Immunolocalization of Muscarinic and VIP Receptor Subtypes and Their Role in Stimulating Goblet Cell Secretion

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PURPOSE. To determine the subtypes of cholinergic muscarinic receptors and receptors for vasoactive intestinal peptide (VIP) present in rat conjunctival goblet cells and whether cholinergic agonists and VIP stimulate goblet cell secretion.

METHODS. Immunofluorescence studies were performed using antibodies against the m1, m2, and m3 muscarinic receptor subtypes and VIP receptors 1 and 2 (VIPR1 and VIPR2). The lectin Ulex europaeus agglutinin I was used to measure glycoconjugate secretion, the index of secretion, from goblet cells in an enzyme-linked lectin assay. In this assay, pieces of conjunctiva were placed on filter paper and incubated for 15 to 120 minutes, with or without increasing concentrations of the cholinergic agonist carbachol or VIP. The muscarinic antagonist atropine and the muscarinic receptor-subtype-selective antagonists pirenzepine (M1), gallamine (M2), and 4-4-diphenylacetoxy-N-(2-chloroethyl)-piperidine hydrochloride (4-DAMP mustard; M3) were incubated with carbachol to determine specificity of receptor activation.

RESULTS. Immunoreactivity to M2 and M3 receptors was found on goblet cell membranes subjacent to the secretory granules. Immunoreactivity to M1 receptor was not on goblet cells but was on the stratified squamous cells. Immunoreactivity to VIPR2 was found on goblet cells with a localization similar to that of the M2 and M3 receptors. VIPR1 was not found on goblet cells or on the stratified squamous cells. Carbachol and VIP induced a time- and concentration-dependent stimulation of glycoconjugate secretion. Carbachol, at 10^-4 M, induced a threefold increase in glycoconjugate secretion, which was completely inhibited by atropine (10^-5 M). Carbachol-induced secretion was inhibited 54% ± 8% by pirenzepine (10^-5 M), 69% ± 14% by gallamine (10^-5 M), and 72% ± 11% by 4-DAMP mustard (10^-5 M). A twofold increase in glycoconjugate secretion was obtained with VIP at 10^-8 M.

CONCLUSIONS. Cholinergic agonists, through M2 and/or M3 muscarinic receptors, and VIP, through VIPR2, regulate conjunctival goblet cell secretion, suggesting that goblet cell secretion in vivo is under the control of parasympathetic nerves. (Invest Ophthalmol Vis Sci. 1999;40:1102-1111)
whereas the M₄ subtype is coupled to inhibition of adenylate cyclase.

Muscarinic receptors have been identified by ³H-QNB (quinuclidinyl benzilate) binding to isolated villus and crypt cells of the rat small intestine and to colon. Autoradiographic mapping and in situ hybridization have revealed M₁ and M₄ receptor subtypes on human airway mucosal glands at a ratio of 1:2. However, muscarinic receptors have not yet been identified specifically in goblet cells.

In addition to cholinergic agonists, the other parasympathetic neurotransmitter vasoactive intestinal peptide (VIP) has been shown to stimulate goblet cell secretion in rat conjunctiva, pancreatic ducts, and two cell lines of colonic goblet cells. To date, two types of VIP receptors, VIPR1 and VIPR2, have been cloned. VIPR2 has a higher affinity for VIP than does VIPR1. Both VIP receptors activate the G protein G₃ζ and thus are coupled to adenylate cyclase. Activation of both types of receptors also increases the intracellular [Ca²⁺⁻].

A recent study from our laboratory showed that parasympathetic and sympathetic, but not sensory, nerves are localized around goblet cells. We have also shown that activation of afferent sensory nerves in the cornea caused conjunctival goblet cell secretion in vivo. However, which nerves and neurotransmitters are the direct stimuli of conjunctival goblet cell secretion is still unknown. Parasympathetic nerves contain the neurotransmitter acetylcholine, a cholinergic agonist, and the neuropeptide VIP.

