Expression of Muscarinic and Adrenergic Receptors in Normal Human Conjunctival Epithelium

Amalia Enríquez de Salamanca,1 Karyn F. Siemasko,2 Yolanda Diebold,1 Margarita Calonge,1 Jianping Gao,2 Mónica Juárez-Campo,1 and Michael E. Stern2

PURPOSE. To study the presence of muscarinic and α- and β-adrenergic receptors in a normal human conjunctival epithelium (IOBA-NHC) cell line.

METHODS. Neurotransmitter receptors were determined in IOBA-NHC cells by flow cytometry, immunofluorescence, and Western blot analysis. Antibodies to M1-, M2-, and M3-muscarinic and to α1A-, α1D-, α2A-, α2B-, α2C-, β1-, β2-, and β3-adrenergic receptor subtypes were used. Different culture media were tested, including the addition of tumor necrosis factor (TNF)-α and/or interferon (IFN)-γ. Normal human conjunctiva biopsy specimens and rat tissues were used in control experiments.

RESULTS. By immunofluorescence microscopy, all receptor subtypes, except the α2A-adrenergic receptor, were detected in control biopsy specimens. By flow cytometry, the M2- and M3-muscarinic receptors and α1A-, α1D-, α2A-, α2B-, α2C-, β1-, and β2-adrenergic receptors were detected intracellularly and in cell membranes of the IOBA-NHC cells. M1-muscarinic and β3-adrenergic receptors were detected only intracellularly, but were mobilized to the cell membrane when cholera toxin and hydrocortisone were omitted from the culture medium. Confocal microscopy detected the M1 and M3-muscarinic and α1A-, α2A-, α2B, β1-; and β2-adrenergic receptor subtypes. Western blot analyses showed bands for all receptors. M3-muscarinic and α2B-adrenergic receptors expression was upregulated when cells were treated with the proinflammatory cytokines IFNγ and/or TNFα.

CONCLUSIONS. The IOBA-NHC cell line maintained expression of the neurotransmitter receptors expressed in normal human conjunctival epithelium. A proinflammatory medium upregulated expression of some receptors. Although the functional state of these receptors is unknown, these findings justify further use of the IOBA-NHC cell line to study the neural component of conjunctival inflammation. (Invest Ophthalmol Vis Sci. 2005;46:504–513) DOI:10.1167/iovs.04-0665

Interaction between the nervous, immune, and endocrine systems plays an important role in inflammatory diseases. In addition to systemic neuroendocrine regulation, there is local regulation at the site of inflammation through the release of proinflammatory neuropeptides and neurotransmitters from peripheral nerves. These neurotransmitters can modulate the activity of both inflammatory and epithelial cells through specific receptors for them.

There is growing evidence that neural alterations occur in several ocular surface diseases—for example, in dry eye syndrome and allergic disorders (Mottele L, et al., IOVS 2003; 44: ARVO E-Abstract 3743). Dry eye syndrome is mediated by an immune-based inflammation in the components of the lacrimal functional unit in which the innervational loop between the lacrimal glands and the ocular surface becomes altered. In a murine model of Sjögren’s syndrome, neurotransmitter release from the lacrimal and salivary gland nerves is impaired. Also, unresponsiveness to cholinergic stimuli and presence of autoantibodies against the M1-muscarinic receptor subtype have been reported in patients with Sjögren’s syndrome.

Neurotransmitters and neuropeptides have many ocular functions. Receptors for these substances are present in the ocular tissues, but the functional consequences of their activation have not always been fully characterized. Sensory, parasympathetic, and sympathetic nerves are present in the conjunctival stroma and epithelium of several species, but only parasympathetic and sympathetic nerves have been detected adjacent to rat conjunctival goblet cells. Cholinergic, adrenergic, and other receptors have also been reported in goblet and nongoblet stratified squamous epithelial cells in rat, mouse, and human conjunctiva. (Diebold Y, et al., manuscript submitted). These nerves and neurotransmitter receptors are important elements in the pathways that integrate the lacrimal functional unit. Thus, we compared the expression of muscarinic and adrenergic receptors in cultured IOBA-NHC cells derived from normal human conjunctiva with expression in vivo. We then assessed changes in receptors when cultured cells were exposed to inflammatory cytokines.

