Cultured Human Trabecular Meshwork Cells Express Functional $\alpha_2A$ Adrenergic Receptors

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Purpose. For the treatment of glaucoma, alpha-2 adrenergic receptor ($\alpha_2$-AR) agonists are thought to lower intraocular pressure primarily by decreasing aqueous humor production. Effects on the outflow pathways, however, also may occur. To begin to examine this possibility, the authors characterized the $\alpha_2$-AR subtypes present in cultures of human trabecular meshwork (HTM) cells using both immunofluorescence microscopy and functional measures of $\alpha_2$-AR activation.

Methods. For immunofluorescence microscopy, subtype-specific polyclonal antibodies that recognize each of the human $\alpha_2$-AR subtypes ($\alpha_2A$, $\alpha_2B$, $\alpha_2C$) were used. Functional studies involved the inhibition of forskolin-stimulated cyclic adenosine monophosphate (cAMP) production, the stimulation of mitogen-activated protein (MAP) kinase activity, and the stimulation of mitotic activity as reflected by the expression of proliferating cell nuclear antigen (PCNA).

Results. From the immunofluorescence microscopy, there was evidence for the presence of the $\alpha_2A$ subtype, but not $\alpha_2B$ or $\alpha_2C$ subtype, on HTM cells. The administration of the $\alpha_2$ agonist, dexmedetomidine, to HTM cells resulted in a 90% inhibition of forskolin-stimulated cAMP formation, a twofold stimulation of MAP kinase activity, and a threefold increase in the expression of PCNA. Additionally, preincubation of cells with either of the $\alpha_2$-AR-selective antagonists, rauwolscine or atipamezole, reversed the functional effects of dexmedetomidine.

Conclusions. Functional $\alpha_2A$-ARs are present on HTM cells where they may affect the outflow pathway during the treatment of glaucoma with $\alpha_2$-AR agonists. Invest Ophthalmol Vis Sci. 1996;37:2426-2433.

In the human eye, the maintenance of intraocular pressure involves a dynamic balance between the secretion of aqueous humor by the ciliary epithelium and its outflow via the trabecular meshwork (TM) and uveoscleral region. Both $\alpha$- and $\beta$-adrenergic agents have been useful in the treatment of elevated intraocular pressure by their effects on these pathways. For example, the formation of aqueous humor by the ciliary epithelium is decreased by both $\beta$-antagonists and $\alpha$-agonists. In addition, outflow can be increased in both human and monkey eyes after the administration of certain catecholamines. Although activation of $\beta_2$-adrenergic receptors (in vivo) located in cells of the TM, the primary route for aqueous outflow.

The activation of alpha-2 adrenergic receptors ($\alpha_2$-ARs) inhibits adenyl cyclase and cAMP formation in a number of secretory and absorptive epithelia, including the ciliary epithelium of the eye. Although $\alpha_2$-ARs have been postulated to be present on the
were permeabilized in saponin buffer (0.05% saponin). Antibodies, specific for each of the α2-AR subtypes, previously were characterized with respect to their morphology, ability to take up acetylated low density lipoprotein, and secretion of tissue plasminogen activator.14

METHODS

Trabecular Meshwork Cells

Human eyes were provided by the Missouri Lions Eye and Tissue Bank from donors who were unidentified except for their gender, age, and time of death. The TM was isolated by blunt dissection, and cells were obtained after digestion of the extracellular matrix and plating.15 Seven strains of HTM cells were prepared from seven pairs of eyes corresponding to ages of 59 years (TM001), 64 years (TM002), 32 years (TM008), 25 years (TM010), 71 years (TM011), 47 years (TM012), and 39 years (TM014). Cell strains were prepared and characterized as described previously.15-16 Seven strains of HTM cells were prepared from seven pairs of eyes corresponding to ages of 59 years (TM001), 64 years (TM002), 32 years (TM008), 25 years (TM010), 71 years (TM011), 47 years (TM012), and 39 years (TM014). Cell strains previously were characterized with respect to their morphology, ability to take up acetylated low density lipoprotein, and secretion of tissue plasminogen activator.14

As described in the following sections, either preconfluent or confluent cells were used for any given experimental condition. This did not, however, influence whether or not the α2A-AR was expressed, but was done according to the requirements of the particular experiment. For example, the use of preconfluent cells for immunofluorescence microscopy was desirable because it was easy to visualize the cellular immunoreactivity on well-separated cells. In contrast, the cAMP assay and Western blots required more material, so the cells were grown to confluence.