The purpose of the present study was twofold: to determine the presence and the cellular distribution of cholinergic muscarinic and VIP receptor subtypes and to determine whether cholinergic agonists and VIP stimulate conjunctival goblet cell glycoconjugate secretion by means of an enzyme-linked lectin assay (ELLA). Our results show that conjunctival goblet cell expressed the M₂ and M₃, but not the M₁, muscarinic receptors, along with VIPR2, but not VIPR1. They also showed conjunctival goblet cell secretion to be under the control of the cholinergic muscarinic pathway activating M₂ and/or M₃ receptors and the VIP pathway using VIPR2.

**Materials and Methods**

**Materials**

_Ulex europaeus_ agglutinin 1 (UEA-1) biotinylated lectin, alkaline phosphatase-conjugated streptavidin, rhodamine-conjugated streptavidin, horseradish peroxidase-conjugated streptavidin, and p-nitrophenylphosphate were obtained from Pierce (Rockford, IL). Fluorescein isothiocyanate (FITC)-conjugated streptavidin was purchased from Molecular Probes (Eugene, OR). Carbamylcholine chloride (carbachol), atropine, bovine submaxillary salivary gland mucin, and phenylmethylsulfonyl fluoride were obtained from Sigma (St. Louis, MO). Pirenzepine hydrochloride, gallamine triethiodide, and 4-diphenylacetoxymethyl-piperidine hydrochloride (4-DAMP mustard) were purchased from Research Biochemicals (Natick, MA).

Polyclonal antibodies against m₃, m₂, and m₁ muscarinic receptor subtypes were obtained from Research and Diagnostic Antibodies (Berkeley, CA). Polyclonal antibodies against VIPR1 and VIPR2 and preabsorbed serum were the generous gift of Edward J. Goetzl (Department of Medicine, Microbiology and Immunology, University of California, San Francisco).

**Immunohistochemistry**

All experiments conformed to the guidelines established by the 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. Male Sprague-Dawley rats weighing between 250 g and 300 g were obtained from Taconic Farms (Germantown, NY). Rats were anesthetized for 1 minute in CO₂ and decapitated, and both eyes were removed. The inferior conjunctiva was carefully removed around the limbus of the cornea from nasal to temporal canthus and fixed for 4 hours at 4°C in 4% buffered formaldehyde solution. The tissue was cryopreserved overnight in 30% sucrose at 4°C in phosphate-buffered saline (PBS) containing 145 mM NaCl, 7.3 mM Na₂HPO₄, and 2.7 mM NaH₂PO₄ (pH 7.2) and frozen in optimal cutting embedding compound (Miles, Elkhart, IN). Cryostat sections (6 µm) were placed on gelatin coated slides and air dried for 2 hours. Sections were processed for lectin histochemistry using biotinylated UEA-I diluted 1:1000 followed by FITC-conjugated streptavidin diluted 1:200, each for 1 hour at room temperature. Antimuscarnic receptor subtype antibodies were diluted 1:1000 in PBS and applied overnight at 4°C. The localization of VIPR1 and VIPR2 was done as described by Hodges et al. Antibodies against VIPR1 and VIPR2 were diluted 1:50 in PBS and applied for 1 hour at room temperature. The secondary antibody, donkey anti-rabbit IgG conjugated to FITC, was diluted 1:100 in PBS and applied for 1 hour at room temperature. For experiments in which biotinylated UEA-I and m₃ receptor antibody were both used, primary probes were incubated together overnight at 4°C. The secondary probes rhodamine-conjugated streptavidin and donkey anti-rabbit IgG conjugated to FITC were then incubated together for 1 hour at room temperature. In selected sections, mounting medium containing propidium iodide (Vectorshied, Vector Laboratories, Burlingame, CA) was used to identify nuclei of conjunctival cells including goblet cells. Sections were viewed by microscope (model UFX II, Nikon, Garden City, NY), equipped for epifluorescence, or by confocal laser scanning microscope (model TCSP4; Leitz Leica, Garden City, NY) equipped with a krypton-argon laser (Leica Lasertechnik GmbH, Heidelberg, Germany). For negative control, the primary antibody was omitted. For VIP receptors, an additional negative control of use of preabsorbed serum was performed.