MATERIALS AND METHODS

All reagents were from Sigma-Aldrich (St. Louis, MO), unless otherwise specified. Table 1 summarizes the information regarding clone, source, and dilution of primary and secondary antibodies. Human recombinant interferon (IFN)-γ and tumor necrosis factor (TNF)-α were purchased from R&D Systems (Minneapolis, MN). Fluorescence antifade mounting medium (Vectashield) was from Vector Laboratories (Burlingame, CA). Propidium iodide (PI) was obtained from Molecular Probes (Leiden, The Netherlands). Bicinchoninic acid (BCA) protein-determinant assay was from Pierce (Rockford, IL). Molecular markers (Rainbow) were from Amersham Biosciences (Buckinghamshire, UK), and unstained precision protein standards and StreptAvidin-HRP solution were from Bio-Rad (Hercules, CA). All the other SDS-PAGE and Western blot reagents were obtained from Bio-Rad.
Table 1. Antibodies Used for Flow Cytometry, Immunofluorescence, or Western Blot Analysis

<table>
<thead>
<tr>
<th>Receptor*</th>
<th>Clone</th>
<th>Dilution (µg/mL)†</th>
<th>Secondary Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>AS-3701S</td>
<td>Serum 1:1</td>
<td>BD-PharMigen Jackson ImmunoRes.</td>
</tr>
<tr>
<td>M2</td>
<td>AS-3721S</td>
<td>Serum 1:1</td>
<td>BD-PharMigen Jackson ImmunoRes.</td>
</tr>
<tr>
<td>M3</td>
<td>AS-3741S</td>
<td>Serum 1:1</td>
<td>BD-PharMigen Jackson ImmunoRes.</td>
</tr>
<tr>
<td>α1A</td>
<td>(C-19) sc-1477</td>
<td>10 4 2</td>
<td>BD-PharMigen Jackson ImmunoRes.</td>
</tr>
<tr>
<td>α1B</td>
<td>(C-18) sc-1476</td>
<td>10 4 2</td>
<td>Zymed Jackson ImmunoRes.</td>
</tr>
<tr>
<td>α1D</td>
<td>(C-19) sc-9352</td>
<td>10 4 2</td>
<td>Zymed Jackson ImmunoRes.</td>
</tr>
<tr>
<td>α2A</td>
<td>(C-19) sc-1478</td>
<td>10 4 2</td>
<td>Zymed Jackson ImmunoRes.</td>
</tr>
<tr>
<td>α2B</td>
<td>(C-19) sc-1479</td>
<td>10 4 2</td>
<td>Zymed Jackson ImmunoRes.</td>
</tr>
<tr>
<td>β1</td>
<td>(A-20) sc-567</td>
<td>10 3 1</td>
<td>BD-PharMigen Jackson ImmunoRes.</td>
</tr>
<tr>
<td>β2</td>
<td>(H-75) sc-9042</td>
<td>10 2 1</td>
<td>BD-PharMigen Jackson ImmunoRes.</td>
</tr>
<tr>
<td>β3</td>
<td>(C-20) sc-1472</td>
<td>10 2 1</td>
<td>BD-PharMigen Jackson ImmunoRes.</td>
</tr>
</tbody>
</table>

FC, flow cytometry; IMF, immunofluorescence; WB, Western blot; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase.

* All primary antibodies were polyclonal and were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), except serum antibodies to M1-, M2-, and M3-muscarinic receptors, which were obtained from R&D Antibodies (Benicia, CA).
† Antibody concentration of muscarinic serum antibodies not provided by the manufacturer; serum dilution used is indicated instead.

Human and Animal Tissues

Biopsy specimens from normal human conjunctiva (n = 4) were obtained, with informed consent, from healthy donors who were undergoing cataract surgery. Rat eyeballs, kidneys, and heart and mouse conjunctiva were surgically excised after the animals were euthanatized and were used as positive control specimens. Experiments conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the Declaration of Helsinki and were approved by the IOBA Research Committee.

Human Conjunctival Epithelial IOBA-NHC Cell Line and Culture Conditions

The IOBA-NHC cell line derived from normal human conjunctiva was used.20 The normal culture medium was DMEM/F12 (Invitrogen-Gibco, Inchinnan, UK) supplemented with 2 ng/mL human epidermal growth factor (EGF), 0.1 µg/mL cholera toxin, and 1 µg/mL bovine pancreatic insulin, plus 10% fetal bovine serum (FBS), 5 µg/mL hydrocortisone, and antibiotics (50 U/mL penicillin, 50 mg/mL streptomycin, and 2.5 µg/mL amphotericin B). Cholera toxin- and hydrocortisone-free medium supplemented with 2% FBS was used when specified. The medium was changed every 2 days. Cells in passages 62 to 75 were used.

Flow Cytometry Assays

Analysis of the Adrenergic and Muscarinic Receptor Expression in the IOBA-NHC Cell Line. IOBA-NHC cells, 1 × 10^5 cells/mL, were washed and resuspended in flow cytometry buffer composed of 1% bovine serum albumin (BSA) and 0.02% sodium azide in ice-cold phosphate-buffered saline (PBS). The cells were stained with anti-adrenergic or anti-muscarinic antibodies (Table 1) at 4°C for 20 minutes in the dark, washed, and incubated with secondary antibody for intracellular staining, cells were fixed with 2% formaldehyde for 15 minutes at 4°C and washed, and primary and secondary antibodies prepared in buffer (0.5% saponin, 1% BSA, and 0.1% sodium azide in PBS) were added at 4°C. Negative control experiments included the omission of the primary antibodies. Samples were analyzed by flow cytometry (FACSCalibur and Cell Quest software; BD Biosciences).

