Muscarinic Receptor Subtypes in Human Iris–Ciliary Body Measured by Immunoprecipitation

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Purpose. To determine the relative levels of the five muscarinic receptor subtypes in the anterior segment of the human eye.

Methods. Antisera selective for each of the five muscarinic receptor proteins were incubated with [3H]-QNB bound receptors solubilized from human iris sphincter, ciliary muscle, and ciliary processes. Precipitation of the radiolabeled receptor–antibody complexes and scintillation counting enabled quantitation of the subtypes in the various tissues. Reverse transcription–polymerase chain reaction was performed on the tissues and cultured smooth muscle cells derived from them.

Results. Approximately 60% to 75% of the muscarinic receptors in the human iris sphincter and ciliary body are the m3 subtype. Lower levels (5% to 10%) of the m2 and m4 receptors are present in these tissues. The m1 receptor (7%) was detected in the ciliary processes and iris sphincter and the m5 receptor (5%), which is usually found only in the central nervous system, was present in the iris sphincter.

Conclusions. The m3 subtype is the predominant muscarinic receptor in the anterior segment of the human eye. The extensive heterogeneity of muscarinic receptors makes it difficult to predict whether subtype-selective drugs will have an improved efficacy and side-effect profile.


The clinical use of cholinergic drugs for many decades has revealed multiple actions mediated by muscarinic receptors in the eye. These include reduction of pupil diameter by contraction of the iris sphincter, accommodation of the lens and enhanced aqueous humor outflow as a result of ciliary muscle contraction, and increased aqueous humor secretion from the ciliary processes. The discovery of muscarinic receptor subtypes that could be differentiated pharmacologically with selective antagonists12 and could couple to different second messenger pathways3 raised the possibility that the various effects of muscarinic drugs on the eye were elicited through different receptors. An M3 receptor that binds pirenzepine with intermediate affinity and 4-DAMP with high affinity and activates phosphoinositol (PI) hydrolysis was demonstrated in the cynomolgus monkey ciliary body,4 human ciliary muscle cells,5 human nonpigmented epi-
thelial cells,6,7 the rabbit iris sphincter,8–10 and human iris sphincter cells.5 Tobin and Osborne11 identified an M2 receptor that inhibits adenylate cyclase activity and has low affinity for pirenzepine in the rabbit ciliary epithelium, providing the first evidence for a second muscarinic receptor in the eye. Its ability to attenuate isoproterenol stimulation of cAMP levels suggested a modulatory role on aqueous humor production.12 A population of M1 receptors that bind pirenzepine with high affinity and elicit PI hydrolysis was also identified in the rabbit ciliary processes.13 Evidence of muscarinic receptor heterogeneity was also found in the rabbit iris sphincter14 and in human ciliary smooth muscle,5 but the particular subtypes involved could not be determined because of the limited selectivity of the available ligands.

Five subtypes of muscarinic receptors have now been characterized by gene cloning,5–17 enabling a more detailed classification of the ocular muscarinic receptors. In the commonly accepted International Union of Pharmacology nomenclature, the genetically defined m1 to m3 receptors correspond to the pharmacologic M1 to M3 receptors. Reverse transcription–polymerase chain reaction (RT–PCR) studies18 and in
were done with iris sphincters from five donors (ages 11, 43, 53, 60, and 64) and ciliary bodies from nine donors (mean age, 34).

Eagle's medium (Gibco, Gaithersburg, MD) supplemented with 10% fetal calf serum and 2000 U/l penicillin and streptomycin. Passage 4 cells were used for immunoprecipitation studies. Reagents and chemicals were purchased from Sigma Chemical Co.