**Electrophoresis and Immunoblot Analysis**

Inferior and superior conjunctiva were removed and homogenized at 4°C in homogenization buffer containing 30 mM Tris-HCl (pH 7.5), 10 mM EGTA, 5 mM EDTA, 1 mM diethiothreitol, 10 mg/ml phenylmethylsulfonyl fluoride, and 5 µM aprotinin. To determine the molecular mass of glycoconjugate detected by UEA-1 lectin, proteins in the homogenate were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 6% gels and transferred to nitrocellulose membrane as described by Towbin et al. The membranes were blocked overnight at 4°C in 5% dried milk in TBST (10 mM Tris-HCl [pH 8.0], 500 mM NaCl, 0.05% Tween-20), and then incubated with biotinylated UEA-1 (1:100) for 1 hour at room temperature. Nitrocellulose membranes were washed three times in TBST and incubated with 1:2500 horseradish peroxidase-labeled streptavidin in TBST for 1 hour. The membranes were washed in TBST three times, and the UEA-1-detectable glycoproteins were visualized.
using the enhanced chemiluminescence method. An aliquot of 100 µg/ml bovine submaxillary mucin was used as a positive control. To determine the specificity of binding, 0.5 M L-fucose was preincubated with the biotinylated UEA-I overnight at 37°C before application to the nitrocellulose membrane.

To determine the presence of muscarinic receptor subtypes in the conjunctiva, conjunctival homogenate was centrifuged at 100,000g for 1 hour at 4°C. The pellet (membrane fraction) was resuspended in homogenization buffer and divided 1:4 with sample buffer. Proteins were resolved by SDS-PAGE using 10% gels and transferred to nitrocellulose membranes. The membranes were incubated with antimuscarinic receptor antibodies diluted 1:1000 in TBST for 1 hour at room temperature. Peroxidase-conjugated antibiotin IgG at 1:2500 was used as the secondary antibody. Immunoreactive bands were visualized using the enhanced chemiluminescence method.

Measurement of Goblet Cell Secretion
Inferior conjunctiva removed from rats as described for immunohistochemistry studies was carefully placed over a 1-cm² filter paper and cut transversely, producing two pieces per eye. The pieces of conjunctiva were preincubated for 5 minutes in keratinocyte basal medium and then incubated in 500 µl of the same medium, with or without the muscarinic agonist carbachol (10⁻⁶ M, 10⁻⁵ M, and 10⁻⁴ M) or VIP (10⁻¹⁰ M, 10⁻⁸ M, and 10⁻⁶ M) for 60 minutes at 37°C. Pieces of conjunctiva were also incubated in the presence of 10⁻⁴ M carbachol or 10⁻³ M VIP for 5 to 120 minutes for time-dependency studies. In some experiments, muscarinic antagonists at 10⁻⁵ M, were added for 10 minutes before addition of carbachol. At the end of the incubation period, the medium was collected, and the tissue homogenized in 1 ml 1% SDS in 0.1 M bicine buffer (pH 9.2) and centrifuged for 2 minutes at 14,000 rpm. The amount of glycoconjugate, our index of mucin secretion, was released in the medium and remaining in the tissue was determined using biotinylated lectin UEA-I in an ELLA. The ELLA was performed according to the manufacturer's protocol (product description number 339916, biotinylated UEA-I, ImmunoPure; Pierce). Biotinylated UEA-I was used at 2 µg/ml, streptavidin conjugated to alkaline phosphatase at 1 µg/ml, and the substrate p-nitrophenyl phosphate at 2.5 mM. A 100-µl aliquot of the tissue homogenate and a 250-µl aliquot of the incubation medium were placed on a titer microplate (MaxiSorb; Nalge Nunc, Napierville, IL) and dried overnight at 40°C. Non-specific binding sites were blocked with 3% bovine serum albumin, 0.05% Tween-20, and 0.15 M NaCl in 0.25 mM Tris-HCl (pH 7.5). Washing buffer contained 0.3% bovine serum albumin, 0.05% Tween-20, and 0.15 M NaCl in 0.25 mM Tris-HCl (pH 7.5). Tris-buffered saline was chosen rather than the phosphate buffer used in a previous study, because the phosphate buffer was incompatible with the alkaline phosphatase detection system. UEA-I detectable glycoconjugates in the supernatant and in the medium were determined in duplicate by means of a microplate reader (model MR 700; Dynatech, West Sussex, UK). A standard curve was constructed using bovine submaxillary gland mucin.

