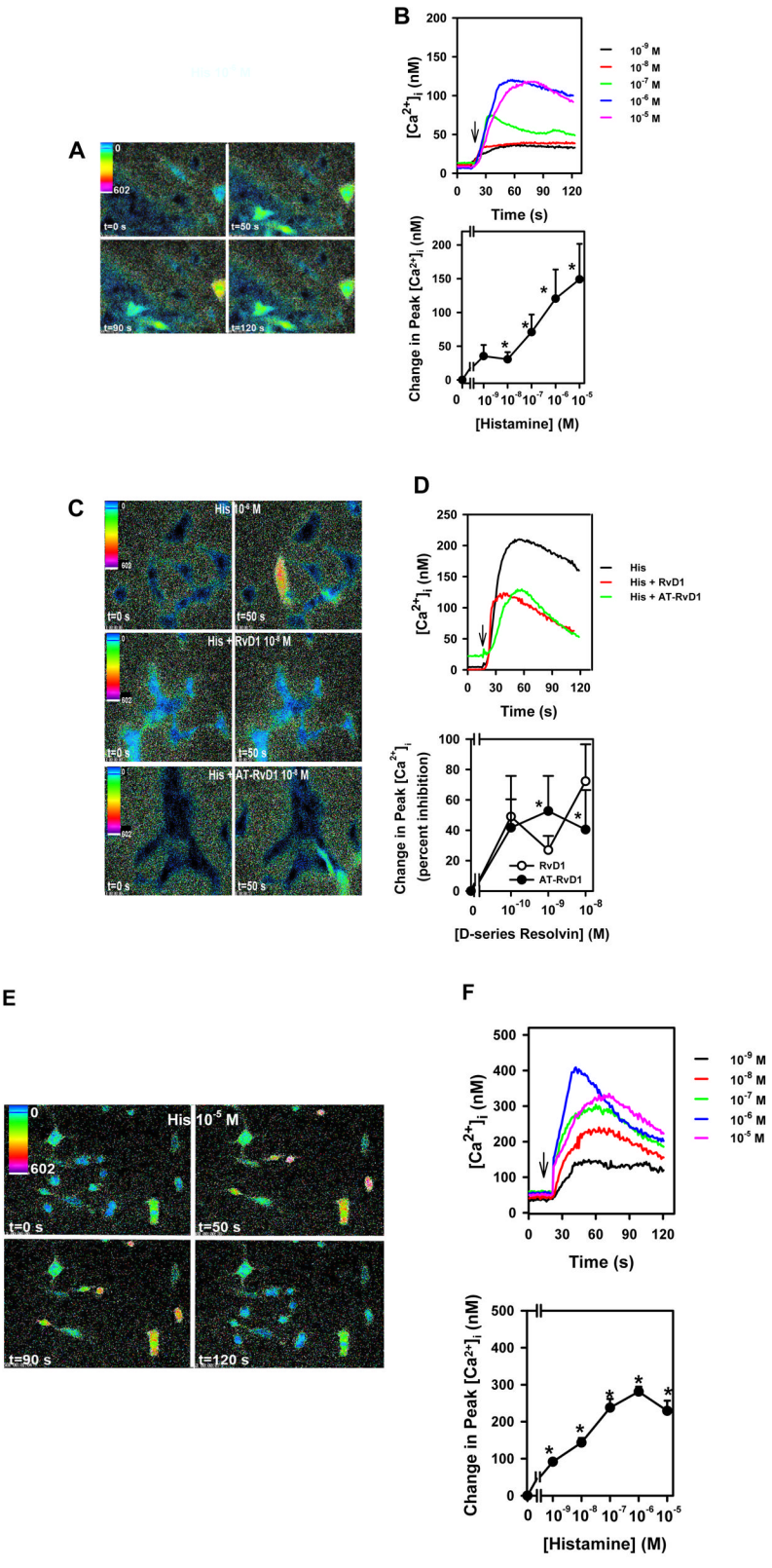


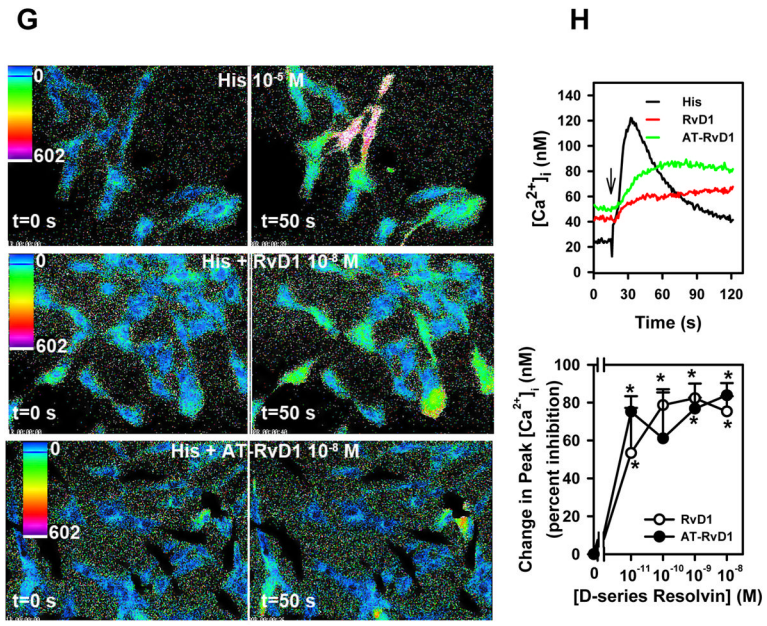
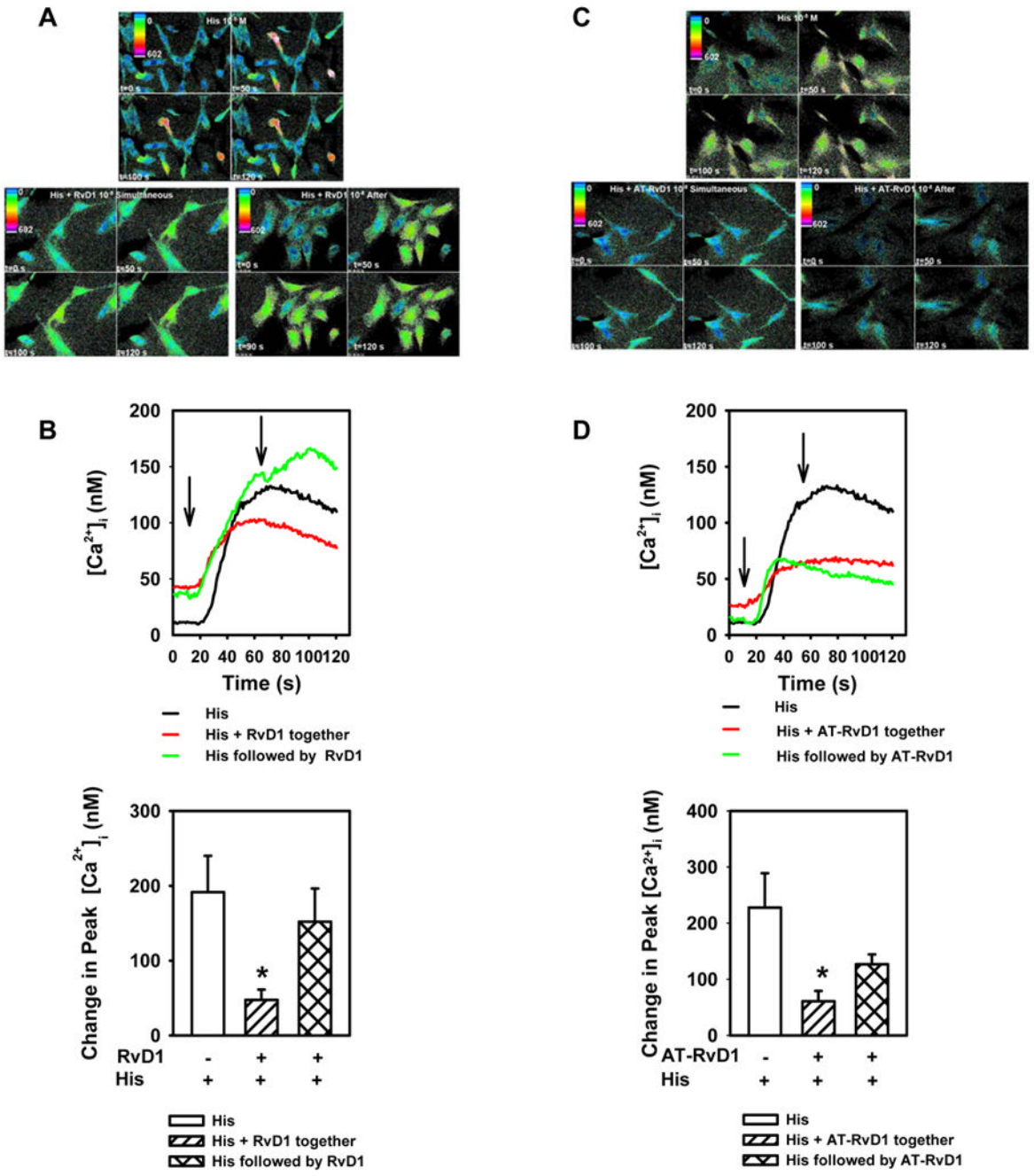