Antisera against the five muscarinic receptor subtypes were graciously provided by Barry Wolfe (Georgetown University, Washington, DC). Receptor labeling, solubilization and immunoprecipitation were carried out essentially as previously described. Briefly, membranes were incubated with 0.5 nM [3H]-QNB for 45 minutes at 32°C in 25 ml of TE buffer containing 5 mM EDTA, pH 7.4, on ice. The homogenates were centrifuged at 23,700g in a Beckman JA-20 rotor for 15 minutes at 4°C. Pellets were resuspended in 2.5 to 6 ml 10 mM Tris, pH 7.4 (TE) containing approximately 10 /µg protein were incubated with 0.5 nM [3H]-QNB (38.8 to 44.9 Ci/mmol; Dupont-New England Nuclear, Boston, MA) for 45 minutes at 32°C. Nonspecific binding was defined in the presence of 5 µM atropine. Reactions were stopped with the addition of 3 ml ice-cold TE buffer and filtered through Whatman (Hillsboro, OR) GF/B glass fiber filters. The filters were washed twice with 3 ml TE, dried, and bound [3H]-QNB was measured by scintillation counting. The Bmax values determined for preparations of rat brain, ciliary muscle, ciliary processes and iris sphincter were 3, 6.6, 5.1 and 2 pmol/mg protein, respectively.

Immunoprecipitation
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METHODS
Tissue Preparation
Chinese hamster ovary cell lines expressing the human m1 to m5 muscarinic receptors were originally constructed by Bonner et al15,17 and were obtained from the National Institutes of Health. The cells were grown in 50% F12 Ham's–50% Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, MD) supplemented with 10% fetal calf serum and 2000 U/ml penicillin and streptomycin. Passage 4 cells were used for all experiments.

Human ocular tissues were obtained from the University of Wisconsin Eye Bank. The iris–ciliary body tissue was cut from the eye immediately after enucleation and frozen at —70°C. Dissections of ciliary body and iris sphincter were attained on ice. The ciliary processes were carefully peeled away from the ciliary body thus separating the muscle and the ciliary epithelial layers. The iris sphincter was cut with fine scissors at the junction of the sphincter and dilator muscle. Iris sphincters from 73 donors (mean age of 46) and ciliary bodies from 10 donors (mean age of 64) were used for immunoprecipitation studies. Reverse transcription–polymerase chain reaction studies were done with iris sphincters from five donors (ages 11, 43, 53, 60, and 64) and ciliary bodies from nine donors (mean age, 34).

For culturing cells from ciliary or iris sphincter muscles, eyes from a 65- and a 56-year-old man, respectively, enucleated 2 to 5 hours after death and kept on ice, were obtained from the Lions–University of California, Irvine Organ and Tissue Bank. Dissection of tissues was performed under sterile conditions using a dissection microscope. The ciliary muscle was exposed by peeling away the ciliary processes. The muscle was then removed and minced into small pieces. The iris was removed from the eye, and the pigmented epithelial layer was scraped off. The sphincter was then separated from the dilator and minced into small pieces. Tissues were placed in a Petri dish and cultured as described previously. Briefly, cells were allowed to migrate from the explants for 10 to 14 days in D-value-containing minimal essential medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 /µg/ml streptomycin, and 25 µg/ml Fungizone (Gibco). Cultures free of fibroblasts were maintained in medium 199 containing 6% glucose (Gibco). Passage 4 and 5 cells were used for RNA isolation.

Dissected frozen tissues or cells were homogenized with a polytron (Brinkmann, Westbury, NY; setting 6: 15 to 20 seconds) in 5 to 12 ml 20 mM Tris, 1 mM EDTA, pH 7.4, on ice. The homogenates were centrifuged at 23,700g in a Beckman JA-20 rotor for 15 minutes at 4°C. Pellets were resuspended in 2.5 to 6 ml 10 mM Tris, 1 mM EDTA, pH 7.4 (TE) using a tissueemizer (Tekmar; Cincinnati, OH; setting 60: 10 to 15 seconds). Protein concentration was measured with the BioRad (Hercules, CA) protein assay. Typically, the homogenate from one rat cortex contained 4 mg protein, ciliary muscle from 2 eyes 1.9 mg protein, the ciliary processes from 2 eyes 0.9 mg protein, and 20 iris sphincters 1 mg protein.

