Characterization of Muscarinic Receptors in Human Lens Cells by Pharmacologic and Molecular Techniques

David J. Collison,1 Robert A. Coleman,2 Rowena S. James,2 Jae Carey,2 and George Duncan1

PURPOSE. Activation of muscarinic receptors has been implicated in an increased risk of cataract after anticholinesterase treatment for glaucoma. The purpose of the present study was to determine the acetylcholine muscarinic receptor subtype(s) present in native human lens epithelial cells (NHLECs) and a human lens cell line, HLE-B3, and to compare the distribution in other ocular cells.

METHODS. Human lens cells were perfused with artificial aqueous humor (35°C) after fura-2 incorporation, and calcium levels were measured using a fluorometric single-cell digital imaging system. Acetylcholine was the primary muscarinic agonist, and the receptor subtypes were elucidated by determining the relative effectiveness of pirenzepine and AF-DX 384 in blocking the agonist-induced response. The levels of expression of mRNA for the receptor subtypes M1 through M5 were determined by quantitative reverse transcription-polymerase chain reaction (QRT-PCR) using a sequence detection system (ABI Prism 7700; Perkin-Elmer, Foster City, CA). This was performed using total RNA extracted from native lens, retina, iris, and sclera and also cultured lens cells.

RESULTS. Acetylcholine induced a similar concentration-dependent increase in peak-amplitude cytosolic calcium in the range 100 nM to 100 μM in both native and HLE-B3 cells. However, the kinetics of the response waveforms to 30-second pulses of acetylcholine were different in the two cell types. At higher concentrations (>1 μM), a second phase appeared in the HLE-B3 cells that was absent in the NHLEC response. The 50% inhibitory concentration (IC50) values for blockade of a 1 μM acetylcholine response by pirenzepine and AF-DX 384 were 30 nM and 230 nM, respectively, for NHLECs, and 300 nM and 92 nM, respectively, for HLE-B3 cells. The QRT-PCR data showed that more than 90% of the total muscarinic receptor mRNA from NHLEC was of M1 origin. In the HLE-B3 cells, however, more than 95% of the mRNA was of M3 origin. mRNA for M3 was also in greatest abundance in other eye tissues, although there was a significant contribution from M1 in iris and sclera.

CONCLUSIONS. Both NHLECs and HLE-B3 cells express muscarinic receptors that produce significant changes in cytosolic calcium in response to acetylcholine. Both pharmacologic and QRT-PCR evidence shows that whereas the M1 subtype predominates in NHLECs, M3 is the major contributor in HLE-B3 cells. In all other eye tissues, M3 appears to be the major contributor. These data should be taken into account when choosing particular models to investigate cataract mechanisms and also when designing muscarinic agonists to treat glaucoma. (Invest Ophthalmol Vis Sci. 2000;41:2633–2641)

The lens is an avascular tissue that derives all its nutrients from the surrounding aqueous and vitreous humors. The lens is also a noninnervated organ and comprises only two cell types—namely, the epithelial cells and their fully differentiated products, the fiber cells. In the mature eye the lens appears to serve no function other than to focus light in a passive manner onto the retina. In spite of this simplicity of form and function, the lens is remarkably well endowed with a range of receptor signaling systems that render it responsive to a number of molecular species in the surrounding humors.1 In this context, several agents used to treat medical conditions in other tissues induce cataract in the lens. Cataract is associated, for example, with the long-term use of corticosteroids to ameliorate inflammation and arthritis2 and anticholinesterases such as echothiophate to treat glaucoma.3,4 Many experimental studies, mainly involving animal models, have been undertaken to investigate the underlying mechanisms, but these have invariably produced conflicting information. For example, Michon and Kinoshita5 reported that, whereas exposure to acetylcholine failed to produce any changes in cultured rabbit lenses, high concentrations of cholinesterase inhibitors induced opacification in this acute model. They concluded therefore that the anticholinesterase effects may arise from some nonspecific interactions of the drug with the lens. However, they pointed out that the lens capsule contains a very high
level of cholinesterase, resembling the true cholinesterase found in neural tissue. Kaufman et al. found evidence for the specific involvement of muscarinic receptors in echothiophosphate-induced cataract, in that atropine inhibited the cataractogenic effect of topically applied echothiophosphate in monkeys. These findings imply either that the lens possesses functional muscarinic receptors or that anticholinesterases modify the humors in some way to render them cataractogenic.