Figure 1. RvD1 and AT-RvD1 reduce histamine-stimulated increase in $[Ca^{2+}]_i$ in conjunctival goblet cells

Block of histamine-stimulated increase in $[Ca^{2+}]_i$ in human (A–D) and rat (E–H) goblet cells. Increase in $[Ca^{2+}]_i$ stimulated by histamine over time is shown in a pseudo color image of $[Ca^{2+}]_i$ from fura-2 loaded single goblet cells in A and E. Intracellular Ca^{2+} response over time, in response to increasing concentrations of histamine (10^{-9}, - 10^{-5} M) from three experiments (human) or a representative experiment (rat) is shown in B and F top panel. Peak $[Ca^{2+}]_i$ over basal for each concentration of histamine from three experiments is shown in B and F bottom panels. *p<0.05 (vehicle vs histamine). Action of RvD1 and AT-RvD1 exposure on histamine (10^{-5} M) stimulated increase in $[Ca^{2+}]_i$, in human (C–D) and rat (G–H) goblet cells. Pseudo color image of single goblet cells is shown in C and G; $Ca^{2+}{}_i$ response over time is shown in D and H top panels in response to histamine (10^{-5} M) alone, or histamine after 30 min exposure with RvD1 (10^{-8} M) or AT-RvD1 (10^{-8} M) from a representative experiment. Peak Ca^{2+} response (D and H bottom panels) to increasing concentrations of RvD1 (open circles) and AT-RvD1 (closed circles) is shown from three experiments. *p<0.05 (histamine vs resolvin plus histamine).



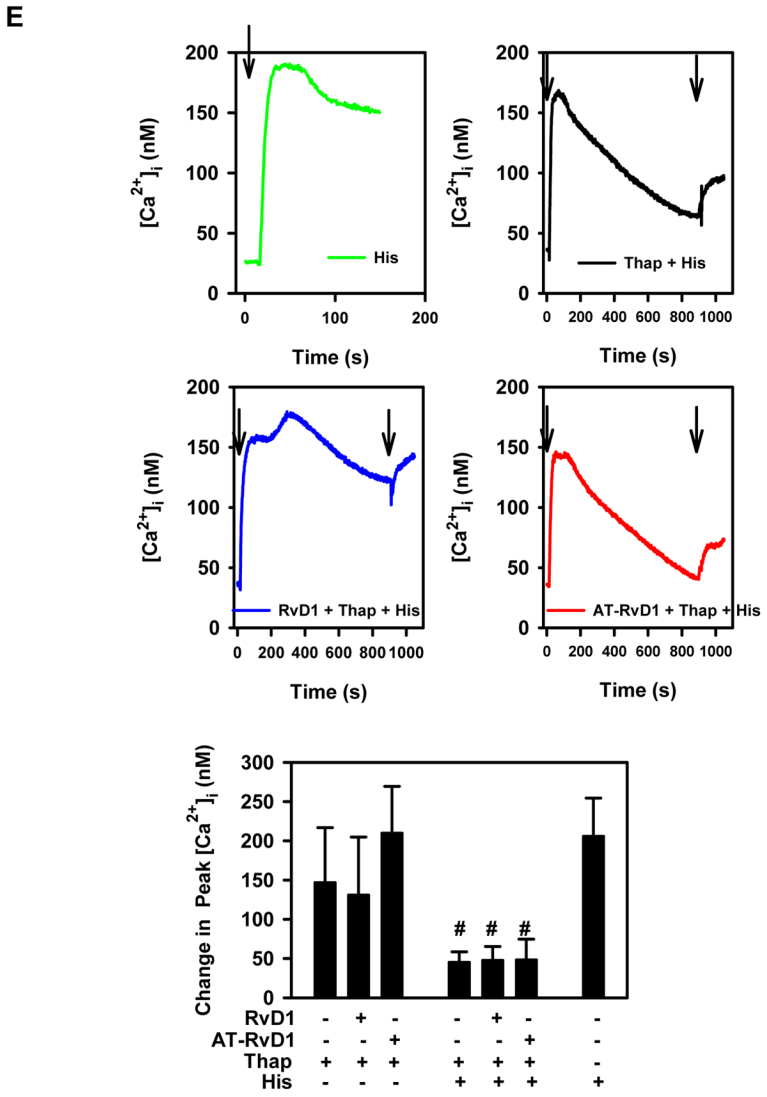


Figure 2. RvD1 and AT-RvD1 reduce histamine-stimulated increase in $[Ca^{2+}]_i$ by preventing release of intracellular Ca^{2+} in conjunctival goblet cells