Data Presentation and Statistical Analysis
Data were expressed according to the formula: secreted glycoconjugate (amount in medium)/total glycoconjugate (amount in medium + amount in tissue) X 100. Data are means ± SEM. When appropriate, data were statistically analyzed using Student's t-test for unpaired values. Values of P < 0.05 were considered to be significant.

RESULTS
Identification of Lectin UEA-I Binding Sites in the Conjunctiva
We have shown that the lectin helix pomatia agglutinin selectively binds to conjunctival goblet cells. However, when helix pomatia agglutinin was used to set up an ELLA to measure goblet cell secretion, it lacked the desired linearity required for increasing concentrations of standard mucin. Thus, we used lectin UEA-I, which binds most strongly to α-L-fucosyl residues in oligosaccharides with type 2 and type 1 structures. Using the ELLA technique, UEA-I detected in a linear manner glycoconjugates from bovine submaxillary mucin ranging from 0.1 µg/ml to 100 µg/ml (data not shown).

We then conducted histochemical studies to determine whether UEA-I selectively binds to goblet cell secretory products. In rat conjunctiva, goblet cells formed clusters that are embedded within the stratified squamous epithelial cells. UEA-I binding was almost exclusively limited to conjunctival goblet cell secretory granules with minimal or no binding in the stratified squamous cells or the underlying stroma (Fig. 1A). Preincubation of UEA-I with L-fucose completely inhibited staining of goblet cells (not shown).

We also performed western blot analyses with inferior and superior conjunctiva and bovine submaxillary mucin as a positive control. UEA-I reacted with a single high-molecular-weight glycoprotein present in bovine submaxillary mucin (Fig. 1B). In inferior and superior conjunctival samples, UEA-I reacted with a high-molecular-weight glycoprotein of more than 220 kDa (Fig. 1B). Preincubation of UEA-I with L-fucose completely inhibited immunoreactivity (not shown).

These results show that UEA-I bound specifically to conjunctival goblet cell secretory products and thus could be used to measure glycoconjugate secretion from these cells in vitro, by means of the the ELLA technique.