Williams et al. first demonstrated that cultured human lens cells possess functional muscarinic receptors and that calcium was released from endoplasmic reticulum (ER) stores on activation of these receptors. More recently, it has been shown that in the intact human lens, muscarinic receptor activation is linked to a change in voltage. Neither of these studies determined receptor subtype, an important question, because the odd numbered receptors M1, M3, and M5 are linked to calcium mobilization, whereas M2 activation produces a voltage change in heart muscle, for example. Because different subtypes are associated with different signaling pathways, it is important to know the totality of subtypes likely to be present in the human lens. Furthermore, this is important in the context of glaucoma therapy, because specific receptor subtypes are now targeted to reduce intraocular pressure.

In the present study, we chose two methods to elucidate the spectrum of muscarinic subtypes present. A pharmacologic approach was used in calcium mobilization studies, because each of the subtypes has different antagonist-binding affinities. Furthermore, molecular cloning studies have identified five distinct genes encoding receptors with distinct primary sequences M1 through M5. In the second approach, quantitative reverse transcription–polymerase chain reaction (QRT-PCR) methods were used to elucidate the totality and levels of the different subtypes in native lens cells and to compare these with the levels in other ocular cell types. Because cell culture techniques are used increasingly to study the molecular mechanisms of lens function, we also believed it important to compare the spectrum of subtypes present in native cells (NHLECs) with that in cultured lens cells.

**METHODS**

**Cell Culture**

The HLE-B3 cell line was kindly provided by Usha Andley (Department of Ophthalmology, Washington University School of Medicine, St. Louis, MO). Cells were grown in 25-cm² culture flasks containing Eagle’s minimum essential medium (EMEM), supplemented with 20% fetal calf serum (FCS), at 37°C under 5% CO₂. Before reaching confluence, the cells were trypsinized, counted (Coulter counter; Coulter, Hialeah, FL), and seeded at a density of 1 × 10⁵ cells/100 μl 10% EMEM on glass coverslips (15 mm diameter). The coverslips formed the base of one of the chambers used for calcium imaging. Cells on coverslips were used for imaging within 6 days. HLE-B3 cells were routinely passaged and regrown to provide material for calcium imaging and RNA extraction, and primary cultures of native cells were produced as described previously. All chemicals were obtained from Sigma (Poole, UK), unless otherwise stated. AF-DX 384 was kindly provided by Boehringer-Ingelheim (Bracknell, UK).

**Native Human Lens Preparations**

Human lenses were obtained from donor eyes. Globes were obtained from the Bristol Eye Bank or the East Anglian Eye Bank within 36 hours of enucleation and after the cornea had been removed for transplantation surgery. Lenses were removed from the globes and dissected from surrounding ciliary, iris, and vitreous bodies. The iris, removed at the ciliary margin, was retained for later analysis. The lens capsule with its adherent epithelium was dissected from the fiber mass and pinned to a plastic base of the plastic chamber used in calcium imaging. Any remaining lens fiber fragments were then removed from the surrounding capsule by irrigation with artificial aqueous humor (AAH) at 30°C. Composition of AAH in mM: 130 NaCl, 5 KCl, 5 NaHCO₃, 1 CaCl₂, 0.5 MgCl₂, 5 glucose, and 20 HEPES, adjusted to pH 7.25 with NaOH. Note that all donor tissues used in this study were from donors aged 50 or more years.

**Measurement of Intracellular Calcium Levels**

Both types of cell preparations were loaded with the acetoxymethylester (AM) form of 3 μM fura-2 for 40 minutes. The cells were then washed in AAH for 20 minutes to allow complete de-esterification. Ratiometric imaging of cytosolic calcium took place on the heated stage of an epifluorescence microscope (Nikon, Melville, NY) fitted with a ×20 objective. Cultured cells were large enough to be imaged singly, whereas native cells were considered too small for individual cell analysis. Therefore, regions of interest consisted of four to five confluent cells. Cells were continuously perfused with AAH and experimental solutions prewarmed to 35°C in a water bath. Solutions were administered by a two-way tap. Turnover time for solutions in the chamber was 5 seconds. Cells were excited alternatively with light of 340- and 380-nm wavelengths. Resultant fluorescent emissions at both wavelengths were collected by a CCD camera (charge-coupled device camera; Photon Technology International, Newark, NJ) at 510 nm sampled every 2 seconds. After background subtraction and calibration, fluorescence ratios (Rs) were converted into real calcium concentrations using the formula