Result of time of addition of RvD1 (10^{-8} M, A and B) or AT-RvD1 (10^{-8} M, C and D) on histamine (10^{-5} M)-stimulated increase in $[Ca^{2+}]_i$ in rat conjunctival goblet cells. A and C are pseudo color images of $[Ca^{2+}]_i$ in single goblet cells in response to histamine alone or with RvD1 or AT-RvD1 added at the same time as histamine or 50 sec after addition of histamine. Intracellular Ca^{2+} response over time to histamine alone, RvD1 or AT-RvD1 added simultaneously with histamine or added 50 sec after histamine is shown in B and D (top panels, from a representative experiment). Peak $[Ca^{2+}]_i$ in response to histamine alone (open bars), RvD1 or AT-RvD1 added simultaneously with histamine (diagonal lines) or added at 60 sec after histamine (cross-hatched lines) is shown in B and D lower panels from seven experiments. *p<0.05 (histamine vs resolvin plus histamine). First arrow indicates addition of RvD1 with histamine. Second arrow indicates addition of RvD1 after histamine. Use of capacitative Ca^{2+} store by RvD1 or AT-RvD1 in rat goblet cells is in E. Left top panel indicates intracellular Ca^{2+} response over time in response to histamine (10^{-5} M) alone. Right top panel indicates thapsagargin (10^{-5} M, first arrow) followed by histamine (10^{-5} Msecond arrow). A 30 min exposure with RvD1 (10^{-10} M) or AT-RvD1 (10^{-11} M)

followed by thapsigargin (10^{-6} M, first arrow) and then histamine (10^{-5} M, second arrow) is indicated in the middle panels. Bottom panel indicates peak $[Ca^{2+}]_i$ in response to histamine (10^{-5} M) alone; exposure of vehicle, RvD1, or AT-RvD1 for 30 min followed by thapsigargin (10^{-6} M) and then followed by histamine (10^{-5} M) from four experiments. # $p<0.05$ (histamine vs resolvin plus thapsigargin plus histamine)

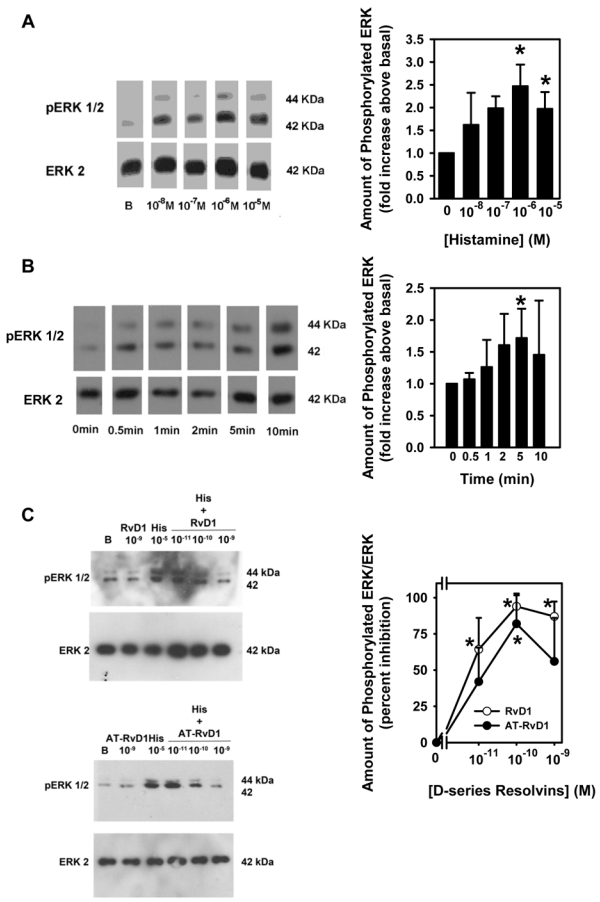


Figure 3. RvD1 and AT-RvD1 reduce histamine-stimulated increase in ERK1/2 activity in conjunctival goblet cells

Block of histamine-stimulated activation of ERK1/2 in rat conjunctival goblet cells. A. is concentration- and B. time-dependence of histamine stimulated increase in ERK1/2 activity measured by western blotting using phosphospecific and total activity antibodies in a representative experiment (left panel) and a mean of three experiments (right panel) *$p<0.05$ (vehicle vs histamine). A. indicates histamine used for 5 min and B. histamine used at 10^{-5} M. C. represents action of 30 min exposure of RvD1 (open circles) or AT-RvD1 (closed circles) on 5-min histamine (10^{-5} M) stimulated increase in ERK1/2 activity in a representative experiment using RvD1 (top left panel) and AT-RvD1 (bottom left panel) and a mean of three experiments (right panel). *$p<0.05$ (histamine vs resolvin plus histamine)

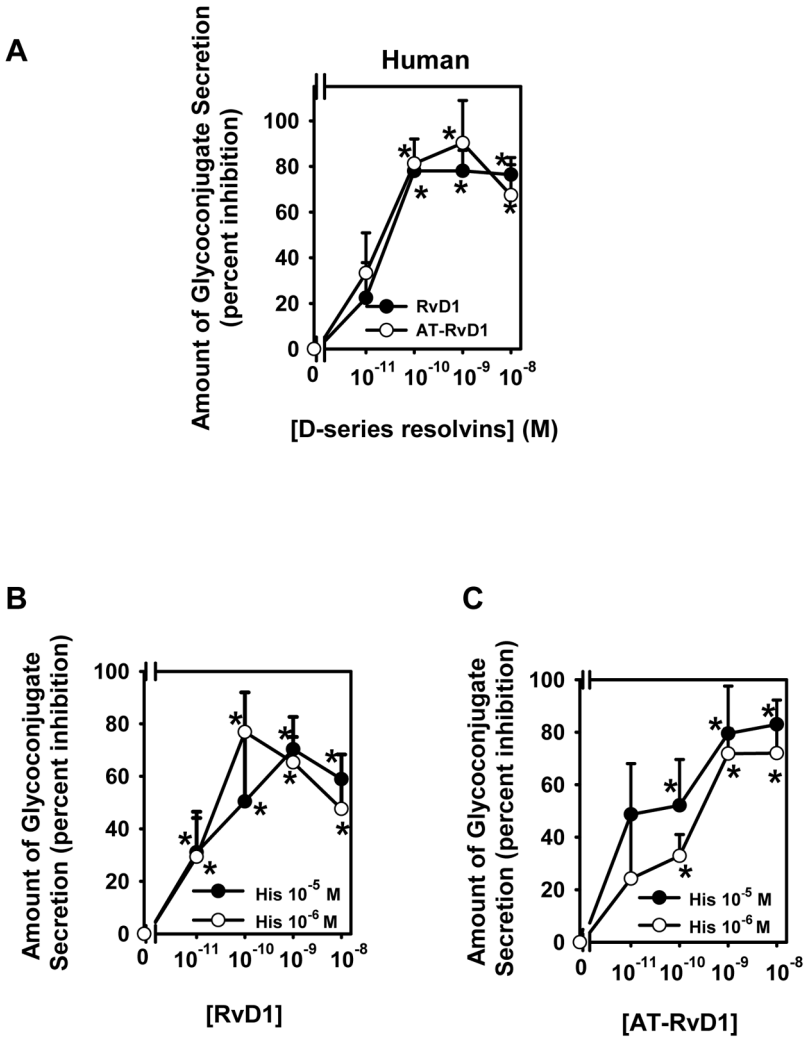


Figure 4. RvD1 and AT-RvD1 reduce histamine-stimulated increase in high molecular weight glycoconjugate secretionin conjunctival goblet cells