To determine receptor density, aliquots containing approximately 10 µg protein were incubated in 1 ml TE containing 0.5 nM [3H]-QNB (38.8 to 44.9 Ci/mmol; Dupont–New England Nuclear, Boston, MA) for 45 minutes at 32°C. Nonspecific binding was defined in the presence of 5 µM atropine. Reactions were stopped with the addition of 3 ml ice-cold TE buffer and filtered through Whatman (Hillsboro, OR) GF/B glass fiber filters. The filters were washed twice with 3 ml TE, dried, and bound [3H]-QNB was measured by scintillation counting. The Bmax values determined for preparations of rat brain, ciliary muscle, ciliary processes and iris sphincter were 3, 6.6, 5.1 and 2 pmol/mg protein, respectively.
cocktail of protease inhibitors (TE/PIC). The labeled membranes were pelleted and washed three times to remove unbound [3H]-QNB. All further steps were done at 4°C to prevent dissociation of the label. The membrane pellet was resuspended in TE containing 1% digitonin and 0.2% cholic acid (1% TEDC) at a final concentration of approximately 1 mg protein/ml, solubilized for 45 minutes, and centrifuged at 80,000g for 45 minutes. Typically, 40% to 45% of the labeled receptors were solubilized with no apparent difference between the subtypes.

The five muscarinic receptor subtypes and a control antisera were incubated in duplicate or triplicate at a concentration of 0.6 mg/ml (0.75 mg/ml for m2) with aliquots of 100 to 200 fmole (4 to 6000 cpm) of solubilized labeled receptors in 0.5 ml TE/PIC for 44 to 48 hours. Residual free [3H]-QNB was removed with a 10-minute incubation in 0.75 ml of a 2.7% serum-treated charcoal solution in 0.1% TEDC.

Supernatant (1.15 ml) was combined with 0.2 ml of 2× stripped Pansorbin (Calbiochem, La Jolla, CA; protein A-coated Staphylococcus aureus cells) that was prepared the previous day. After a 30-minute incubation with constant gentle rotation, the receptor-antibody-Pansorbin complex was pelleted at 13,000g. The supernatant and pellet were separated, 0.2 ml of 0.1 N NaOH-3% sodium deoxycholate was added, and the counts per minute of [3H]-QNB labeled receptor in each fraction determined by scintillation counting.

Non-specific precipitation by the control antisera represented 2% to 3% of total labeled receptors and was subtracted from the pellet values. Control samples that had been labeled in the presence of 5 μM atropine demonstrated that non-specific [3H]-QNB binding accounted for less than 5% of the total counts per minute. Percentage of precipitation by each antibody was calculated by dividing the adjusted pellet counts per minute by the combined pellet and supernatant counts per minute.

Reverse Transcription–Polymerase Chain Reaction
To avoid artifact bands caused by contaminating genomic DNA, tagged subtype-specific antisense primers with a 5’ 30 bp tag were used for reverse transcription. Subsequent polymerase chain reaction (PCR) amplification was carried out with a subtype-specific sense primer and the unique tag sequence as the antisense primer. This “RNA template-specific PCR” has been described previously by Shuldiner et al.13

Total RNA was prepared from cells and ocular tissues that had been frozen no more than 5 hours after death by guanidinium thiocyanate–phenol–chloroform extraction.21 Reverse transcription was carried out on 1 to 2 μg total RNA using the first strand synthesis kit from Boehringer Mannheim (Indianapolis, IN). A cDNA aliquot was denatured at 95°C for 5 minutes and combined in a 50-μl volume with 1.5 mM MgCl2, 100 μM dNTPs, 0.625 units Ampli Taq polymerase (Perkin Elmer, Foster City, CA), 25 μM primers and PCR buffer (50 mM KCl; 100 mM Tris–HCl, pH 8.3) for 40 cycles of PCR in a Perkin Elmer 9600 thermal cycler. The standard cycle included a 94°C, 30-second denaturation; a 60°C to 63°C, 1-minute annealing; and a 72°C, 1-minute extension. There was a 6-minute extension step at the end. Products were analyzed by agarose gel electrophoresis.