\[
[Ca^{2+}] = K_d(R - R_{min})/(R_{max} - R)[S1/S2]
\]

where \(K_d\) is the dissociation constant, \(R_{min}\) is the fluorescence ratio in zero calcium, \(R_{max}\) is the maximum fluorescence ratio, and \(S1/S2\) is the fluorescence intensity. Calibration involved permeabilizing the cells at the end of the experiment with ionomycin (5 μM) and bathing the cells in calcium-free AAH that contained 1 mM EGTA, 1 μM thapsigargin, 150 mM KCl, and 100 μM plasma membrane (PM) Ca-adenosine triphosphatase (ATPase) inhibitor, W7. This permitted a measurement of the fluorescence ratio in zero calcium (\(R_{min}\)). The same cells were then exposed to a similar solution that had 10 mM calcium replacing EGTA to obtain a maximal ratio (\(R_{max}\)). The factor \((S1/S2)\) is the fluorescence intensity at 380 nm, when all the fura-2 is in the calcium-free form divided by the fluorescence intensity when the fura-2 is in the bound form. As for \(R_{min}\) and \(R_{max}\), this factor was determined from calibration experiments. The \(K_d\) for fura-2 was taken as 224 nM.\(^16\)

**Pharmacology**

Concentration–response curves were fitted with the equation\(^17\).
where $A$ is the response amplitude corresponding to agonist concentration $C$, $A_{max}$ is the amplitude for the maximal response with 1 mM acetylcholine, $EC_{50}$ is the concentration of agonist required to elicit a half-maximal response, and $x$ is the Hill coefficient describing co-operativity and receptor occupancy. In the present study, the best-fit was obtained when $x = 1$.

Inhibition curves were fitted with the equation

$$A/A_{max} = 1 - C/(C + EC_{50}^b)$$  \hspace{1cm} (3)

where $C$ is the agonist concentration (1 μM) and, again, best fits were obtained with $x = 1$. Antagonist potency is normally expressed in terms of $pK_b$, where $K_b$ is the apparent dissociation constant generated from the Cheng–Prusoff equation$^{17}$

$$K_b = IC_{50}/(1 + A/EC_{50})$$  \hspace{1cm} (4)

where, $IC_{50}$ is the antagonist concentration required to reduce the agonist response to 50%, and $A$ is the agonist concentration (1 μM). $pK_b$ values are a negative logarithm of the dissociation constant $K_b$ and represent the affinity of each antagonist for a particular receptor subtype. See Caulfield$^{18}$ for a detailed review of $pK_b$ values (summarized in Table 1). Twenty-two capsules were used for the calcium mobilization studies.

### Quantitative Reverse Transcription PCR

**Isolation of Total RNA.** All tissues (including iris, retina, and sclera) were snap frozen in liquid nitrogen immediately after dissection. Note that after the lens was dissected from the eye, the epithelium was removed by cutting the posterior capsule. No attempt was made in this case to remove tightly adherent fibers. To obtain sufficient RNA for analysis, both lenses from each donor were pooled. In one case a whole eye, the epithelium was removed by cutting the posterior capsule after dissection. Note that after the lens was dissected from the eye, the epithelium was removed by cutting the posterior capsule.

Total RNA was extracted from snap frozen tissues and cultured cells using TriZol, a commercially available solution of phenol and guanidine isothiocyanate, according to the protocol described by the manufacturer (Life Technologies, Grand Island, NY). The concentration and purity of the RNA were determined by measurement of the optical density at 260 and 280 nm. The RNA was extracted from snap frozen tissues and cultured cells using TriZol, a commercially available solution of phenol and guanidine isothiocyanate, according to the protocol described by the manufacturer (Life Technologies, Grand Island, NY). The concentration and purity of the RNA were determined by measurement of the optical density at 260 and 280 nm. The RNA solutions were diluted to a working concentration of 1 μg/μl in nuclease-free water with the addition of RNase inhibitor (Perkin–Elmer, N808-0119).