Block of 2-hr histamine (10^{-5} M)-stimulated high molecular weight glycoconjugate secretion in A. human and B. and C. rat conjunctival goblet cells by a 30 min prior exposure of RvD1 or AT-RvD1. A. indicates mean of four experiments using increasing concentrations of RvD1 (closed circles) and AT-RvD1 (open circles). Mean of four experiments with stimulation by histamine at 10^{-6} M (open circles) and 10^{-5} M (closed circles) after 30 min exposure to RvD1 (B) and AT-RvD1 (C). *p<0.05 (histamine vs resolvin plus histamine).

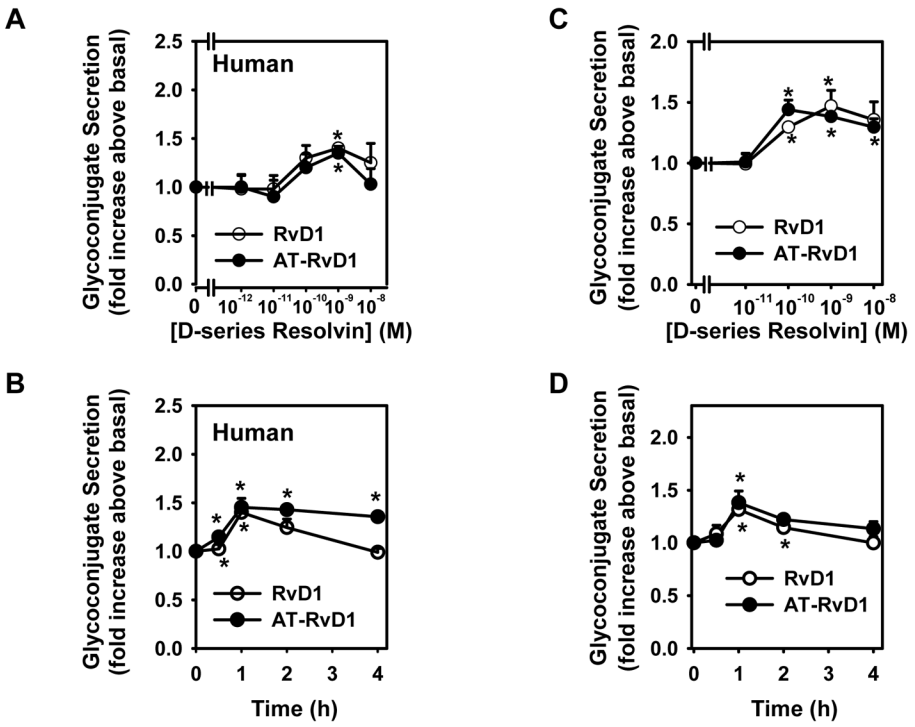
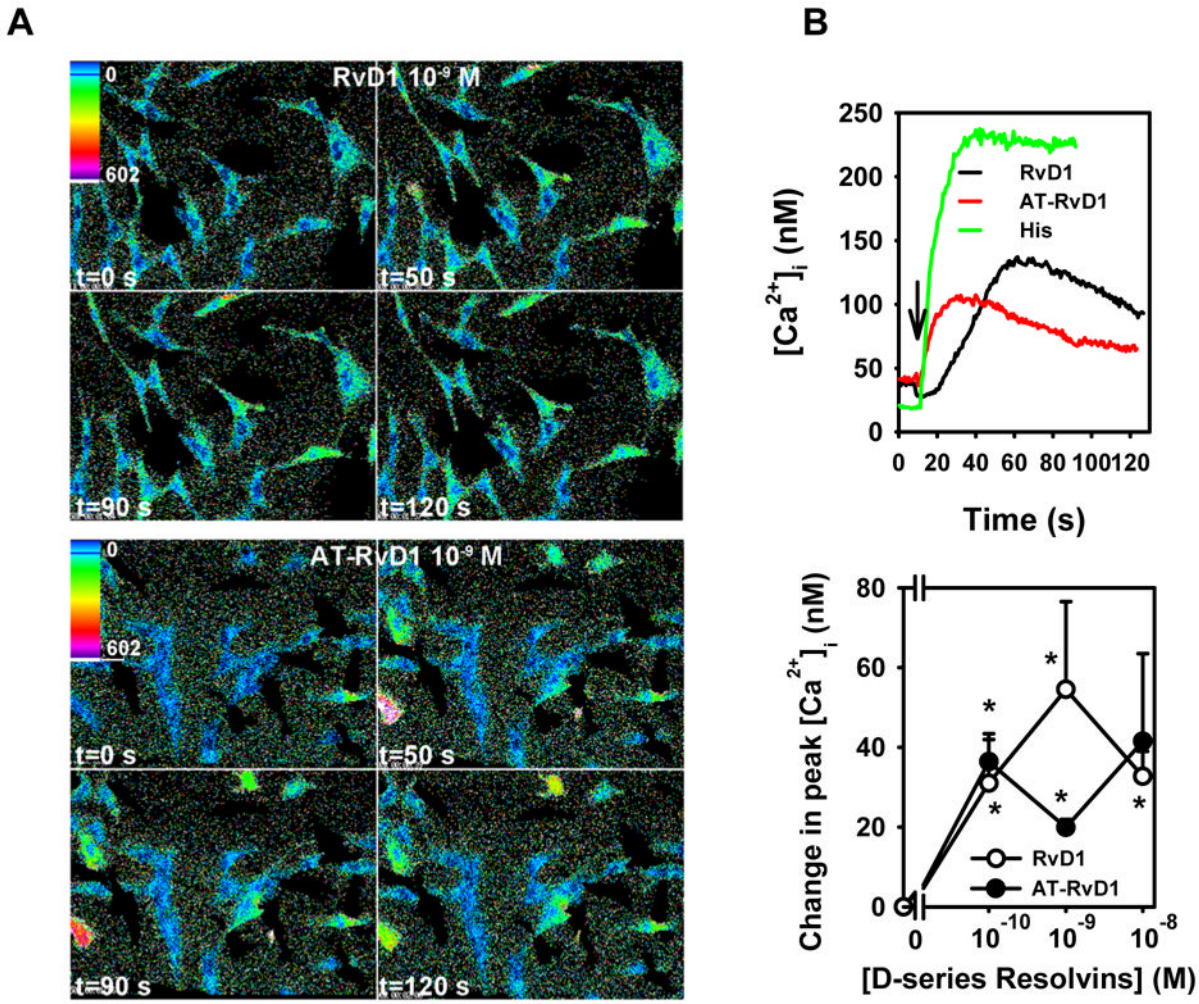
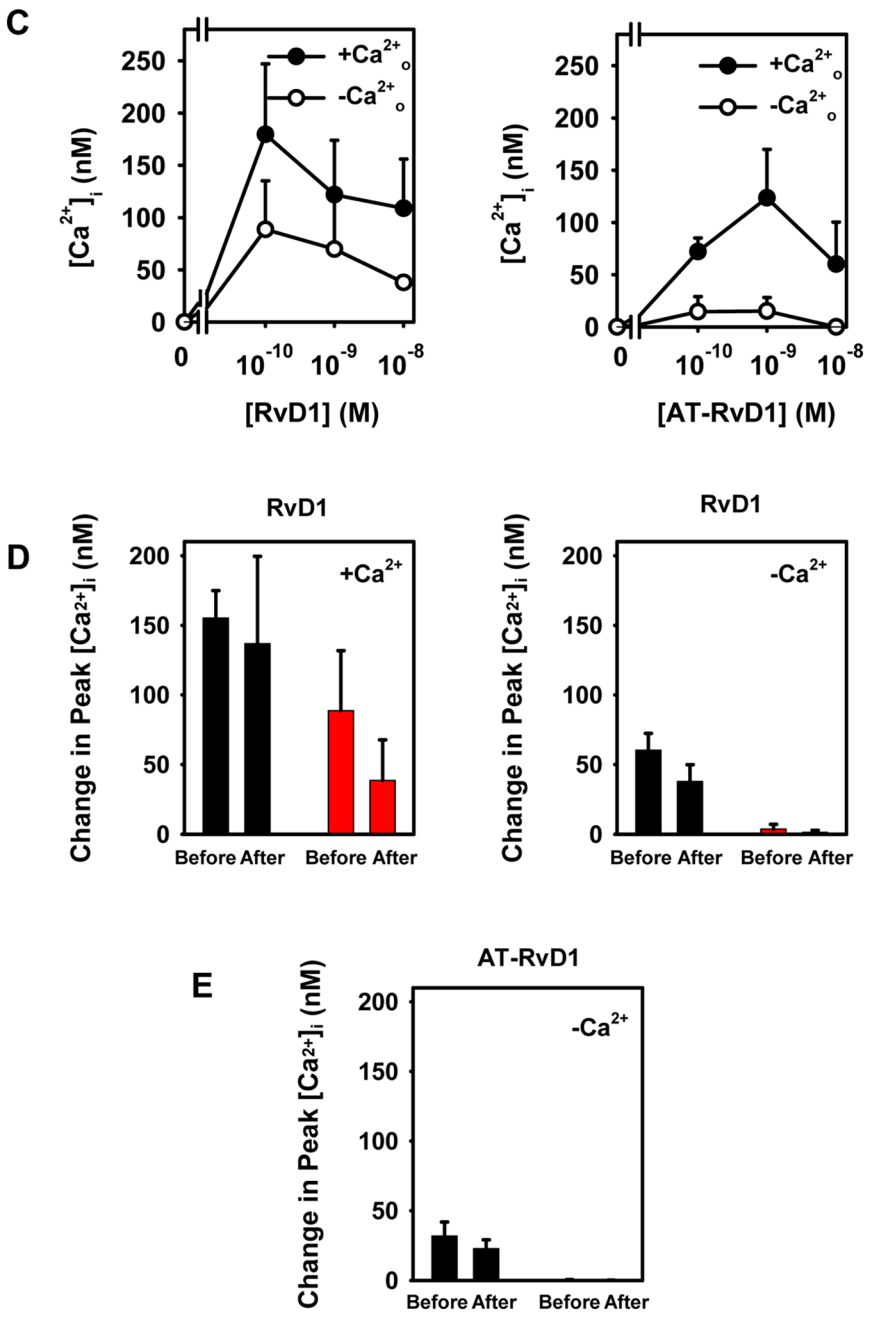