The tag sequence was 5’ GAA CAT CGA TGA CAA GCT TAG GTA TCG ATA 3’. The subtype-specific antisense sequences to which the tag was appended were, m1: 3’ CAT GGA GCC TTC GTC CTC T (nt 837 to 855); m2: 3’ AAC ACA GTT TTC AGT CAC A (nt 807 to 825); m3: 3’ GGT TTC CAG CTC TTG (nt 947 to 965); m4: 3’ ACG CTC TGC TTC ATT AGT G (nt 745 to 761); and m5: 3’ CGC AAG CAG GAT CTG AAG A (nt 746 to 764). The subtype-specific sense primers were, m1: 5’ CAA CAT CAC CGT CCT GCC A (nt 33 to 51); m2: 5’ CCT CCG TCT TAC AAG TCC T (nt 33 to 51); m3: 5’ CCT GCA CAA TAA CAG TAC A (nt 6 to 24); m4: 5’ CAA CTT CAC ACC TGT CAA T (nt 6 to 24); and m5: 5’ TCT TAC CAC AAT GCA ACC A (nt 13 to 31). The expected sizes of the PCR products were, 823 bp for m1, 793 bp for m2, 960 bp for m3, 756 bp for m4 and 752 bp for m5. Nucleotides are numbered from the beginning of the coding sequence. The Genbank accession numbers for the m1, m2, m3, m4, and m5 receptor sequences are X15263, X15264, X15266, X15265, and M80333, respectively.

RESULTS

Immunoprecipitation
Muscarinic Receptor Expressing Chinese Hamster Ovary Cell Lines. The specificity and sensitivity of each antisera were tested on solubilized membranes from CHO cell lines that stably express each of the five muscarinic receptors.10,17 The m1, m2, m3, m4, and m5 receptor antisera bound 90%, 50%, 78%, 80%, and 78%, respectively, of the [3H]-QNB labeled muscarinic receptors from the corresponding CHO cell lines (Fig. 1). The m1, m2, and m4 antisera did not precipitate muscarinic receptors from any of the other cell lines. The m3 antisera bound 2% of the muscarinic receptors from the m1– and m2– expressing cells and the m5 antisera bound 2% of the receptors from the m2 and m3 CHO cells.

To confirm the sensitivity of these antisera in a
mixed receptor population, defined mixtures of m3 and m5 CHO cell membranes were immunoprecipitated with m3 and m5 antisera. When the relative amount of m3 receptor was changed from 0% to 5%, the percentage of immunoprecipitation by the m3 antisera increased from 0% to 7%. Likewise a 0% to 5% change in the m5 receptor density resulted in a 2% to 5% increase in immunoprecipitated receptor. Further increases in receptor density resulted in a linear increase in the amount of receptor immunoprecipitated (Fig. 2A). Similar results were seen when the m1 and m3 receptors were mixed in defined ratios. Five percent of the m3 receptor was detected as 5%, and 5% of the m1 receptor was detected as 8% (Fig. 2B).

Rat Cortex. The antisera were then tested on solubilized rat cortical membranes. The density of each subtype expressed as a percentage of total muscarinic receptors was 41% m1, 19% m2, 17% m3, 24% m4, and 0% m5 (Fig. 3).