**Principles of TaqMan Technology.** A quantitative analysis of specific mRNA expression was performed using a sequence detection system (ABI Prism 7700; Perkin–Elmer, Foster City, CA). The use of this system to determine specific mRNA expression has been described.$^{19,20}$ The system uses a fluorogenic probe to generate sequence specific fluorescent signals during PCR. The probe is an oligonucleotide positioned between the forward and reverse PCR primers with fluorescent reporter and quencher dyes attached. While the probe is intact, the intensity of reporter fluorescence is suppressed by the quencher. If the probe forms part of a replication complex, the fluorescent reporter is cleaved from the quencher by the 5’→3’ exonuclease activity inherent in Taq polymerase.

The starting copy number of an mRNA target sequence ($C_0$) is established by determining the fractional PCR cycle number ($C$) at which the fluorescent signal generated during the replication process passes above a threshold baseline. Quantification of the amount of target mRNA in each sample is established through comparison of experimental $C$ values with a standard curve.

**Transcription Detection.** Pairs of primers and TaqMan probes were designed by computer (Primer Express software; Perkin–Elmer) to amplify specific small fragments from the human muscarinic receptors (M1–M5; Table 2). The specificity of the primer and probe set was verified by running the products from a PCR reaction performed on human genomic DNA on a 4% agarose gel. All sets generated a single PCR product of the expected size. In addition, a pair of primers and a TaqMan probe were designed that span an intron and amplify a 78-bp portion of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. GAPDH, a ubiquitously expressed housekeeping gene, was used to provide an internal marker of mRNA integrity within the experiment. The muscarinic receptor probes were labeled with the fluor FAM (6-carboxy-fluorescein), the GAPDH probe with the fluor JOE (2,7-dimethoxy-4,5-dichloro-6-carboxy-fluorescein). Both fluoros were quenched with TAMRA (6-carboxy-tetramethyl-rhodamine).

Total RNA samples were treated with RNase-free DNase (amplification grade; Gibco, Paisley, UK) according to the manufacturers’ instructions. For each RNA sample 100 ng was then used as template for first-strand cDNA synthesis. The RNA in a volume of 4 μl and in the presence of reverse primers for M1 through M5 and GAPDH, 1× PCR buffer II (Perkin–Elmer) and 5 mM MgCl$_2$ was heated to 72°C for 5 minutes and cooled slowly to 55°C. After addition of all other reagents, the 6-μl reaction was incubated at 37°C for 30 minutes followed by an enzyme inactivation step of 90°C for 5 minutes. The final

### Table 1. Relative Affinities of the Antagonists Used for Each Muscarinic Receptor Subtype

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
<th>M5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methoctramine</td>
<td>8.3</td>
<td>6.6</td>
<td>6.9</td>
<td>7.0</td>
<td>6.1</td>
</tr>
<tr>
<td>AF-DX 384</td>
<td>7.4</td>
<td>8.7</td>
<td>7.5</td>
<td>8.7</td>
<td>8.3</td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>9.0</td>
<td>8.4</td>
<td>9.1</td>
<td>8.9</td>
<td>9.0</td>
</tr>
</tbody>
</table>

Data are expressed as average $pK_b$ values derived from functional binding studies.$^{18}$ See also equation 4.

### Table 2. GenBank Accession Numbers for the Human Muscarinic Receptor Subtypes That Have Been Sequenced

<table>
<thead>
<tr>
<th>Receptor Subtype</th>
<th>GenBank Accession No.</th>
<th>Nucleotides*</th>
<th>Amplicon Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>M55128</td>
<td>1272–1372</td>
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<td>M2</td>
<td>X15264</td>
<td>1379–1495</td>
<td>117</td>
</tr>
<tr>
<td>M3</td>
<td>X15266</td>
<td>1445–1536</td>
<td>92</td>
</tr>
<tr>
<td>M4</td>
<td>M16405</td>
<td>2343–2447</td>
<td>105</td>
</tr>
<tr>
<td>M5</td>
<td>M80333</td>
<td>257–329</td>
<td>73</td>
</tr>
</tbody>
</table>

The start and finish nucleotide numbers of the amplicons and the amplicon sizes of the unique sequences from which the primers were designed are also shown.