Figure 5. RvD1 and AT-RvD1 increase high molecular weight glycoconjugate secretion in conjunctival goblet cells

Increase in high molecular weight glycoconjugate secretion in human (A and B) and rat (C and D) conjunctival goblet cells. Concentration- and time-dependence of RvD1 (open circles) or AT-RvD1 (closed circles) stimulation of high molecular weight glycoconjugate secretion. A. and C. represent 2 hr stimulation with increasing concentrations of RvD1 and AT-RvD1. C and D. show 10^{-8} M resolvin stimulation for 0–4 hrs. Mean of three experiments for A – D. *p<0.05 (vehicle plus resolvin)





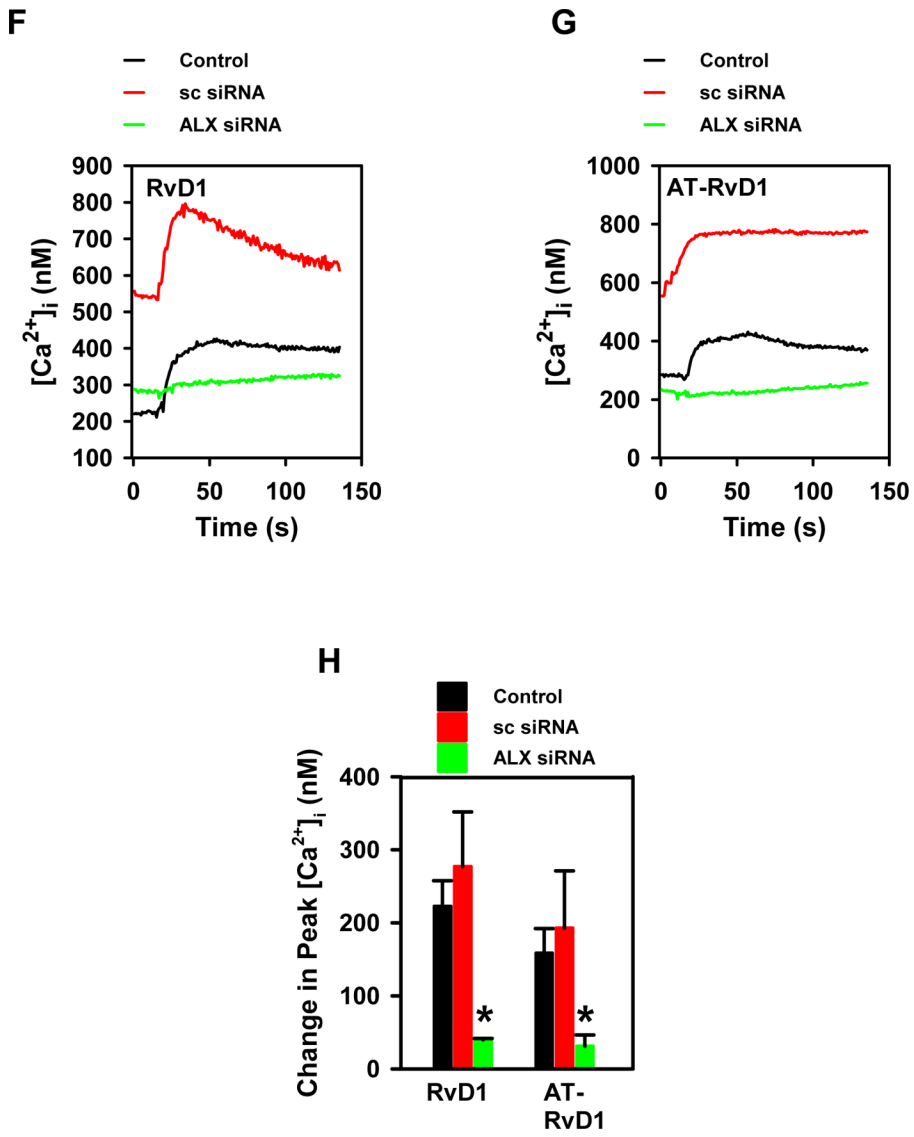


Figure 6. RvD1 and AT-RvD1 increase in $[Ca^{2+}]_i$, in conjunctival goblet cells

Increase in $[Ca^{2+}]_i$ using fura-2 loaded rat conjunctival goblet cells stimulated by RvD1 and AT-RvD1, A. is pseudo color images of $[Ca^{2+}]_i$ in single goblet cells stimulated by RvD1 (top panels) or AT-RvD1 (bottom panels). Intracellular $[Ca^{2+}]_i$ over time in response to RvD1 (10^{-9} M), AT-RvD1 (10^{-8} M), or histamine (10^{-5} M) from a representative experiment (B top panel), and peak $[Ca^{2+}]_i$ response to increasing concentrations of RvD1 (open circles) and AT-RvD1 (closed circles) from four experiments are shown in B bottom panel. *p<0.05 (vehicle vs resolvin). C. is peak $[Ca^{2+}]_i$ in response to RvD1 (left panel) and AT-RvD1 (right panel) in the presence (closed circles) and absence (open circles) of extracellular Ca^{2+} from three experiments, *p<0.05 (resolvin in presence vs absence of extracellular Ca^{2+}) D. and E. represent peak $[Ca^{2+}]_i$ in response to thapsigargin (10^{-6} M) added either before or after RvD1 (10^{-8} M) in the presence (left panel of D) or absence (right panel of D) of extracellular Ca^{2+} or before or after AT-RvD1 (10^{-8} M) addition in the absence of extracellular Ca^{2+} (E). F and G represent mean response of $[Ca^{2+}]_i$ over time from three experiments to RvD1 (F) and AT-RvD1 (G) in fura-2 loaded conjunctival goblet

cells treated with transfection reagent only (control), a scrambled sequence siRNA (sc siRNA) or ALX/FPR2 siRNA. H represents mean of peak $[Ca^{2+}]_i$ from three experiments.

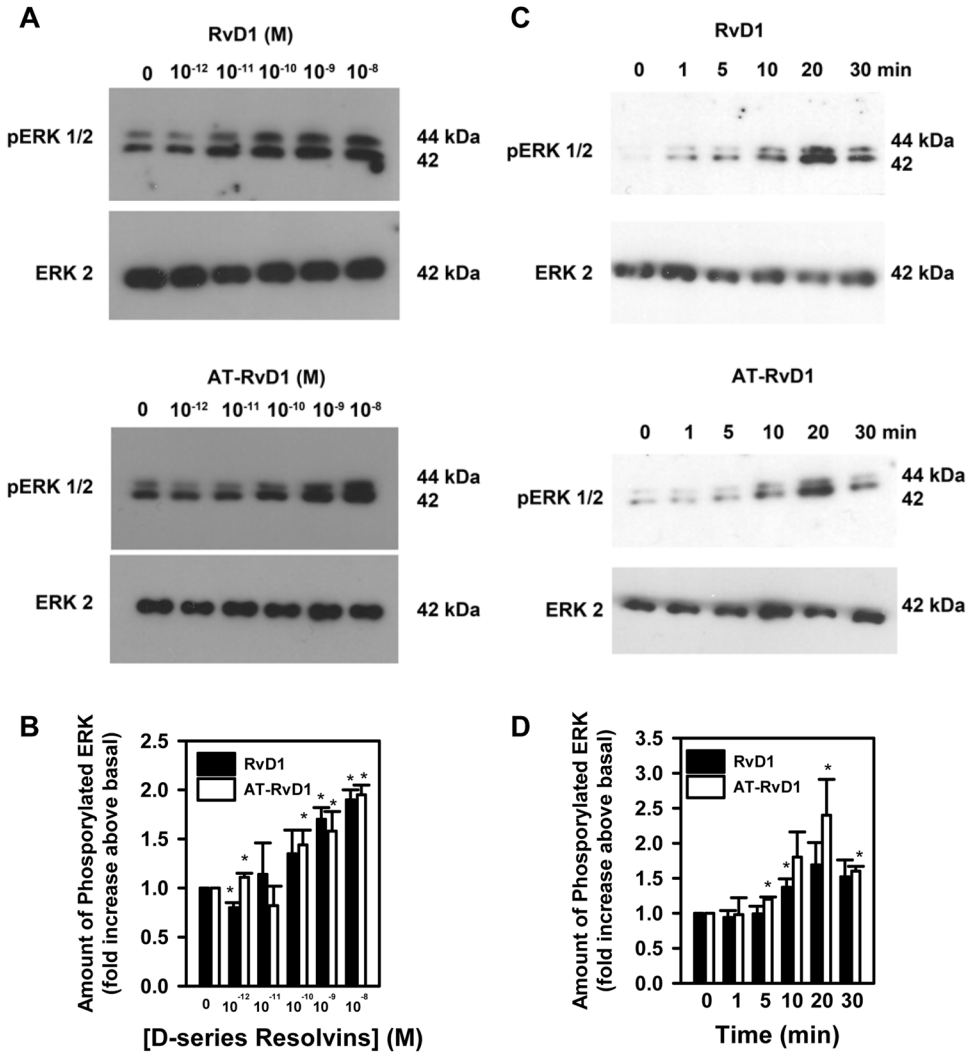


Figure 7. RvD1 and AT-RvD1 increase ERK1/2 activity conjunctival goblet cells

Response of ERK1/2 activity to RvD1 (upper panels in A and C) or AT-RvD1 (bottom panels in A and C) in rat conjunctival goblet cells. Concentration (left panels)- and time (right panels)-dependence of ERK1/2 activity in a representative western blotting experiment. Mean of three experiments using RvD1 (closed bars) and AT-RvD1 (open bars) for 5 min in B and RvD1 (10^{-9} M, closed bars) and AT-RvD1 (10^{-10} M, open bars) in D. *p<0.05 (vehicle vs resolvin)