Effect of Inflammatory Cytokines on Adrenergic and Muscarinic Receptor Expression in the IOBA-NHC Cell Line. The effect of the inflammatory cytokines IFN-γ and TNF-α on adrenergic and muscarinic receptor subtype expression level was analyzed in IOBA-NHC cells by flow cytometry. Cells were plated at 1 × 10^5 cells/mL and incubated for 48 hours in the absence or the presence of IFN-γ (500 U/mL), TNF-α (25, 50, and 100 ng/mL), and a combination of IFN-γ (500 U/mL) and TNF-α (25 ng/mL). Untreated or stimulated cells were harvested after 48 hours, resuspended in buffer, and analyzed as described in the prior section.

Immunofluorescence Assays

Normal human conjunctiva biopsy specimens and rodent conjunctiva, kidney, brain, and heart were fixed in 4% formaldehyde for at least 4 hours, rinsed in 5% sucrose dissolved in PBS, placed overnight at 4°C in 30% sucrose dissolved in PBS, embedded in optimal cutting temperature (OCT) compound, and frozen. Cryostat sections (7 µm) were collected in poly-l-lysine-treated slides and kept at −80°C until used. IOBA-NHC cells in passages 62 to 65 were seeded onto glass coverslips. When confluence was reached, they were fixed in ice-cold methanol for 10 minutes, washed in PBS, and kept frozen until use. On the day of use, human conjunctiva, rodent tissue cryosections, and IOBA-NHC cell coverslips were hydrated for 30 minutes and blocked in PBS containing 1% BSA, 4% PBS, and 0.2% to 0.3% Triton X-100 for 1 hour. Antibodies to muscarinic and adrenergic receptor subtypes diluted in blocking buffer (Table 1) were incubated overnight at 4°C. Cells were washed three times. Secondary antibodies were incubated for 1 hour at room temperature. After they were washed, cells were counterstained with PI; 1:12,000, mounted, and viewed in a confocal laser scanning microscope (model LSM310; Carl Zeiss Meditec, Jena, Germany), equipped with a krypton-argon laser. FITC and PI were excited with 488 and 543-nm emission laser beams, respectively, and detected with a band-pass emission barrier filter. Digital images were taken. Negative control experiments included the omission of primary antibodies. All images were obtained using a 63× objective, except Figure 2E, which was obtained with a 40× objective.

Electrophoresis and Western Blot Analysis

Cells and tissues were homogenized in ice-cold radioimmunoprecipitation assay (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) plus protease inhibitors (100 µL/mL phenylmethylsulfon fluoride, 6 µL/mL aprotinin, and 100 mM sodium orthovanadate). After homogenization, samples were incubated for 30 minutes on ice and centrifuged at 14,000 rpm for 30 minutes at 4°C. Total cell protein in the supernatant was measured by the BCA method, which was compatible with the calibration curve of bovine serum albumin (Sigma, St. Louis, MO). Protein concentration was determined with the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Tyrosine hydroxylase, VIP, cholera toxin, and calcitonin gene-related peptide (CGRP) were detected by Western blot analysis. Immunoblots were probed with antibodies specific for the following proteins: α-subunit of the G protein (BD Pharmingen), α1A (North East Biolabs), α1B (Biologics), α1D (Cayman), α2A (Cayman), α2B (Cayman), α2C (Cayman), β1 (CosmoBio), β2 (MBL), and β3 (MBL). Blots were analyzed with the chemiluminescent substrate ECL (Amersham Biosciences). Blots were visualized on X-ray film (Kodak, Rochester, NY). The intensity of the bands was quantified using the ImageQuant software (Raytest, Germany) and normalized to the expression of the loading control GAPDH.
with the buffer used for homogenization. Proteins in the homogenate were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gels according to the method of Laemmli. Proteins were transferred to nitrocellulose membranes according to Towbin and blocked in Tris-buffered saline (5% dried milk, 4% FBS, and 0.05% Tween-20 in Tris; TBS-T) overnight. Membranes were incubated with antibodies to muscarinic and adrenergic receptor subtypes for 1 hour at room temperature in blocking buffer. They were then washed three times with TBS-T buffer and incubated with secondary HRP-conjugated antibodies. StrepTactin-HRP solution (1:5000; Bio-Rad) was added for chemiluminescence protein standards detection. Membranes were incubated with HRP reagent according to the manufacturer's protocol (Immunostar; Bio-Rad). Immunoreactive bands were visualized by the chemiluminescence method (Chemidoc XRS; Bio-Rad), and images were analyzed on computer (Quantity One software; Bio-Rad). Rat conjunctiva, kidney, aorta, and brain were used as control tissues.