Ocular Tissues. Solubilized membranes prepared from human iris sphincter, ciliary muscle, and ciliary processes were immunoprecipitated with each of the antisera (Table 1). In the iris sphincter, the density of each subtype was 7.4% m1, 7.8% m2, 59.1% m3, 11.4% m4, and 5.4% m5. In the ciliary muscle the relative densities were 0.8% m1, 5.4% m2, 73.5% m3, 4.9% m4, and 2.4% m5. In the ciliary processes, there were 6.6% m1, 4.9% m2, 57.6% m3, 4.6% m4, and 2% m5.

Reverse Transcription–Polymerase Chain Reaction

The specificity of each set of PCR primers was tested using RNA prepared from the muscarinic receptor containing CHO cell lines. Each primer pair amplified a single fragment of the expected size from CHO cell cDNA expressing the corresponding receptor (Fig. 4). Only faint nonspecific products less than 400 bp in size were amplified by these primers when incubated with cDNAs from CHO cells expressing the other four subtypes (data not shown).

The expression of mRNA encoding the muscarinic receptors from Chinese hamster ovary (CHO) cell lines stably expressing single subtypes. Muscarinic receptors from five cell lines that express single subtypes were labeled with [3H]-QNB, solubilized, and immunoprecipitated using the antisera for the five muscarinic receptors. The counts per minute for [3H]-QNB–labeled receptor immunoprecipitated from the cell lines indicated on the x-axis is expressed as a percentage of total counts per minute of labeled receptors in each incubation.

**FIGURE 1.** Immunoprecipitation of muscarinic receptors from Chinese hamster ovary (CHO) cell lines stably expressing single subtypes. Muscarinic receptors from five cell lines that express single subtypes were labeled with [3H]-QNB, solubilized, and immunoprecipitated using the antisera for the five muscarinic receptors. The counts per minute for [3H]-QNB–labeled receptor immunoprecipitated from the cell lines indicated on the x-axis is expressed as a percentage of total counts per minute of labeled receptors in each incubation.
rinic receptor subtypes in the human ocular tissues was determined by RT-PCR (Fig. 5). In addition to the tissues used for immunoprecipitation studies, cultures of smooth muscle cells isolated from the human iris sphincter and human ciliary muscle were studied. In the iris sphincter sample (Fig. 5A), primers specific for the m1, m2, m4 and m5 receptors amplified PCR products, with the m2 particularly abundant. In samples from iris smooth muscle cells (Fig. 5B), only products from reactions with m2, m3, and m4 primers were apparent. Products of PCR were amplified by primers selective for the m2, m3, and m4 subtypes from both ciliary muscle tissue (Fig. 5C) and ciliary muscle cell samples (Fig. 5D).

RAT CORTEX

FIGURE 3. Quantitative immunoprecipitation of muscarinic receptor subtypes from rat cortex. Rat cortex homogenate was labeled with [3H]-QNB, solubilized, and immunoprecipitated using antisera selective for the subtypes indicated on the x-axis. The counts per minute of [3H]-QNB-labeled receptor immunoprecipitated by each antisera are indicated by the solid bar and the counts per minute of [3H]-QNB-labeled receptor not recognized by each antisera, representing the other four subtypes, are indicated by the hatched part of the bars. For each antisera, the total counts per minute were approximately 4250. The percentage of immunoprecipitation calculated from the data is indicated above the bars. The data are representative of two separate experiments.

DISCUSSION

Data collected in previous pharmacologic and molecular biologic studies on muscarinic cholinergic receptors in anterior ocular tissues have led to the conclusion that there is subtype heterogeneity. The m3 and m2 subtypes appear to be the most prevalent, but there is also evidence for the expression of the other three subtypes (m1, m4, and m5). The lack of highly selective pharmacologic agents and the qualitative nature of the studies on gene expression results in a somewhat confusing picture to date.