Immunocytochemistry

Antibodies, specific for each of the α2-AR subtypes, were prepared and characterized as described previously.15,16 The HTM cells (TM001, TM002, TM008, TM010, TM011, TM012, or TM014) were seeded onto gelatin-coated (1%) glass coverslips and were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) (2.7 mM potassium chloride, 1.5 mM KH2PO4, 0.5 mM magnesium chloride, 8.1 mM Na2HPO4, pH 7.4) for 15 minutes at 22°C and were washed (3 × 5 minutes) with PBS. Cells then were permeabilized in saponin buffer (0.05% saponin in PBS containing 10% goat serum) for 30 minutes at 22°C and were placed upside-down onto 40 μl of saponin buffer containing the chicken anti-α2A, α2B, or α2C antibodies (1:800, 1:50, and 1:50 dilution, respectively) for 2 hours at 22°C. The cells were washed (3 × 5 minutes) with PBS and were incubated with 40 μl of fluorescein-conjugated rabbit antichicken secondary antibody (Pierce, Rockford, IL) at a 1:1000 dilution in saponin buffer for 2 hours at 22°C. Finally, cells were washed (3 × 5 minutes) in PBS, mounted, and visualized using a Leica TCS-4D confocal microscope equipped with SCANWARE version 4.2a (Southwest Environmental Health Sciences Center, Experimental Pathology Service Core, Tucson, AZ).

Membrane Preparation and Immunoblot Analysis

The HTM cells (TM002, TM011, or TM014) or COS-7 cells were scraped into Tris–magnesium–EDTA (TME) buffer (50 mM Tris, 10 mM magnesium chloride, 1 mM EDTA, pH 7.5), homogenized using a Polytron homogenizer (Brinkman Instruments, Westbury, NY), and centrifuged at 2500g for 20 minutes. The membrane pellets were resuspended in TME buffer with a Duall homogenizer and were centrifuged at 13,000g for 6 minutes. Pellets were resuspended in loading buffer (4% sodium dodecyl sulfate, 125 mM Tris, 8% glycerol) and were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis according to the manufacturer’s instructions for a 12% gel (Mini-protein II; BioRad, Hercules, CA). After electrophoresis, proteins were transferred to nitrocellulose using a Transblot system (BioRad). The blots were preincubated for 1 hour at 22°C in Tris-buffered saline (10 mM Tris, 154 mM sodium chloride, pH 8), containing 5% nonfat powdered milk and 0.2% Tween (TBS-T), and then were probed with the affinity-purified anti-α2A-AR antibodies (1:1000 dilution) overnight at 4°C. The blots were washed (3 × 5 minutes) in TBS-T and were incubated with a 1:100 dilution of horseradish peroxidase-conjugated secondary antibody for 2 hours at 22°C (goat antichicken immunoglobulin G [IgG], Sigma, St. Louis, MO). The blots were visualized using chemiluminescence (Pierce) on Kodak X-OMAT film (Kodak, Rochester, NY) according to manufacturer’s instructions.

Cyclic Adenosine Monophosphate Assay

The HTM cells (TM001, TM002, TM008, TM010, or TM011) were seeded onto 24-well plates at a density of 50,000 to 100,000 cells per well and were grown until confluent in humidified air with 5% carbon dioxide in media 199 (Gibco, Grand Island, NY) containing 15% defined fetal bovine serum (Hyclone, Logan, UT), 90 μg/ml porcine heparin (Sigma), 20 U/ml endothelial cell growth supplement (containing crude basic fibroblast growth factor [bFGF]), 1.7 mM L-glutamine (Sigma), and 50 mM Hepes (Sigma) without added antibiotics. After 24 hours, the media were removed and the cells were rinsed once and then cultured with serum-free media 199 for an additional 24
FIGURE 1. Immunofluorescence microscopy of cultured human trabecular meshwork cells (TMO11) probed with antibodies specific for the alpha-2A adrenergic receptor (α2A-AR). A shows total fluorescence, and B shows the background labeling that was obtained after preincubation of primary antibodies with a 10-fold excess of the glutathione S-transferase/α2A fusion protein for 16 to 18 hours at 4°C. C shows the fluorescence when the primary antibody was omitted. Bar = 10 μm.