RESULTS
Flow Cytometry Analysis
Flow cytometry of IOBA-NHC cells revealed constitutive expression on cell membranes of the M2- and M3-muscarinic receptors and the β1, β2, α1A, α1B, α1D, α2A, α2B, and α2C-adrenergic receptors as well as expression in intracellular locations (Fig. 1A). M1-muscarinic and β2-adrenergic receptors were detected only intracellularly when normal culture medium was used (Fig. 1A). However, cell membrane expression of both these receptors was detected when cells were cultured

![Cell Membrane](image1)
![Intracellular](image2)

**Figure 1.** Flow cytometry analysis of muscarinic and adrenergic receptor subtypes in the IOBA-NHC cell line. Shaded traces: negative control; open traces: receptor expression. (A) Normal culture medium. (Left) Cells expressed detectable levels of all cell-membrane–bound muscarinic receptors except M1 and all adrenergic subtypes except β2. (Right) Cells expressed detectable intracellular levels of all muscarinic and adrenergic receptor subtypes. (B) When cultured for 72 hours in cholera toxin–and hydrocortisone-free medium, cells expressed detectable levels of cell membrane-bound M1-muscarinic (left) and β2-adrenergic (right) receptors. The primary antibody was omitted from negative control experiments. At least three independent experiments were performed. Each flow cytometry histogram corresponds to a representative experiment. FL1-H, relative fluorescence intensity; MFI, mean fluorescence intensity; CT, cholera toxin; H, hydrocortisone.
FIGURE 2. Confocal microscopy immunofluorescence detection of muscarinic and β-adrenergic receptor subtypes in the IOBA-NHC cell line. Cryosections of normal human conjunctiva biopsy specimens were used as control tissues. Both control (A–C, G–I) and IOBA-NHC (D–F, J–L) cells were double labeled with FITC-conjugated IgG secondary antibody to primary antibody for the M1, M2, and M3 muscarinic and β1, β2, and β3 adrenergic receptors (green) and with PI (red) to identify nuclei. Immunoreactivity to the M1-, M2-, and M3-muscarinic receptors was detected in all conjunctival epithelial cells in normal human conjunctiva (A–C). Immunoreactivity to the M2- and M3-muscarinic receptors was detected in IOBA-NHC cells, showing a cytosolic distribution (E, F). No detectable immunofluorescence was obtained for the M1-muscarinic receptor (D).

Immunoreactivity to the β2 and β3-muscarinic receptors was detected in IOBA-NHC cells, showing a cytosolic distribution (J, K). No detectable immunofluorescence was obtained for the β3-adrenergic receptor subtype in the IOBA-NHC cell line (L). Micrographs are representative of at least three different experiments. Magnification: (A–D, F–L) ×239; (E) ×204.
FIGURE 3. Confocal microscopy immunofluorescence detection of α₁- and α₂-adrenergic receptor subtypes in the IOBA-NHC cell line. Cryosections of normal human conjunctiva biopsy specimens were used as control tissues. Both control (A–C, G–I) and IOBA-NHC (D–F, J–L) cells were double labeled with FITC-conjugated IgG secondary antibody to primary antibody for α₁A-, α₁B-, α₁C-, α₂A-, α₂B- or α₂C-adrenergic receptors (green) and with PI (red) to identify nuclei. Immunoreactivity to α₁A-, α₁B-, and α₁D-adrenergic receptors was detected in human conjunctiva (A–C). Immunoreactivity to the α₂A-adrenergic receptor was detected in IOBA-NHC cells, showing a specific intracellular location (D). Neither α₁B- nor α₁D-adrenergic receptor subtypes were detected in IOBA-NHC cells (E, F). Immunoreactivity to α₂A- and α₂B-adrenergic receptor subtypes was detected in human conjunctiva (G, H). The α₂C-adrenergic receptor subtype was not detected in normal human conjunctiva (I). Immunoreactivity to α₂A- and α₂B-adrenergic receptors was detected in IOBA-NHC cells (J, K). The α₂C-adrenergic receptor was not detected in the cell line (L). Micrographs are representative of at least three experiments. Magnification, ×239.
in cholera toxin- and hydrocortisone-free medium supplemented with 2% FBS for 72 hours before analysis (Fig. 1B).

**Immunofluorescence Assays**

The cellular localization of muscarinic and adrenergic receptor subtypes in normal human conjunctiva biopsy specimens and in IOBA-NHC cells was studied by confocal microscopy. Immunoreactivity to the M1-, M2-, and M3-muscarinic receptors was detected in all epithelial cells in conjunctiva cryosections (Figs. 2A–C). Confocal microscopy revealed the presence of the M1-, M2-, and M3-muscarinic receptor subtypes in the cytosol of the IOBA-NHC cell line (Figs. 2E, 2F). No immunoreactivity was found for the M4-muscarinic receptor (Fig. 2D).