To determine which are the significant muscarinic receptor subtypes in the iris and ciliary body that may serve as targets of selective drug action, we have quantified receptor protein levels using subtype-selective antibodies. These antibodies were generated against fusion proteins containing fragments of the third intracellular loop regions of these receptors, except for the m3 antisera that is directed against a carboxyl terminal synthetic peptide. These domains have the least sequence similarity between the subtypes. Results of previous studies have shown that these antibodies could quantitatively immunoprecipitate rat-brain muscarinic receptors that have been solubilized and radiolabeled with the nonselective ligand, [3H]-QNB.

Control experiments carried out on solubilized membranes from CHO cell lines that stably express
TABLE 1. Percent Immunoprecipitation of Muscarinic Receptors by Subtype-Selective Antisera

<table>
<thead>
<tr>
<th>Cell/Tissue</th>
<th>m1</th>
<th>m2</th>
<th>m3</th>
<th>m4</th>
<th>m5</th>
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<tr>
<td>HISM cells</td>
<td>7.4±0.8</td>
<td>7.8±5.5</td>
<td>59.1±7.8</td>
<td>11.4±4.1</td>
<td>54±3.1</td>
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<tr>
<td>Iris sphincter</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td></td>
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<td>(n=2)</td>
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<td>(n=2)</td>
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<tr>
<td>Iris sphincter</td>
<td>+</td>
<td>++</td>
<td>+</td>
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<tr>
<td>(n=3)</td>
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<td>(n=3)</td>
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<tr>
<td>HCSM cells</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Ciliary muscle</td>
<td>0.8±1.3</td>
<td>5.4±2.6</td>
<td>73.5±0.9</td>
<td>4.9±4.7</td>
<td>2.4±2.1</td>
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<td>(n=3)</td>
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<td>(n=3)</td>
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<tr>
<td>Ciliary muscle</td>
<td>+</td>
<td>++</td>
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<td>(n=3)</td>
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<td>Ciliary processes</td>
<td>6.6±2.1</td>
<td>4.9±2.4</td>
<td>57.6±5.5</td>
<td>4.6±5.5</td>
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HISM = human iris sphincter smooth muscle; HCSM = human ciliary smooth muscle.
Values are mean ± SD from two to four independent experiments as indicated in parentheses. Abundance of fragments amplified by RT-PCR from mRNA: + = low; ++ = medium; +++ = high.

Each of the human muscarinic receptor subtypes confirmed the selectivity and sensitivity of these antibodies on human receptors (Fig. 1). Each antibody, except the m2 receptor antibody, immunoprecipitated 78% to 90% of the appropriate receptor protein and less than 2.5% of any of the other receptors. The m2 receptor antibody was less effective, only immunoprecipitating approximately 50% of the m2 receptors. Thus, the levels of m2 receptor determined in various ocular tissues could be underestimated by half.

The sensitivity and selectivity of these antibodies suggested that they could detect a population of muscarinic receptors that represented as little as 5% of the total muscarinic receptors in a tissue. To simulate such a situation, m3 and m5 receptors, or m1 and m3 receptors, were mixed together in various ratios and immunoprecipitated with their respective antibodies. The results demonstrate that the antibodies can distinguish between a mixture that contains no target receptor or 5% target receptor (Fig. 2). Also, there is a linear relationship between the amount of receptor and the amount that the antibodies precipitate.

Control experiments on solubilized rat cortical tissue (Fig. 3) resulted in values for each receptor subtype that were similar to those previously determined and consistent with results from in situ hybridization studies that showed that the m1 and m4 receptors were the most abundant. The experiments also demonstrated that we could quantitatively immunoprecipitate muscarinic receptor subtypes from solubilized tissues.