hours. After replacement with fresh serum-free media, the HTM cells were preincubated with 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX; Sigma) for 1 minute at 37°C. The medium was aspirated and drugs plus 0.5 mM IBMX and 10 μM forskolin were added for 15 minutes at 37°C. The drug solutions were aspirated, the plates were transferred to ice, and 150 μl of ice-cold Tris-EDTA (50 mM Tris/4 mM EDTA, pH 7.5) was added to each well. The cells were scraped, transferred to microfuge tubes, boiled for 10 minutes, and centrifuged in a microcentrifuge at maximum speed for 2 minutes (~12,000 g). The supernatant (50 μl) was added to 50 μl [3H]cAMP (Dupont-NEN, Boston, MA) and 100 μl of cold protein kinase A (PKA, Sigma) solution (0.06 mg PKA/ml of Tris-EDTA). After a 2-hour incubation at 4°C, 100 μl of activated charcoal solution (20 mg/ml activated charcoal containing 2% bovine serum albumin in Tris-EDTA) was added, and the mixture was vortexed and centrifuged at maximum speed for 1 minute at 22°C. The samples were placed on ice, and 200 μl of each supernatant was transferred to scintillation vials for counting. A standard curve was generated by adding 50 μl of cAMP standards (0.25 to 32 pmol, Sigma), instead of cytosol, to PKA solution with [3H]cAMP.

Mitogen-Activated Protein Kinase Assay
The HTM cells (TM011, TM012, or TM014) were seeded onto 6-well culture plates and grown to confluence as described above. The cells were washed once and grown with serum-free media 199 for 24 hours. Cells were stimulated with drug solutions for 10 minutes at 37°C, drug solutions were aspirated, and cells were transferred to ice and washed with ice-cold PBS. The preparation of cell lysates and measurement of mitogen-activated protein (MAP) kinase activity were performed as described previously.17 Cells were scraped into 300 μl of ice-cold lysis buffer (50 mM β-glycerophosphate, 1 mM EDTA, 2 mM magnesium chloride, 100 μM sodium vanadate, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM diethiothreitol, 20 μM pepstatin, and 20 μM leupeptin, pH 7.2) and centrifuged in a microcentrifuge at maximum speed (~12,000 g) for 15 minutes at 4°C. Cell lysate (10 μl) was added to 30 μl MAP kinase assay buffer (48 mM magnesium chloride plus 4 mg/ml myelin basic protein, Gibco), 10 μl of 100 μg/ml protein kinase A inhibitor (PKI, Sigma), and 10 μl of [γ-32P]ATP (2200 cpm/pmol; DuPont-NEN) and incubated at 37°C for 15 minutes. The reaction was stopped by precipitation with 10 μl of 25% (wt/vol) trichloroacetic acid, and 25 μl of the resulting mixture was spotted onto P-81 phosphocellulose filter paper (Whatman, Maidstone, UK) and air dried. The filters were washed four times with 75 mM phosphoric acid for 5 minutes and once with acetone for 2 minutes and were air dried before counting. Specific counts for each sample were calculated as total counts for that sample minus the background binding of γ-32P-ATP to filters in the absence of cell lysate. For comparisons, specific counts for each sample were normalized to protein concentration.

Proliferating Cell Nuclear Antigen Immunolabeling
The HTM cells (TM011) were seeded onto coverslips in six-well culture plates and cultured as described above. Cells were grown in serum-free media 199 for 24 hours and then were incubated for 30 hours in one of the following: serum-free media 199, media 199 supplemented with 15% serum and bFGF (20 U/ml), serum-free media 199 plus 100 nM dexmedetomidine, serum-free media 199 containing rauwolscine (10 μM) plus dexmedetomidine (100 nM), or serum-free media 199 containing atipamezole (10 μM) plus dexmedetomidine (100 nM). After treatment, the cells...
were washed once in PBS and fixed in 5% glacial acetic acid in ethanol for 15 minutes at 22°C and washed (3 × 5 minutes) in PBS. Cells were incubated for 10 minutes with sodium borohydride (1 mg/ml), washed (3 × 5 minutes) in PBS, and were permeabilized in Triton buffer (0.1% Triton X-100 in PBS with 10% goat serum) for 30 minutes. The coverslips (inverted) then were placed in 40 μl of fluorescein isothiocyanate-conjugated anti-proliferating cell nuclear antigen (PCNA) antibody (Accurate Chemical and Scientific, Westbury, NY) at a 1:100 dilution in Triton buffer and incubated for 60 minutes at 22°C. Cells were washed (3 × 5 minutes) with PBS, mounted, and visualized with an Olympus BH-2 microscope (Olympus, Melville, NY) equipped with a vertical fluorescence illuminator and high-performance dichroic filters (excitation 475 nm, emission 525 nm). The PCNA expression was quantified by counting PCNA-labeled and unlabeled nuclei in 10 random visual fields and expressing the data as the percent PCNA-labeled nuclei per total nuclei (PCNA-labeled plus unlabeled). Counts were recorded and averaged by two viewers that were masked to the nature of treatment.