The β1-, β2-, and β3-adrenergic receptor subtypes were detected in human conjunctiva biopsy specimens, as previously shown. All epithelial cells were positive (Figs. 2G–I). The β1- and β2-adrenergic receptor subtypes were also present in IOBA-NHC cells (Figs. 2J, 2K) and had a predominantly cytosolic localization. No immunoreactivity was found for the β3-adrenergic receptor subtype (Fig. 2L).

Most of the α-adrenergic receptor subtypes were detected in normal human conjunctiva biopsy specimens (Figs. 3A–C, 3G, 3H), as previously described (Diebold Y, et al., 2003 Singapore Eye Research Institute/Association for Research in Vision and Ophthalmology meeting; Diebold Y, et al., manuscript submitted). Immunoreactivity for α1A-adrenergic receptors was detected in both goblet and nongoblet cells (Fig. 3A), and, in the apical cell layer, it was present in clusters. The α1B-adrenergic receptor was present in the basal epithelial cell layer (Fig. 3B). Immunoreactivity for the α1D-adrenergic receptor was detected more intensely in the goblet cells and was weaker in nongoblet cells (Fig. 3C). The α2A-adrenergic receptor was detected in all epithelial cells. In contrast, the α2C-adrenergic receptor was clearly located in basal epithelial cells but only weakly in the rest of the epithelium (Figs. 3G, 3H). No immunoreactivity was detected for the α2C-adrenergic receptor in normal human conjunctiva (Fig. 3I).

In the IOBA-NHC cell line, α1A-, α2A- and α2C-adrenergic receptors were detected. The α1A-adrenergic receptor was clustered about the nucleus, whereas α2A- and α2C-adrenergic receptors were in the cell membranes and cytosol (Figs. 3D, 3J, 3K). No immunoreactivity was found for the α1D-, α1E-, and α2C-adrenergic receptors (Figs. 3E, 3F, 3L).

A change in the distribution of immunofluorescence of the α1A- and α2C-adrenergic receptors was observed when cells were cultured for 48 hours in cholera toxin- and hydrocortisone-free medium supplemented with 2% FBS. A slight mobilization from perinuclear clusters toward the cell membrane occurred in the α1A-adrenergic receptor (Fig. 4A). The α2C-adrenergic receptor, which was not detected under the standard culture conditions, appeared in the cell membranes of IOBA-NHC cells in cholera toxin- and hydrocortisone-free medium supplemented with 2% FBS (Fig. 4B). No change in immunofluorescence distribution was observed for any of the other receptors studied (data not shown).

**Electrophoresis and Western Blot Analysis**

Western blot analysis of IOBA-NHC cell homogenates subjected to SDS-PAGE electrophoresis (Table 2) had immunoreactive bands for the M1-, M2-, and M3-muscarinic receptors (Fig. 5). A major 58-kDa band was detected for each. Weaker immunoreactive bands were also detected at 68 kDa. Immunopositive bands were also detected for the β1-, β2-, and β3-adrenergic receptors (Fig. 5). A major band at ∼60 kDa was present for the β1-, β2- and β3-adrenergic receptor subtypes in IOBA-NHC cells, along with a weaker 70-kDa band.

**Table 2. Molecular Weight of Immunoreactive Bands**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Reported MW*</th>
<th>C+</th>
<th>IOBA-NHC Cell Line</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>55</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68</td>
<td>68</td>
</tr>
<tr>
<td>M2</td>
<td>55</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68</td>
<td>68</td>
</tr>
<tr>
<td>M3</td>
<td>55</td>
<td>109</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68</td>
<td>68</td>
</tr>
<tr>
<td>α1A</td>
<td>60</td>
<td>115</td>
<td>115</td>
</tr>
<tr>
<td>α1B</td>
<td>67</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>α1D</td>
<td>140</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>68</td>
<td>67</td>
</tr>
<tr>
<td>α2A</td>
<td>64</td>
<td>115</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td></td>
<td>67</td>
<td>67</td>
</tr>
<tr>
<td>α2B</td>
<td>64</td>
<td>115</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td></td>
<td>70</td>
<td>67</td>
</tr>
<tr>
<td>α2C</td>
<td>64</td>
<td>115</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td></td>
<td>67</td>
<td>67</td>
</tr>
<tr>
<td>β1</td>
<td>50–55</td>
<td>120</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td></td>
<td>70</td>
<td>67</td>
</tr>
<tr>
<td>β2</td>
<td>68</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>70</td>
<td>80</td>
</tr>
<tr>
<td>β3</td>
<td>65</td>
<td>120</td>
<td>116</td>
</tr>
<tr>
<td></td>
<td></td>
<td>70</td>
<td>67</td>
</tr>
</tbody>
</table>

* Data provided by the manufacturers, C+ (control tissue) for M1, M2, M3: rat conjunctiva; for α1A, α1B, α1D, α2A, α2B, β1, and β2: rat kidney; for β3: aorta; for α2C: rat brain.; MW, molecular weight.