*From 5’ end of the forward primer to the 3’ end of the reverse primer.
Reaction conditions were as follows: 1× PCR buffer II (Perkin-Elmer); 5 mM MgCl₂; 1 mM dATP, dGTP, dCTP, and dTTP; and 12.5 units MuLV reverse transcriptase (Gibco). The resultant cDNA was subjected to PCR amplification in the sequence detection system in multiplex reactions in which the copy numbers of the individual muscarinic receptors were determined in conjunction with those of the GAPDH transcript. Final reaction conditions were 4% glycerol, 1× TaqMan buffer A (Perkin-Elmer); 6.25 mM MgCl₂; 430 μM dATP, dUTP, dGTP, and dCTP; 2.5 units AmpliTaq Gold; and 0.1 units AmpErase.

**Figure 1.** Time-lapse fluorometric ratio images of acetylcholine-induced calcium mobilization in native (a) and HLE-B3 cells (b). Typical phase images of the two types of cells are given in (c) and (d). Note that the time course of the response is longer in HLE-B3 cells and also there is a greater heterogeneity in the number of cells that respond to acetylcholine.

**Figure 2.** Calcium mobilization characteristics of NHLECs (a) and HLE-B3 cells (b) to increasing concentrations of acetylcholine. Acetylcholine was administered each time as a 30-second pulse. There was a clear difference in response kinetics and response shape between the two cell types. Tissue-cultured HLE-B3 cells showed a pronounced second phase at higher acetylcholine concentrations that was undetectable in NHLECs. The tracings were generated from two donor capsules and 3 HLE-B3 cultures, and the data are representative of eight donors and 25 HLE-B3 cell cultures, respectively.
UNG (uracil-N-glucosylase; both Perkin–Elmer). An initial enzyme activation step of 94°C for 12 minutes was followed by 45 cycles of 94°C 15 seconds and 60°C 1 minute of PCR.

**Transcription Quantification.** Standard curves have been generated for many different targets using 3, 6, 15, 30, and 60 ng of sheared genomic DNA, equivalent to 1,000, 2,000, 5,000, 10,000, and 20,000 copies of the human genome, respectively. Reactions were performed in duplicate under conditions identical with those just described. The criteria for successful primer and probe design are extremely rigorous, and it has been our experience that the efficiency of amplification with sets that conform is very high. We have determined the frequency distribution of the data points from a large number of standard curves generated with different primer–probe sets and have found that they are very tightly grouped (Coleman et al. unpublished data, 1997). For that reason, we have now used these data to generate a global standard curve that is used to quantify all target mRNA copy numbers. The fractional PCR cycle ($C_T$) at which the accumulated fluorescent signal exceeded a predetermined level above background was compared with the global standard curve to generate a starting copy number for the M1 through M5 transcripts. Data for muscarinic receptor gene expression were only accepted when the associated level of GAPDH expression in the sample was 10,000 copies or more. Failure to achieve this level of GAPDH expression is suggestive of significant loss of mRNA integrity. Twenty capsules were used to generate native lens data.

No animals were used in this study and because no donor details were revealed other than age, sex, cause, and time of death, this study conformed with principles embodied in the Declaration of Helsinki.

**RESULTS**

**Spatial and Temporal Signaling Characteristics**

Figure 1a shows time-lapse images of NHLECs responding to 10 μM acetylcholine. Of the cells in the field of view, 95% were seen to respond, whereas in a confluent patch of HLE-B3 cells only 60% to 65% of cells responded to the same concentration of agonist (Fig. 1b). When the purinergic receptor agonist adenosine triphosphate (ATP) was applied, all the HLE-B3 cells in the field of view respond by mobilizing calcium (data not shown). This heterogeneity in responses elicited after activation of different G-protein–linked receptors is characteristic of lens cells in culture, whereas the NHLECs respond with much more homogeneity to a range of agonists (data not shown). The duration of response exhibited by both cell types to 10 μM acetylcholine are also markedly different. NHLECs consistently have much shorter response times than HLE-B3 cells, not only to acetylcholine but also to other agonists, such as histamine (data not shown). The time course of the responses to pulses of increasing concentrations of acetylcholine for both NHLECs and HLE-B3 cells are further explored in Figures 2a and 2b. Note that at low concentrations (100 nM), the HLE-B3 response had a long latency. These kinetics have been reported in human primary lens cells. At higher acetylcholine concentrations, the native cell responses were more symmetrical in form, whereas those from the transformed cell line showed evidence of a second component. However, when the peak response heights were plotted as a function of agonist concentration, the response characteristics appeared more similar (Fig. 3). The concentration of agonist required to produce a half-maximal response ($EC_{50}$) in HLE-B3 cells was 0.9 μM. Similarly in NHLEC, the $EC_{50}$ was 0.4 μM.