Immunolocalization of Cholinergic Muscarinic and VIP Receptor Subtypes in Goblet Cells
In a first set of experiments, we performed western blot analyses to determine which subtypes of cholinergic muscarinic receptors were present in the conjunctiva. A membrane fraction was prepared from conjunctival homogenate by high-speed centrifugation, and the proteins in this fraction were subjected to SDS-PAGE followed by western blot analysis using sera specific to m₁, m₂, and m₃ muscarinic receptor subtypes. These antibodies had been generated against peptide sequences from the nonhomologous regions of the carboxyl terminus of m₁, m₂, and m₃ receptor subtypes. The specificity of these antibodies was determined by enzyme-linked immunosorbent assay and western blot analysis, with elimination of the primary antibody and preincubation of the primary antibody with the immunogenic peptide serving as controls. One major band was detected for each antibody indicating that m₁, m₂, and m₃ muscarinic receptor subtypes were all present in the conjunctival membrane fraction (Fig. 2). All three proteins migrated with an apparent molecular weight of approx-
FIGURE 1. (A) Localization of UEA-1 binding sites in rat conjunctiva. Sections from inferior conjunctiva were labeled with biotinylated UEA-1 lectin. Shown is the conjunctival epithelium that contained several layers of stratified squamous cells with clusters of variable numbers of goblet cells (arrows) intercalated between the stratified squamous cells. Goblet cells extended from the basement membrane, which is adjacent to the stroma, to the tear film. The cell bodies of the goblet cells, which contact the basement membrane, were not stained, whereas the secretory granules, which account for a large volume of the apical area of the goblet cells, were intensely stained. Sections were printed at low contrast so that the location of positive staining within the conjunctiva can be seen. Epi., epithelium. (B) Conjunctival homogenate was subjected to SDS-PAGE and western blot analysis using biotinylated UEA-1 as a primary antibody. Mucin from bovine submaxillary glands was used as a positive control. Lane 1: 100 μg/ml mucin standard; lane 2: inferior conjunctival homogenate; lane 3: superior conjunctival homogenate. Standard molecular weight markers (in kilodaltons) are indicated. Similar results were obtained in at least two other experiments. Magnification, (A) X20.

approximately 55 kDa, which is in agreement with the report of Ndoye et al.52

These results show that rat conjunctiva expressed all three subtypes of the muscarinic receptor. However, they did not indicate which cell type expressed these receptors. To determine this, we performed immunohistochemical studies using the same antibodies as were used in the western blot analysis experiments. M1 immunoreactivity was found in the stratified squamous cells, but not in goblet cells, whereas immunoreactivity to the M2 and M3 muscarinic receptor subtypes was found mainly in goblet cells (Fig. 3). M2 immunoreactivity, compared with that of M3, was not found on all goblet cells, suggesting that not all goblet cells expressed the M2 muscarinic receptor, whereas they all seemed to express the M3 subtype. M2 immunoreactivity was also seen in the stratified squamous cells (Fig. 3).

In another set of experiments, we used antibodies against VIPR1 and VIPR2 to determine their cellular distribution in the conjunctiva. These antibodies had been raised against the amino terminus of the receptors.17 Immunoreactivity to VIPR2 was seen surrounding the basal region of goblet cells and in the conjunctival stroma (Fig. 3). Use of preabsorbed serum substantially reduced the fluorescence (data not shown). Similar to the M2 muscarinic receptor, VIPR2 did not seem to be expressed on all goblet cells. VIPR1 could not be detected in the conjunctiva (not shown). M2 and M3 muscarinic receptors and VIPR2 did not seem to be expressed on the basolateral membranes of goblet cells as was expected. Instead, the antibody binding had a cuplike localization immediately subjacent to the secretory granules. This localization was confirmed in double-labeling experiments. Sections were incubated with anti-M3 muscarinic receptor antibody and a biotinylated UEA-1 lectin to stain goblet cell secretory granules. M3 muscarinic receptor was visualized with FITC and UEA-1 with rhodamine.

We also used propidium iodide to stain cell nuclei including those of goblet cells. M4 muscarinic receptors seemed to be localized immediately subjacent to the secretory granules and above the nuclei (Fig. 4). Combining our results from western blot analysis and immunocytochemistry experiments, we concluded that M2 and M3 receptors are on goblet cell membranes located subjacent to the secretory granules and above the nuclei, but we did not identify these membranes. Furthermore, muscarinic and VIP receptors were localized in the same area (midportion rather than apical or basal) of the goblet cells, as were VIP-containing nerves (which are parasympathetic and contain a muscarinic neurotransmitter and VIP), described in Dartt et al.20

These results show that conjunctival goblet cells expressed the M2 and M3, but not the M4, muscarinic receptor subtypes. They also show that VIPR2, but not VIPR1, was expressed in conjunctival goblet cells.
selective antagonists. Pirenzepine is an M₃ selective antagonist, gallamine an M₂ antagonist, and 4-DAMP mustard an irreversible M₁ antagonist. Pirenzepine at 10⁻⁵ M inhibited carbachol-induced secretion 54% ± 8% (n = 5), gallamine at 10⁻⁵ M 69% ± 14% (n = 7), and 4-DAMP mustard 72% ± 11% (n = 9; Fig. 6). Thus, the order of selectivity of antagonist inhibition was M₃ > M₂ > M₁.