**Figure 4.** Confocal microscopy immunofluorescence detection of α1C- and α2C-adrenergic receptors in the IOBA-NHC cell line cultured 72 hours in cholera toxin- and hydrocortisone-free medium. Cells were double labeled with FITC-conjugated IgG secondary antibody (green) to primary antibody for the α1C- or α2C-adrenergic receptors and with PI (red) to identify nuclei. (A) Immunoreactivity to the α1C-adrenergic receptor subtype was detected in IOBA-NHC cells, showing a slight mobilization from previous specific intracellular location shown in Figure 3D toward the cytosol. (B) The α2C-adrenergic receptor subtype was predominantly located in the cytosol. These micrographs are representative of at least three different experiments. Magnification, ×218.
The IOBA-NHC cell line contained immunoreactive bands for \( \alpha_{1A} \), \( \alpha_{1B} \), and \( \alpha_{1D} \)-adrenergic receptor subtypes (Fig. 5). Major bands were present at 55, 60 and 59 kDa for the \( \alpha_{1A} \), \( \alpha_{1B} \), and \( \alpha_{1D} \)-adrenergic receptor subtypes, respectively. Weaker bands were detected at 67 kDa in the three cases.

The \( \alpha_{2A} \), \( \alpha_{2B} \), and \( \alpha_{2C} \)-adrenergic receptor subtypes were also present in the IOBA-NHC cell line (Fig. 5). Major bands were located at 58, 57 and 58 kDa, respectively. Weaker bands were again detected at 67 kDa for the \( \alpha_{2A} \) and \( \alpha_{2B} \)-adrenergic receptors. For the \( \alpha_{2C} \)-adrenergic receptor, a strong band was detected at 32 kDa, which was also weakly present in rat kidney homogenates.

For all receptors analyzed, immunoreactive bands at higher molecular weights (~110 to 120 kDa) were present. These may correspond to receptor dimers. Table 3 summarizes the results obtained with the three different techniques used: flow cytometry, immunofluorescence, and Western blot analysis.

### Effect of Inflammatory Cytokines on Muscarinic and Adrenergic Receptor Expression in IOBA-NHC Cell Line

To study the expression of muscarinic and adrenergic receptors under inflammatory conditions, we exposed the IOBA-NHC cells to a proinflammatory medium resembling a diseased ocular surface. IFN-\( \gamma \) upregulated M1-muscarinic receptor expression (Fig. 6A) but had no effect on the other receptors examined (data not shown). TNF-\( \alpha \) did not change the expression levels of either the muscarinic or adrenergic receptors (data not shown) at any of the doses tested. When cells were incubated with the combination of cytokines, \( \alpha_{2B} \) and \( \alpha_{2C} \)-adrenergic receptor cell surface expression was enhanced (Figs. 6B, 6C).

### DISCUSSION

This study demonstrates by flow cytometry, immunofluorescence microscopy, and Western blot analysis that IOBA-NHC cells express the muscarinic and adrenergic neurotransmitter...
receptors that are also expressed in normal human conjunctiva epithelium (Table 5). The expression of some of these receptors can be upregulated when cells are treated with proinflammatory cytokines. The $M_1$-muscarinic and $\alpha_{1A}$-, $\alpha_{1D}$-, $\alpha_{2C}$-, or $\beta_2$-adrenergic receptor subtypes were detected by flow cytometry but not by microscopy, perhaps because of the different sensitivities of the two techniques and/or the tissue fixation necessary for microscopy.

We found $M_1$, $M_2$, and $M_3$-muscarinic receptors in all epithelial cells of the conjunctiva cryosections. Previously, we described these receptors to be localized in occasional epithelial cells throughout the human conjunctival epithelium, including the goblet cells, with $M_2$ and $M_3$-muscarinic receptors especially prominent in the basal epithelial cell layer. The differences between findings in that study and in the present one may be attributed to the use of cadaveric conjunctival tissues in the previous work, different localization of biopsies tissue, and different batches of antibodies.

Western blot analysis of IOBA-NHC homogenates subjected to SDS-PAGE revealed the presence of immunoreactive bands for all the receptors studied (Fig. 5). The immunoreactive bands obtained with rat control tissues had a slightly higher molecular weight than previously reported (Table 2). Western blot analysis of the $\beta_2$, $\alpha_2$, $\alpha_3$, $\alpha_4$, and $\alpha_5$-adrenergic receptors in the IOBA-NHC cell line showed the major immunoreactive bands at slightly lower molecular weight than that reported for these receptors. In addition, a weaker immunoreactive band was detected in all cases in the same position as that of the positive control (Fig. 5, Table 2). The differences observed in the molecular weight of the bands in control tissues and the IOBA-NHC cell line compared with the reported molecular weight of the receptors may be due to differences in the protein glycosylation/palmitoylation and/or phosphorylation pattern between different species and/or different tissues. In addition, many receptors, such as the $\alpha_2$ and the $\beta_2$-adrenergic receptors, occur in different isoforms. In the case of IOBA-NHC cells, another possibility is that non-mature or truncated forms of the receptors are expressed. It is possible that some of the bands detected by Western blot analysis correspond to the pool of intracellularly expressed receptors detected by flow cytometry and confocal microscopy that could correspond to partially degraded receptors.