**Pharmacology**

To compare the pharmacologic sensitivity of the two types of cells, the same concentration of agonist was used throughout. The concentration chosen was 1 μM, because this level elicited...
a submaximal response in both cases, and repeated applications at 15-minute intervals did not lead to loss in responsiveness (Fig. 4 and data not shown). Figure 4 shows a continuous 40-minute record of repeated exposure of NHLECs to short pulses of 1 μM acetylcholine. Pretreatment with the M1-specific muscarinic antagonist pirenzepine at a concentration of 10 nM had no effect on responsiveness to acetylcholine, but 100 nM caused complete inhibition. Recovery was slow after exposure to high concentrations of pirenzepine. There was 50% recovery after 5 minutes and total recovery after a further 20 minutes (data not shown). The effect of different concentrations of AF-DX 384 on responses of HLE-B3 cells to 1 μM acetylcholine is shown in Figure 5, and the full inhibition curves for both types of cell are given in Figure 6. Invariably, the inhibition curves were sigmoidal in shape. Because NHLECs preparations are scarce, only two antagonist curves were generated to pirenzepine and AF-DX 384, respectively (Fig. 6a). In the case of HLE-B3 cells it was possible to generate four inhibitory curves with additional data from 4-DAMP and methoctramine (Fig. 6b). The antagonist potency order of pKᵦ for NHLECs was pirenzepine > AF-DX 384 (Table 3). For HLE-B3 cells, the potency order was 4-DAMP > AF-DX 384 > methoctramine > pirenzepine, (Table 4). This potency profile of antagonists indicates that the M1 subtype mediated the acetylcholine effect in NHLECs, whereas M3 mediated the response to acetylcholine in HLE-B3 cells.

Quantitative Reverse Transcription PCR

It is possible that the lens may express more than one subtype of muscarinic receptor, which could greatly complicate the interpretation of the pharmacologic inhibition curves. Furthermore, muscarinic receptor subtypes that are not coupled to calcium mobilization (such as M2 and M4) could also be present.21

Therefore, QRT-PCR was used in conjunction with calcium imaging studies to quantify the expression of such receptors at the mRNA level. Because the M1 and M3 subtypes are found with greatest frequency in a variety of cell types22 and were identified by pharmacologic techniques in native and HLE-B3 cells, respectively (see above), our initial efforts were

Table 3. IC₅₀ and pKᵦ Values for Pirenzepine and AF-DX 384 in NHLECs

<table>
<thead>
<tr>
<th></th>
<th>Pirenzepine</th>
<th>AF-DX 384</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC₅₀ (nM)</td>
<td>30</td>
<td>230</td>
</tr>
<tr>
<td>pKᵦ</td>
<td>8.1</td>
<td>7.2</td>
</tr>
</tbody>
</table>

Values were derived by applying equation 4 to the data given in Figure 6a. The best agreement in both cases coincides with the pKᵦ for the M1 receptor (refer to Table 1).
DISCUSSION

The present comparative studies concerning muscarinic receptor expression and function were performed predominantly on native lens cells and the lens cell line, HLE-B3 (Tables 1 through 4). The results are fully consistent, in that native cells contained the highest level of mRNA for the M1 receptor, and the pharmacologic characterization based on calcium release also identified the same receptor. Similarly, mRNA for the M3 receptor was in highest abundance, whereas in HLE-B3 cells, M3 mRNA was predominant. In neither cell type was there appreciable expression of M2, M4, or M5 (Table 4). It is interesting to note that cultured primary lens epithelial cells had increased the copy number for M5 receptors fourfold over their native counterparts and that of the M1 receptor had been reduced to 15% of the native value. The M3 expression in cultured lens primaries had increased so that it approached that of the HLE-B3 value. In the whole eye, M3 expression levels dominated, and this was also true of dissected retina, iris, and sclera (Table 5).