When conjunctival pieces were incubated in the presence of VIP, there was a time-dependent stimulation of glycoconjugate release (Fig. 7A) that was approximately linear for 60 minutes. Thus a 1-hour incubation period was used to study the effect of increasing concentrations of VIP on goblet cell glycoconjugate secretion. The effect of VIP on conjunctival goblet cell glycoconjugate secretion was concentration-dependent, reaching a maximum at 10⁻⁴ M (twofold increase above baseline; Fig. 7B). VIP increased the amount of glycoconjugate secreted from 1.8% ± 0.6% to 4.1% ± 0.9% at 10⁻⁴ M (Fig. 7B; n = 5–6).

These results show that parasympathetic agonists are able to stimulate conjunctival goblet cell secretion with the cholinergic muscarinic pathway being more effective than the VIPergic one.

**DISCUSSION**

In the present study, we showed that conjunctival goblet cells expressed the M₂ and M₃ muscarinic receptor subtypes along with VIPR2. Muscarinic receptors have been identified by ³H-QNB binding to isolated villus and crypt cells of the rat small intestine and to colon. Autoradiographic mapping with in situ hybridization have revealed M₁ and M₃ subtypes on human airway mucosal glands. Autoradiographic mapping and non-subtype-selective VIP receptor antibodies have detected VIP receptors on rat and rabbit conjunctiva, on mucous cells of human tracheal submucous glands, and on the human, rat, and rabbit intestinal epithelium. However, to our knowledge, this is the first study showing that muscarinic and VIP receptors are directly localized on goblet cells.

Muscarinic and VIP receptors had a surprising location on goblet cells. In lacrimal gland acini, using the same antisera, we found that M₃ receptors and VIPR1 and VIPR2 were located on the basolateral membranes, as expected. In contrast, in conjunctival goblet cells, muscarinic and VIP receptors were located on membranes above the nuclei and subjacent to the mucin granules. These membranes were not identified and could be plasma membranes, intracellular membranes, or both. M₂, M₃, and VIPR2 had a similar location, except that VIPR2 was located on a portion of the lateral membranes that separate the individual goblet cells within a cluster. The localization of these receptors in the midportion rather than apical or basal area of goblet cells seems to correspond to that of VIP-containing nerves described in Darrt et al. The localization of receptors adjacent to the secretory granules could decrease the latency between activation of receptor and the beginning of exocytosis, or because all secretory granules are released on activation of a cell, it could ensure activation of many secretory granules at the same time. It has been shown in exocrine gland acinar cells including the lacrimal, that the first increase in the intracellular [Ca²⁺] initiated by activation of M₃ receptors occurs in the apical portion of the cell near the secretory granules rather than in the basolateral portion of the cell near the receptors.
To determine the role of cholinergic agonists and VIP on goblet cell secretion, we developed an ELLA using the lectin UEA-I. Our results showed that lectin UEA-I could be used to measure specifically goblet cell secretion in vitro. This conclusion is based on the following findings: In the histochemical experiments, UEA-I specifically stained goblet cell secretory granules; in the western blot analysis, UEA-I reacted with a single high-molecular-weight glycoprotein (>220 kDa) in conjunctival homogenate that migrated to the same position as a standard mucin from the submaxillary gland; the binding of UEA-I is specific, because it can be inhibited by preincubating the lectin in the presence of fucose; and in the ELLA experiments, UEA-I detected increasing concentrations of mucin from the submaxillary gland in a linear manner.