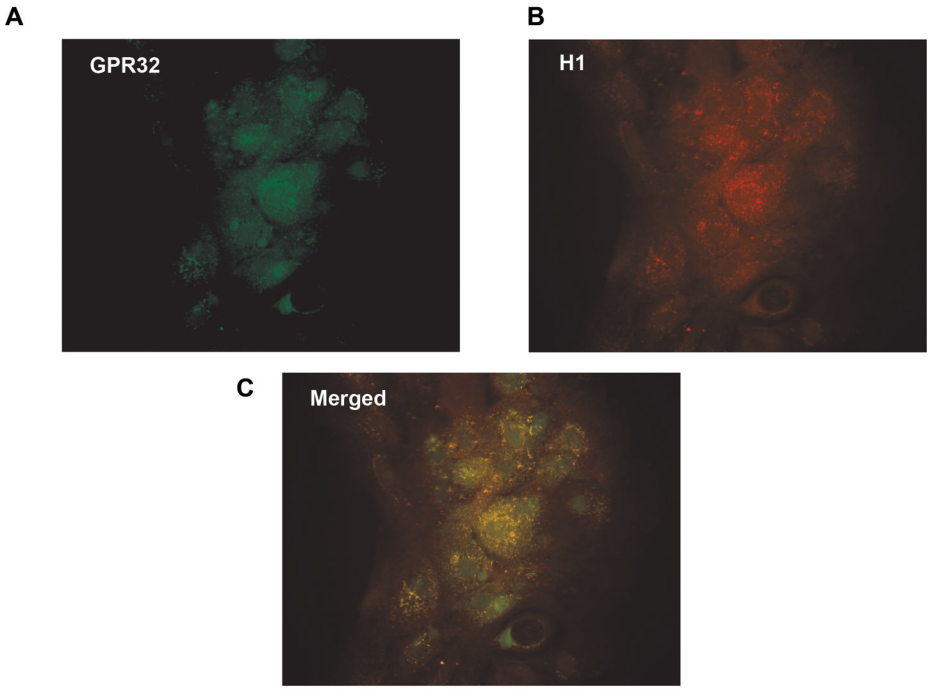


Figure 8. GPR32 and H1 receptor have overlapping localization in conjunctival goblet cells

Immunofluoresence microscopy of the location of the GPR32 receptor (green) in A, the H_1 receptor (red) in B, or a merged image C in human conjunctival goblet cells. Magnification 400x.

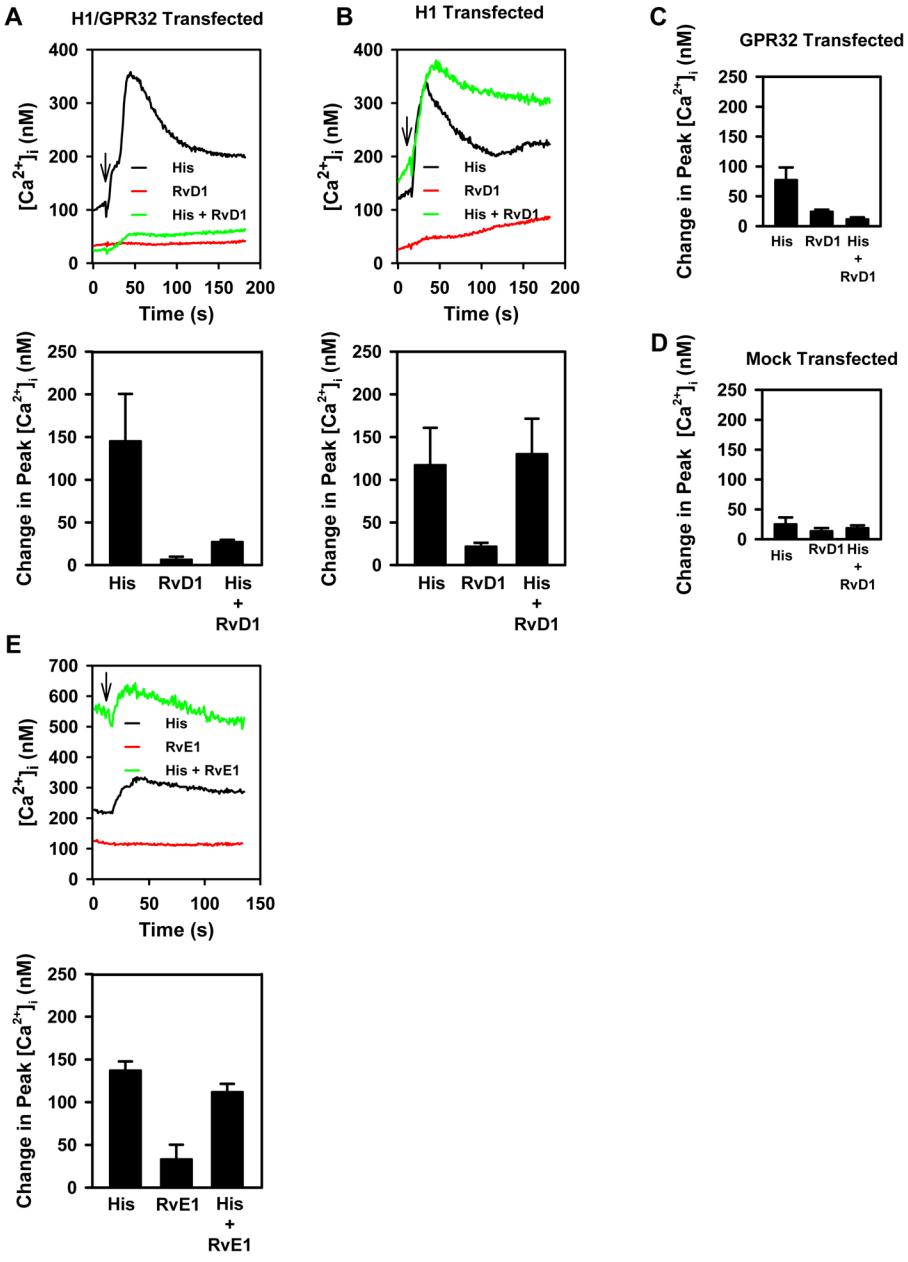


Figure 9. RvD1 uses the GPR32 receptor to block histamine activation of the H_1 receptor by phosphorylating the H_1 receptor in CHO cells

A–D represent response of $[Ca^{2+}]_i$ to histamine (10^{-5} M), RvD1 (10^{-8} M), or 30-min RvD1 exposure followed by histamine in fura-2 loaded CHO cells that had been transiently transfected with A. H_1 and GPR32 receptors, B. H_1 receptor, alone C. the GPR32 receptor alone, or D. mock transfected. A. and B. indicate intracellular $[Ca^{2+}]_i$ over time (upper panels) or peak $[Ca^{2+}]_i$ (lower panels) in response to histamine (10^{-5} M), RvD1 (10^{-8} M), or addition of RvD1 30 min before addition of histamine. C. and D. show peak $[Ca^{2+}]_i$ in response to histamine, RvD1, or addition of RvD1 30 min before addition of histamine. E indicates intracellular $[Ca^{2+}]_i$ over time (upper panel) or peak $[Ca^{2+}]_i$ (lower panel) in response to histamine (10^{-5} M), RvE1 (10^{-8} M), or addition of RvE1 30 min before addition of histamine.