It is interesting that the relative frequency of subtype expression in the human brain is similar to that in native lens cells, in that both have high levels of M1 relative to M3.26 In this limited context, the lens more closely resembles the brain than does the retina, where M3 predominates (Table 5). Defining the functions of each receptor subtype in the brain has been largely speculative and based mainly on quantitative distribution of protein within a given tissue. A major problem in elucidating the function of each receptor type in neuronal tissue has been the absence of a good experimental model system. For example, in the cultured neuronal P19 cell line, the predominant muscarinic subtypes are M2, M3, and M5.27 Because the M1 and M4 subtypes are largely absent, these cells are fundamentally different from native tissues. There again of Shepard and Rae23 who reported the M5 receptor as the only muscarinic receptor present in human lens cells. They identified the receptor in a cDNA library made from tissue-cultured cells, but we have found that mRNA levels corresponding to the different types in tissue-cultured cells do not necessarily reflect the native state (Table 5). In cultures from older donors, M3 was the predominant subtype, with minor contributions from M1 and M5. Shepard and Rae23 cultured very young tissue (<1 year), and it is possible that the native expression may differ between young and mature tissue. It is also possible that very young cells respond to culture conditions differently from older cells. The physiological effects of activating either M1 or M3 appear to be similar. Both efficiently mediate phosphoinositol (PI) hydrolysis and inhibit K+ current.24 However, there are differences in their mitogenic effects: M1 activation produces a much greater stimulation of DNA synthesis than M3.25 This could be of importance in the lens where a tight control of DNA synthesis is maintained. As far as the present study is concerned, M1 or M3 activation seemed to produce different cytosolic calcium-increase kinetics. The main difference appears to be a greater second phase associated with the M3 subtype. Riach et al.1 have pointed out in a study of primary cultured human lens cells that the second phase arises from a calcium influx, in that it is absent in calcium-free medium. The differences in kinetics are therefore probably largely due to differences in the coupling of the ER to the capacitative calcium entry pathway in the two cell types, rather than differences in coupling of the receptor to calcium release.

It is of interest that the message for M5 was downregulated in cultured human lens cells, with little or no expression of M1 or M3. A limited study was therefore made of M4 and M5 expression in native and HLE-B3 cells. In the present QRT-PCR studies we found that in native cells, M1 mRNA was in greatest abundance, whereas in HLE-B3 cells, M3 mRNA was predominant. In neither cell type was there appreciable expression of M2, M4, or M5 (Table 4). It is interesting to note that cultured primary lens epithelial cells had increased the copy number for M5 receptors fourfold over their native counterparts and that of the M1 receptor had been reduced to 15% of the native value. The M3 expression in cultured lens primaries had increased so that it approached that of the HLE-B3 value. In the whole eye, M3 expression levels dominated, and this was also true of dissected retina, iris, and sclera (Table 5).

| TABLE 4. IC₅₀ and pKb Values for Four Muscarinic Antagonists Applied to HLE-B3 Cells |
|---------------------------------|-------|-------|-------|-------|-------|
|       | 4-DAMP | AF-DX 384 | Pirenzepine | Methoctramine |
| IC₅₀ (nM) | 1.9   | 92     | 300    | 1.3*  |
| pKb     | 9.0   | 7.4    | 6.6    | 6.2   |

The data were derived from Figure 6b, and the closest correspondence is with the pKb for M3 receptor (see also Table 1).

* In micromolar.

concentrated on these. However, during the course of this study Shepard and Rae23 reported that the message for M5 predominated in cultured human lens cells, with little or no expression of M1 or M3. A limited study was therefore made of M4 and M5 expression in native and HLE-B3 cells. In the present QRT-PCR studies we found that in native cells, M1 mRNA was in greatest abundance, whereas in HLE-B3 cells, M3 mRNA was predominant. In neither cell type was there appreciable expression of M2, M4, or M5 (Table 4). It is interesting to note that cultured primary lens epithelial cells had increased the copy number for M5 receptors fourfold over their native counterparts and that of the M1 receptor had been reduced to 15% of the native value. The M3 expression in cultured lens primaries had increased so that it approached that of the HLE-B3 value. In the whole eye, M3 expression levels dominated, and this was also true of dissected retina, iris, and sclera (Table 5).