High-molecular-weight immunoreactive bands were observed in all cases, probably corresponding to dimers, as the formation of homodimers occurs for many receptors, including the $\beta_2$-adrenergic and muscarinic receptors. The $\beta_2$-adrenergic receptor dimers are stable, even under the denaturing conditions applied for analysis by SDS gel electrophoresis. The differences in the protein glycosylation/palmitoylation and/or the reported molecular weight of the receptors may be due to the functional significance of muscarinic and adrenergic receptors in the plasma membrane is well established, they are also present in subcellular locations in several types of cells. Modifications in the quantity of receptors at the plasma membrane help modulate responses to stimulation. Thus, intracellular locations may include both newly synthesized and recycled stores of receptors in amounts that vary with the rate of synthesis and the shedding and desensitization processes. Receptor desensitization controlled by PKA-dependent phosphorylation results in a generalized downregulation of all the receptors that regulate cAMP production, regardless of the state of receptor occupation. The presence of cholera toxin in the culture medium of IOBA-NHC cells could induce cAMP accumulation, resulting in the phosphorylation of receptor molecules that have the PKA substrate consensus motif. Furthermore, in polarized cells, the mobilizing of newly synthesized proteins to the apical surface is under the control of a cholera toxin-sensitive protein. These factors could account also for the intracellular localization observed for the receptors. When IOBA-NHC cells were cultured for 72 hours in cholera toxin- and hydrocortisone-free medium supplemented with 2% FBS, cell membrane receptor expression of the $M_1$-muscarinic and $\beta_2$-adrenergic receptors increased (Fig. 1B). In addition, some mobilization toward the cell cytosol of the $\alpha_2$- and $\alpha_5$-adrenergic receptors was observed by confocal microscopy (Fig. 4). Other mechanisms could also contribute to the intracellular presence of muscarinic and adrenergic receptors. For instance, the cell cholinergetic environment and the presence of a pair of extracellular cysine residues in the $M_1$-muscarinic receptor, N-glycosylation of receptors, and temperature can also alter the distribution of cellular receptors. All these factors could work independently or in conjunction with cAMP-dependent redistribution of muscarinic and adrenergic receptors.

Based on ligand binding and Western blot analyses, Hurt et al. proposed that the intracellular pool of $\alpha_2$-adrenergic receptors in normal rat kidney cells have some functional significance. Also, a role in cellular growth regulation has been proposed for nuclei muscarinic binding sites in rabbit corneal cells. Intracellular receptors may have functions not related to signal transduction, as well. For instance, they can act as ligand buffers, as some of them bind ligand in a specific way.

The autonomic nervous system is implicated in neural regulation of conjunctival cell functioning, both in electrolyte and water transport and in goblet cell protein and mucin secretion. More recently, the role of the autonomic nervous system and the endocrine system in inflammatory disorders has become apparent. There is growing evidence that neural alterations have a role in some inflammatory immune diseases, such as dry eye syndrome and allergic diseases (Moterle L,
et al., IOVS 2003;44:ARVO E-Abstract 3743). In dry eye syn-
drome, excessive nervous stimulation can provoke the activa-
tion of T cells and subsequently the release of inflammatory
cytokines into the lacrimal glands, tear film, and conjunctiva.54
During ocular allergic reactions several neurotransmitters are
released from nerves at the ocular surface into the tear film.55
Epithelial cells from conjunctiva can also participate directly in
inflammatory processes by secreting several cytokines after
stimulation 55,56.

In this study we showed that under a simulated inflamma-
tory condition with the proinflammatory cytokine INF-γ, IOBA-
NHC cells responded by upregulating M₃-muscarinic receptors (Fig. 6A). In the presence of INF-γ+TNF-α, the α₁B- and α₂B-
adrenergic receptors were upregulated (Figs. 6B, 6C).