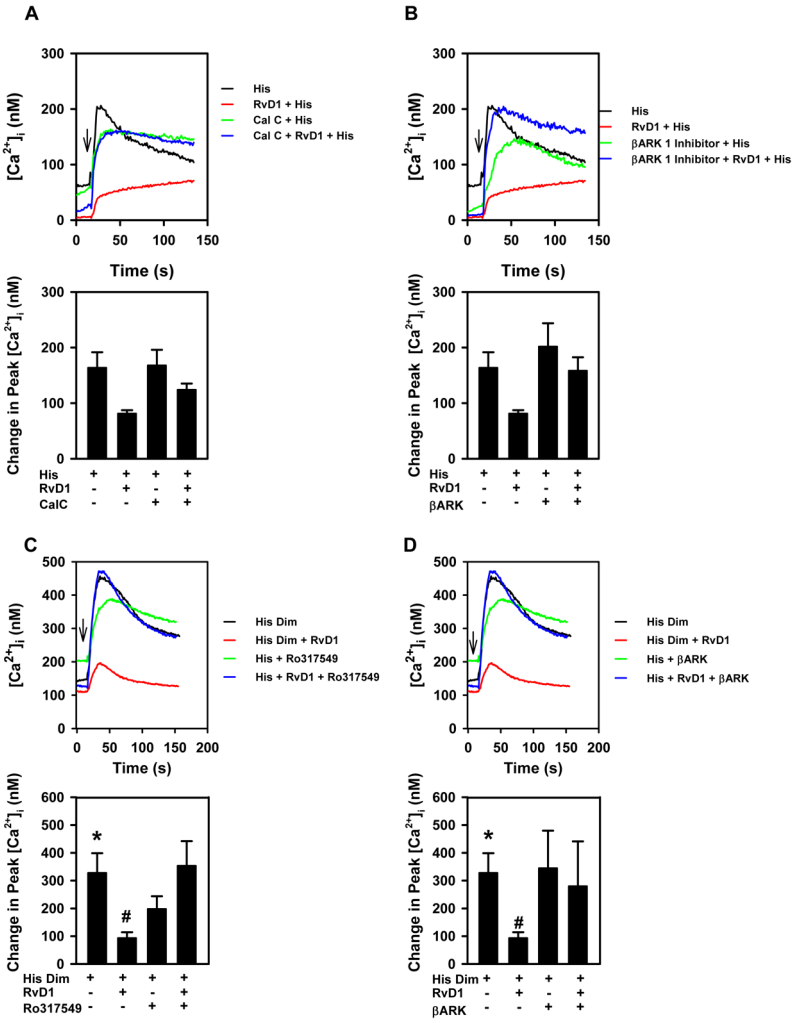


Figure 10. RvD1 uses PKC and β-adrenergic receptor kinase 1 to block histamine activation of the H1 receptor in CHO cells and conjunctival goblet cells

A. and B. demonstrate action of PKC inhibitor calphostin C (10^{-7} M) (A) or βARK1 inhibitor (10^{-7} M) (B) on RvD1 (10^{-8} M) block of histamine (10^{-5} M) on the $[Ca^{2+}]_i$ in fura-2 loaded CHO cells that had been transiently transfected with the H_1 and GPR32 receptors. C. and D. demonstrate action of PKC inhibitor Ro-317549 (10^{-7} M) (C) or βARK1 inhibitor (10^{-7} M) (D) on RvD1 (10^{-8} M) block of histamine dimaleate (10^{-6} M) on the $[Ca^{2+}]_i$ in fura-2 loaded rat goblet cells. Intracellular $[Ca^{2+}]_i$ over time (upper panels in A and B) or peak $[Ca^{2+}]_i$ (lower panels in A and B) in response to histamine (10^{-5} M), RvD1 (10^{-8} M) added 30 min before addition of histamine; calphostin C or βARK inhibitor (10^{-7} M) added 45 min before histamine; or calphostin C or βARK inhibitor (10^{-7} M) added 15 min before addition of RvD1 (10^{-8} M) which was added 30 min before histamine. Intracellular $[Ca^{2+}]_i$ over time (upper panels in C and D) or peak $[Ca^{2+}]_i$ (lower panels in C and D) in response to histamine dimaleate (10^{-6} M), RvD1 (10^{-8} M) added 30 min before addition of histamine dimaleate; Ro-317549 or βARK inhibitor (10^{-7} M) added 45 min before histamine; or Ro-317549 or βARK inhibitor (10^{-7} M) added 15 min before addition of RvD1 (10^{-8} M) which was added 30 min before histamine dimaleate. Seventeen - twenty cells were selected for each experimental condition.

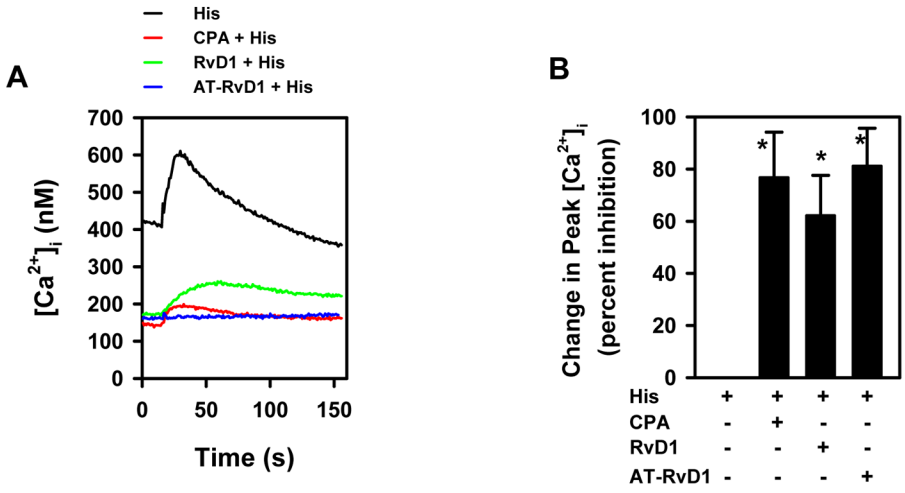


Figure 11. H_1 histamine antagonist, RvD1, and AT-RvD1 each block histamine-stimulated increase in $[Ca^{2+}]_i$ to the same extent in rat goblet cells

A. indicates mean intracellular $[Ca^{2+}]_i$ over time and B. percent inhibition of peak $[Ca^{2+}]_I$ in response to histamine alone (10^{-5} M) or following a 30 min exposure with the H_1 antagonist chlorpheniramine (CPA, 10^{-4} M), RvD1 (10^{-8} M) or AT-RvD1 (10^{-8} M) from four experiments *$p<0.05$ (histamine vs inhibitor plus histamine).