RESULTS

Seven HTM cell strains (TM001, TM002, TM008, TM010, TM011, TM012, and TM014) were screened with antibodies specific for the three α2-AR subtypes (α2A, α2B, and α2C). Figure 1 shows that immunofluorescence labeling of preconfluent HTM cells was obtained with antibodies against α2A subtype. Panel A shows the total fluorescence and panel B shows the background fluorescence that remained after preincubation of primary antibody with a 10-fold excess of glutathione S-transferase-α2A fusion protein. Panel C shows the background that was observed when the primary antibodies were omitted. Similar studies using antibodies against the α2B and the α2C subtypes did not show any specific immunofluorescence (data not shown).

In Figure 2, immunoblotting was used to additionally characterize the labeling of HTM cells by the anti-α2A antibodies. Lanes 3 and 4 represent positive controls and show membranes from COS-7 cells that were transfected with the α2A subtype. The membrane preparation used in lane 4 was diluted 100-fold relative to lane 3. Lane 1 shows membranes prepared from confluent HTM cells (TM002), and the diffuse band at Mr ~ 70,000 is consistent with the presence of the glycosylated form of α2A-AR. Lane 2 represents a negative control and shows membranes prepared from COS-7 cells that were transfected with the α2B subtype.

To determine if this α2A-like immunoreactivity could be correlated with functional evidence, cAMP accumulation, MAP kinase activity, and PCNA expression were determined in the presence and absence of α2-adrenergic agents. Figure 3A shows a concentration-response curve for the inhibition of forskolin-stimulated cAMP formation by the α2-selective agonist, dexmedetomidine in confluent HTM cells (TM002). At a concentration of 1 μM, there was approximately a 90% inhibition of forskolin-stimulated cAMP formation, and an EC50 of 10 nM was observed. In addition, Figure 3B shows the ability of the α2-AR antagonists, rauwolscine (10 μM) and atipamezole (10 μM), to significantly reverse the inhibition of forskolin-stimulated cAMP formation by dexmedetomidine (100 nM).

In addition to the inhibition of adenylyl cyclase, it has been shown recently that the activation of α2 ARs can stimulate MAP kinase activity. To examine this possibility in preconfluent HTM cells (TM011, TM012, TM014), dexmedetomidine was tested for its ability to stimulate MAP kinase activity, and the results are shown in Figure 4. In the presence of dexmede-
B

FIGURE 3. The effect of the alpha-2 adrenergic receptor (α2-AR) agonist, dexmedetomidine, on forskolin-stimulated cyclic adenosine monophosphate (cAMP) accumulation (A) and its blockade by antagonists (B) in human trabecular meshwork (HTM) cells (TM002). A shows a concentration-response curve for the inhibition of forskolin-stimulated (FSK; 10 μM) cAMP formation with increasing concentrations of dexmedetomidine. B shows the results obtained from coincubation of dexmedetomidine (DMT; 100 nM) with either atipamezole (AT, 10 μM) or rauwolscine (RW; 10 μM) on forskolin-stimulated cAMP accumulation in HTM cells.

Cells were treated for 10 minutes at 37°C, and cell lysates were analyzed for cAMP content as described in Methods. Untreated cells reflect basal (BSL) levels of cAMP accumulation. The data represent the mean (± standard error of the mean, n = 4 observations) for one experiment, which has been repeated a total of seven times for A and nine times for B using five cell strains (TM001, TM002, TM008, TM010, and TM011). Compared to forskolin-stimulated control specimens, statistical significance was found (*P < 0.05; **P < 0.01) using the Student’s t-test assuming unequal variance.

dexmedetomidine (100 nM), MAP kinase activity was stimulated to a level comparable to that obtained with phorbol ester (PMA, 100 nM), a known activator of this pathway. In addition, coincubation of dexmedetomidine with the α2-AR-specific antagonists, rauwolscine (10 μM) or atipamezole (10 μM), completely blocked the stimulation of MAP kinase activity by dexmedetomidine.