| TABLE 5. QRT-PCR mRNA Copy Number of the Five Muscarinic Receptor Subtypes in Different Ocular Tissues |
|---------------------------------|-------|-------|-------|-------|-------|
| Ocular Tissue | M1     | M2     | M3     | M4     | M5     |
| NHLECs         | 1249.5 ± 398.7 | 130.9 ± 87.4 | 8.7 ± 7.39 | 1.5    | 27.7   |
| HLE-B3 cells   | 1.0 ± 0.4 | 0.04 ± 0.04 | 3177.0 ± 1133.2 | 27 ± 11.1 | 12.0 ± 6.16 |
| Lens primary cultures | 7.2 ± 7.2 | 0.5 ± 0.5 | 1196.8 ± 108.5 | 9.5 ± 6.4 | 107.2 ± 50.0 |
| Iris           | 585.7   | 0      | 3853.9 | 516.2  | 19.0   |
| Retina         | 176.1 ± 113.6 | 6.2 ± 5.3 | 7345.8 ± 3650.6 | 726.4 ± 717.5 | 330.5 ± 0 |
| Sclera         | 1336.2  | 0.04   | 4471.4 | 3.7    | 37.9   |
| Whole eye      | 2081.0  | 340.4  | 4868.5 | 191.3  | 80.1   |

Number (n) as follows for the corresponding replicates on independent samples used in the determination of M1–M3 and M4 and M5. For NHLECs, n = 8 and 2, respectively. For HLE-B3 cells, n = 7 and 5; cultured lens primary cells, n = 5 and 2; iris, n = 2 and 2; sclera, n = 2 and 2; retina, n = 5 and 2, respectively. Only one completely intact eye was sampled. Data are expressed as copy number/100 ng RNA and are expressed as mean ± SEM when appropriate, and simply as the mean when only two samples were available. Note that the RNA extracted from the lens capsule may also have a small contribution from adherent fibers. Also the data for the cultured cells were derived from different cultures in each case.
appears to be a similarity in the behavior of lens cells in culture, in that M1 expression shifts to M3 in the cultured cell line.

High levels of M3 mRNA have been found in native and cultured ciliary muscle, ciliary epithelium, iris, cornea, and trabecular meshwork. Immunoprecipitation of muscarinic receptors in human iris and ciliary body revealed that 60% to 75% of all subtypes present were of the M3 variety, whereas M1, M2, M4, and M5 accounted for less than 10% of total muscarinic receptors. A similar pattern, in which all subtypes are present, seems to be emerging from in situ hybridization and Northern blot analysis studies performed on ciliary muscle, but functional contractile responses seem to be mediated by M3 muscarinic subtypes, with M1 having far less importance. When muscarinic receptor expression in human eye was studied by a number of techniques, the M3 subtype appeared to be the most abundant, and the present PCR determinations performed on the whole eye, iris complex, retina, and sclera support these findings (Table 5).

The present findings in native lens cells and in muscarinic receptor distribution in the whole eye help clarify formerly identified diseases associated with disturbing the ocular acetylcholine signaling system. For example, the presence of relatively high levels of mRNA for the M1 receptor in the lens helps explain the association of anticholinesterase treatment for glaucoma with an increased incidence of cataract.

The findings also help in a more rational development of new strategies for intervention in certain eye diseases. It has been suggested that specific muscarinic agonists could be used to treat glaucoma and because the lens and iris profiles are very different (Table 5 and References 31 and 33), we suggest that such an agonist should clearly discriminate between M3 and M1 subtypes. Another area in which muscarinic intervention is clinically relevant is in the area of form-deprivation eye elongation, in which muscarinic receptors appear to play a critical role in driving eye growth. Muscarinic agonists, including atropine and pirenzepine, can also inhibit the axial growth characteristics of chick form-deprivation myopia. Although pirenzepine is a more powerful M1 antagonist, it should be noted that scleral cells express higher levels of the M3 subtype (Table 5). The present data indicate that it would be prudent to target the M3 subtype in eye growth experiments to see whether this system alone is responsible for driving growth.

In conclusion, pharmacologic and gene-expression data suggest that the contributions of the various subtypes of muscarinic receptors in human lens epithelial cells differ in native and immortalized cells, with M1 predominating in the former and M3 in the latter. Furthermore, preliminary gene expression data with primary cultured human lens epithelial cells demonstrates an apparent transition state, with clear expression of both M1 and M3 receptor mRNAs. These findings provide a cautionary note to those interested in the effects of modulators of muscarinic receptors in human ocular function and who intend to use human epithelial cell lines for their studies.

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