Using the ELLA, we found that the cholinergic agonist carbachol induced a time- and concentration-dependent stimulation of goblet cell secretion. Conjunctival goblet cell secretion was not detected until a relatively large concentration of carbachol (10^-5 M) was used, and maximum secretion was not obtained even at 10^-4 M. Colonic goblet cell lines, cultured tracheal goblet cells, and nasal goblet cells respond similarly to high concentrations of carbachol, with maximum secretion occurring at 10^-4 M to 10^-3 M carbachol. In contrast, goblet cells in the rabbit nictitans and pancreatic ducts responded to carbachol at 10^-3 M, and the latter had a maximal response at 10^-5 M. Diffusion into tissue compared with isolated cells cannot explain the differences in sensitivity, because colonic cell lines were less sensitive to carbachol than were goblet cells in the nictitans and pancreatic duct tissue.

There are three possible explanations for this difference in sensitivity. First, the number of muscarinic receptors on goblet
FIGURE 4. Immunolocalization of the M₃ muscarinic receptor subtype in conjunctival goblet cells. Sections from inferior conjunctiva were processed for immunohistochemical studies using a polyclonal antibody against the M₃ muscarinic receptor subtype. The same sections were double-labeled with UEA-I to stain goblet cell secretory granules (left) or propidium iodide (PI) to stain cell nuclei including those of goblet cells (right). These are confocal micrographs of 12 images stacked together. Micrographs in the left column contain a single cluster of goblet cells with individual goblet cells indicated by arrows. Intense green staining indicates the location of M₃ receptors. Diffuse red staining indicates the location of secretory granules. Note that green fluorescence is subjacent to red fluorescence. Note that M₃ receptors were not located on the basolateral membranes of goblet cells indicated by an arrowhead. Micrographs in the right column contain three clusters of goblet cells with individual goblet cells indicated by arrows. Intense green staining indicates the location of M₃ receptors. Intense red staining indicates cell nuclei. Note that green fluorescence is just above red fluorescence. Micrographs were printed at low contrast so that the location of positive staining within the conjunctiva could be seen. Similar results were obtained in at least two other experiments. Magnification, ×40.
Regulation of Conjunctival Goblet Cell Secretion

Cells could vary among tissues. Second, the subtypes of muscarinic receptors present on goblet cells could vary among tissues. This could lead to differential sensitivity to agonist because the efficiency of coupling to phospholipase C is receptor subtype-specific. Finally, the ratio of stimulatory (M1, M3, and M5) compared with inhibitory (M2 and M4) muscarinic receptor subtypes could vary between tissues. Further experimentation is necessary to decide between these possibilities.

The secretory response of rat conjunctival goblet cells to carbachol was completely inhibited by the muscarinic antagonist atropine, showing that carbachol is acting primarily through activation of muscarinic receptors. Using subtype-selective muscarinic receptor antagonists, we found that activation of M1, M2, and M3 receptors each stimulate goblet cell secretion, with M3 receptors being the most effective and M1 receptors being the least effective. Activation of M2 and M3 receptors to stimulate goblet cell secretion is consistent with these receptors being located on goblet cells. M2 receptors, however, were detected only on the stratified squamous cells, and activation of these receptors would not be expected to stimulate goblet cell secretion. One explanation is that the receptor-subtype-selective antagonists used could have nonspecifically inhibited the other receptor subtypes. Specifically, M1 receptor antagonists can inhibit M2 and M4 receptors in addition to M1 receptors. Comparative dose–response curves, which we are not able to obtain using conjunctival tissue, would have helped resolve this issue. Another possible explanation for this result is that cholinergic agonists could have activated M1 receptors on the stratified squamous cells to secrete a paracrine factor that in turn stimulated goblet cell secretion. A final possibility is that our ELLA for secretion detected stratified squamous cell secretion in addition to goblet cell secretion. This latter possibility is very unlikely, given that UEA-I binding was limited to goblet cells.