The effect of α2-AR activation on PCNA expression also was examined in the preconfluent HTM cells (TM011). PCNA is a protein whose expression is associated with the mitotic activity of cells and can be quantified by immunofluorescence microscopy as described in the Methods section. Figure 5 shows that in HTM cells treated with dexmedetomidine (100 nM), there was a threefold increase in PCNA expression over that of untreated cells. Additionally, coincubation of HTM cells with either of the α2-AR antagonists, rauwolscine (10 μM) or atipamezole (10 μM), prevented the increase of PCNA expression by dexmedetomidine. As a positive control, cells were treated with 15% serum and bFGF (20 U/ml), which also stimulated the expression of PCNA (fourfold over the serum-free treated cells).

DISCUSSION

The HTM cells participate in the regulation of aqueous outflow and, therefore, have been a target for the pharmacologic treatment of glaucoma. One approach has involved use of adrenergic agents (e.g., the application of epinephrine to human eyes increases aqueous outflow).2-4 As shown in cultured HTM cells, the mechanism of action of epinephrine involved the activation of β2-ARs and the stimulation of intracellular cAMP formation.5 Using this same model, we have investigated the possibility that α2-ARs also might be present. Using subtype-specific antibodies and functional assays, we have characterized the presence of α2A-AR subtype in HTM cells.

Although it had been speculated previously that these receptors are present in the human TM,12 this is the first demonstration of α2-ARs in HTM cells. The finding that it is the α2A subtype that is present in HTM cells is interesting in light of our previous findings of the α2B and α2C subtypes in the human ciliary epithelium.16 This means that, at least for the human eye, there are marked differences in the cellular localization and possible function of the α2-AR subtypes.
Thus, in the ciliary epithelium, the activation of \( \alpha_2B \) or \( \alpha_2C \) subtypes or both are involved with inhibiting the secretion of aqueous humor, in contrast to the \( \beta \)-ARs, which stimulate secretion. In the TM, the activation of \( \beta_2\)-ARs increases outflow, and the possibility exists that activation of the \( \alpha_2A\)-AR in this tissue might limit outflow. These potentially counteracting effects, in principle, could limit the efficacy of \( \alpha_2\)-AR agonists that activate all three subtypes. In fact, recent clinical studies show that although both of the \( \alpha_2\)-selective agonists, \( \beta_2\)-adrenergic agonist, decreased cAMP accumulation. This inhibition was blocked by the \( \alpha_2\) adrenergic antagonists, rauwolscine and atipamezole,
and was consistent with the known ability of the α2A subtype to inhibit cAMP formation. In previous studies of HTM cells, cAMP formation was increased by the stimulation of β-ARs, suggesting that the α2-ARs functionally antagonize the effects of β-AR activation in the TM.

A novel activity that has been ascribed recently to the activation of recombinant α2-ARs is the stimulation of MAP kinase activity.10,22,23 We examined this pathway in the HTM cells and found that dexmedetomidine produced a stimulation of MAP kinase activity that could be blocked with α2-adrenergic antagonists. These results indicate that the endogenous α2-ARs of HTM cells are coupled to a second messenger pathway that is involved potentially with cellular proliferation. Further support for this possibility was obtained in our studies of PCNA expression, which is a marker for DNA replication and cellular mitosis.24 Thus, after treatment of HTM cells with dexmedetomidine, there was an increase in PCNA expression that was blocked by rauwolscine and atipamezole. This is similar to the findings of a recent study in which clonidine, an α2-adrenergic agonist, increased the incorporation of tritiated thymidine into cultured HTM cells.25 Again, functional antagonism of β-ARs is suggested because it has been reported that epinephrine inhibits the mitotic activity of cultured HTM cells through a mechanism that involves β-AR stimulation.26

It has been thought previously that the main effect of the activation of α2-ARs in the eye was to decrease aqueous secretion via the inhibition of cAMP formation in ciliary epithelial cells. Although this certainly is important, we have now shown by immunofluorescence microscopy the presence of the α2A subtype in cultured HTM cells and have shown functional α2-AR responses on cAMP formation, MAP kinase activity, and PCNA expression. This suggests the possibility that α2-ARs are present in the human TM where they may functionally oppose the effects of β-AR activation on aqueous outflow and the mitotic activity of this tissue. Further studies, however, will be required to confirm the presence of functional α2-ARs in the human TM.

Key Words
adenyl cyclase, cyclic adenosine monophosphate, glaucoma, mitogen-activated protein kinase, proliferating cell nuclear antigen

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