Both adrenergic and muscarinic receptors are present in
lymphocytes and macrophages and can modulate immune
functioning when activated 57–62. Both IL-1β and TNF-α treat-
ment provoke a downregulation of the α₁a-adrenergic receptor
mRNA expression in monocytes (THP-1) and in human umbil-
cinal endothelial cells (HUVECs).63 In the THP-1 cell line, the
cytokines cause α₁a-adrenergic receptor mRNA upregulation,
whereas α₂B-adrenergic receptor mRNA expression is not mod-
ified. In HUVECs, this treatment induces a downregulation of
α₂B-adrenergic mRNA expression.63 This cytokine-dependent
regulation of α₁-adrenergic subtype expression could play a
role in the pathogenesis of inflammatory diseases, such as
juvenile chronic arthritis.65 Investigators from this laboratory
proposed that the increased TNF-α and/or IL-β production
observed during chronic inflammation may be responsible for
induction of α₁a-adrenergic receptor expression in cells of the
immune system in these patients.65,66 Cytokine upregulation of
M₃-muscarinic and α₁B and α₂B-adrenergic receptor expression
in our IOBA-NHC cells by INF-γ and INF-γ+TNF-α, respec-
tively, suggests that cytokine-dependent regulation of neuro-
transmitter receptors in conjunctival epithelium plays a role in
the pathogenesis of inflammatory ocular surface diseases.

In summary, we have shown that the IOBA-NHC cell line
expresses all the muscarinic and adrenergic receptor subtypes
that are present in the human conjunctival epithelium in vivo
and that proinflammatory cytokines upregulate the expression
of some of them. More studies about the functionality of these
receptors in IOBA-NHC cells, as well as in the normal human
conjunctiva, are undoubtedly needed. Our findings support the
use of the IOBA-NHC cell line as a tool for further study of the
neural regulation of conjunctival epithelium and its possible
role in neurogenic inflammation.

Acknowledgments

The authors thank Jose M. Herrera, MD, for providing the humanconjunctival biopsy specimens, Sagrario Callejo, PhD, for confocal
microscopy assistance, Victoria Saéz, and Carmen García-Vázquez
for excellent technical assistance, and Miguel Jarrín, MSc, for animal han-
dling.

References

1. Sternberg EM. Neuroendocrine regulation of autoimmune/inflam-
2. Stern ME, Beuerman RW, Fox RI, et al. The pathology of dry eye:
the interaction between the ocular surface and lacrimal glands.
conjunctival provocation of allergic subjects with allergen. J Al-
4. Gao J, Schwab TA, Addeo JV, Ghosh CR, Stern ME. The role of
apoptosis in the pathogenesis of canine keratoconjunctivitis sicca:
the effect of topical cyclosporine A therapy. Cornea. 1998;17:
654–663.

5. Stern ME, Gao J, Schwab TA, et al. Conjunctival T-cell subpopu-
lations in Sjögren’s and non-Sjögren’s patients with dry eye. Invest
651.
7. Zouari D, Kublin CL. Impaired neurotransmission in lacrimal and
salivary glands of a murine model of Sjögren’s syndrome. Adv Exp
to cholinergic stimuli in primary Sjögren’s syndrome. Ann Rheum
against lacrimal gland M3 muscarinic acetylcholine receptors in
patients with primary Sjögren’s syndrome. Invest Ophthalmol Vis
10. Humphreys-Behr MG, Peck AB. New concepts for the develop-
ment of autoimmune exocrinopathy derived from studies with the
11. Nietgen GW, Schmidt J, Hesse L, Hönnemann CW, Durieux ME.
Muscarinic receptor functioning and distribution in the eye: mo-
lecular basis and implications for clinical diagnosis and therapy.
of alpha-2B adrenergic receptor subtypes in bovine ocular tissue
receptor subtypes in human ocular tissue homogenates. Invest Ophthal-
2-adrenoceptor subtypes in the porcine eye: identification of alpha
2A-adrenoceptors in the choroid, ciliary body and iris and alpha2A
and alpha2C-adrenoceptors in the retina. Exp Eye Res. 1996;63:
57–66.
16. Matsu T, Cynader MS. Localization of alpha-2B adrenergic recep-
adrenergic receptor subtypes in the anterior segment of the hu-
adrenergic binding sites in the human eye: an autoradiographic
19. Schmitt CJ, Gross DM, Share NN. Beta-adrenergic receptor sub-
types in iris-ciliary body of rabbits. Graefes Arch Clin Exp Oph-
20. Lind GJ, Cavanagh HD. Nuclear muscarinic acetylcholine recep-
34:2943–2952.
21. Rios JD, Zoukhi D, Rawe IM, Hodges RR, Zieske JD, Dart DA.
Immunolocalization of muscarinic and VIP receptor subtypes and
their role in stimulating goblet cell secretion. Invest Ophthalmol Vis
22. Diebold Y, Rios JD, Hodges RR, Rawe I, Dartt DA. Presence of
nerves and their receptors in mouse and human conjunctival goblet
molology; Vol 2.
25. Dartt DA, McCarthy DM, Mercer HJ, Kessler TL, Chung EH, Zieske
JD. Localization of nerves adjacent to goblet cells in rat conjunc-
Development of conjunctival goblet cells and their neuroreceptor
2137.
27. Kessler TL, Mercer HJ, Zieske JD, McCarthy DM, Dartt DA. Stimu-
lation of goblet cell mucous secretion by activation of nerves in rat