M2 muscarinic receptors are coupled to a pertussis toxin-sensitive inhibitory guanosine triphosphate-binding protein, Gi, and inhibit adenylate cyclase. In contrast, M1 and M3 muscarinic receptors are coupled to the activation of the phosphoinositide-specific phospholipase C which cleaves phos-
FIGURE 7. Effect of VIP on glycoconjugate secretion from conjunctival goblet cells. Conjunctiva was incubated for 1 hour at 37°C, with or without VIP. (A) Time dependency of VIP-induced glycoconjugate secretion. •, Baseline; ○, VIP (10^{-8} M). Data are mean ± SEM of six independent experiments. (B) Concentration dependency of VIP-induced glycoconjugate secretion. Data are mean ± SEM of five to six independent experiments. *Significant difference from results without VIP.

In a recent study, we found that increasing the cytosolic 
[Ca^{2+}] using a Ca^{2+} ionophore or activation of protein kinase C using phorbol esters stimulates goblet cell secretion (Dartt et al. unpublished data, 1998). These studies suggest that M_3 muscarinic receptors in conjunctival goblet cells are likely to be coupled to the phospholipase C pathway. Although M_2 receptors are also located on goblet cells, the signaling mechanism used by these receptors to stimulate goblet cell secretion is unknown.

In addition to carbachol, VIP also stimulated conjunctival goblet cell secretion. Because acetylcholine and VIP are contained in parasympathetic nerves and stimulate secretion, parasympathetic nerves seem to play a major role in conjunctival goblet cell secretion. In the present study we found that maximum secretion was induced by 10^{-8} M VIP, but that a supramaximal concentration of VIP caused submaximal secretion. We previously obtained an identical concentration-response curve measuring the effect of VIP on goblet cell secretion in vivo. Using an ELLA with the lectin helix pomatia agglutinin, we also found that VIP stimulated conjunctival goblet cell secretion in vitro with a maximum effect at 10^{-8} M VIP, but a supramaximal concentration did not cause a submaximal response. In goblet cells from other tissues, a similar concentration of VIP induces a maximal secretory response. In colonic goblet cells in culture and in nasal and pancreatic duct goblet cells, VIP at 10^{-7}M to 10^{-6} M stimulates secretion. In contrast, tracheal goblet cells do not contain VIP receptors and do not secrete in response to VIP. Thus, goblet cells that contain VIP receptors and respond to VIP, including conjunctival goblet cells, have similar sensitivity to VIP, unlike goblet cell muscarinic receptors that vary among tissues in sensitivity to carbachol.

In a previous report we noted that the number of conjunctival goblet cells per unit area varied considerably among subjects and between groups of animals. This was reflected in the variability in our results in experiments in vivo. The same variability occurred in experiments in vitro in the present study. This variability is most notable when the amount of goblet cell secretion is compared between the time- and concentration-dependency studies for VIP. The percentage of glycoconjugates secreted under basal conditions in the time-dependency experiments was greater than in the concentration-dependency experiments. This was also true for the VIP-stimulated conditions. Even though the percentage of glycoconjugates secreted varied, the fold-stimulation did not vary as dramatically.

Another drawback of in vitro experiments on conjunctival goblet cells is also related to the same variability among animals. All concentration-dependency studies and those studies using antagonists had to be performed with all conditions in each animal. Because we were able to divide the inferior conjunctiva into only four pieces, only four conditions could be used in a given experiment. We were limited to three concentrations of agonist or one concentration of antagonist.

In summary, we showed that M_2 and M_3, but not M_1, muscarinic receptors and VIPR2, but not VIPR1, were directly localized on goblet cells. We also showed that activation of these receptors stimulated goblet cell secretion. Thus we conclude that conjunctival goblet cell secretion, in vivo, is under the control of parasympathetic nerves.
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References