In all the ocular tissues and cells tested, the m3 receptor is the predominant subtype (Table 1), confirming the previous pharmacologic findings using the antagonist, 4-DAMP. The heterogeneity that has been seen previously appears to be caused by a complex tissue-specific mixture of the other subtypes. In the ciliary muscle, as well as in smooth muscle cells cultured from ciliary muscle, small populations of the m2 and m4 receptors are also present. Since the m2 antibody binds less well than the other antibodies, the m2 receptors may represent 10% of the total muscarinic receptors. The results of Zhang et al using in situ hybridization on ciliary muscle tissue and cell lines are consistent with our data. They reported that m3-expressing cells were ubiquitously distributed while m2 expression was more discretely localized to single cells or small clusters of cells. They also detected a small population of m5-expressing cells. Although

Figure 4. Reverse transcription–polymerase chain reaction of muscarinic receptor mRNAs from Chinese hamster ovary cell lines stably expressing single subtypes. Sets of oligonucleotide primers that are specific for each of the muscarinic receptor subtypes were used to prepare cDNAs and to amplify DNA fragments from cells expressing the corresponding subtype mRNA (below). Products were analyzed by agarose gel electrophoresis with a lambda HindIII DNA size marker (left lane). The predicted sizes of the polymerase chain reaction products are 823 bp (base pairs; m1), 793 bp (m2), 960 bp (m3), 756 bp (m4), and 752 bp (m5).
there was some receptor immunoprecipitated by the m5 antisera in the current data, the value (2.4%) is too small to be considered conclusive. The ciliary processes express the m1, m2, and m4 subtypes in addition to the m3 receptor. Both the m1 and m2 receptors have been detected previously in second-messenger studies conducted on rabbit ciliary processes, which suggests that these subtype populations, though small, are functional. Since m1 mRNA has not been detected by RT–PCR in cultured NPE cells, it is possible that m1 receptors are expressed in the pigmented epithelium. Their distribution and their function in aqueous humor secretion remain to be determined. The location and role of the m4 subtype is also unknown. Transcripts encoding m4 were previously detected in bovine ciliary processes by Northern blot analysis.

All five subtypes were detected in the iris sphincter. Because of the small size of this tissue, however, it is possible that contamination with receptors from other cell types associated with the tissue is more significant. The m3 mRNA has been detected in human iris sphincter, as described for m2 and m5 receptors in the midbrain of rats. Outside the central nervous system, m5 mRNA has been detected in human macrophages and in the human ciliary muscle. The presence of m5 protein in a small number of human cells outside the central nervous system is demonstrated for the first time in these results.

The same subtypes were detected by RT–PCR and immunoprecipitation with one surprising exception. Even though the m3 subtype is the predominant muscarinic receptor at the protein level in iris sphincter, no m3 transcripts were detected (Fig. 5A). Control experiments on m3—expressing CHO cells (Fig. 4) indicate that this primer set is not as sensitive as the other primers, but it did detect m3 transcripts in the ciliary muscle and iris sphincter cell lines (Fig. 5). Interestingly, in the Northern blot studies of Honkanen et al., they also did not detect any m3 mRNA (m4 in their nomenclature) in the iris sphincter. Perhaps the level of m3 transcripts in the iris sphincter is low and not easily detected. One explanation for this result is the recent finding that m3 receptor protein is degraded very slowly. There is no need, therefore, for cells to express much m3 mRNA. Such disparities between mRNA and protein levels demonstrate the value of measuring levels of receptor protein, as we have done.

The coexistence of multiple subtypes, some that activate the same second messenger pathways, in such apparently homogeneous tissues as smooth muscle is puzzling. The current data and the in situ hybridization study on ciliary muscle of Zhang et al. do not determine whether receptor subtypes coexist in a single cell. But the in situ results clearly demonstrate that cells within the ciliary muscle have unique patterns of muscarinic receptor expression. This tissue and the cell lines derived from it should be useful models for determining the physiological significance of the various receptor subtypes. The longitudinal muscle of the rat ileum has both the m2 and m3 receptor subtypes, and it has been suggested that inhibition of adenylate cyclase by the m2 subtype antagonizes β-adrenergic-mediated relaxation, thereby enhancing m3–induced muscle contraction. It will be interesting to see whether the m2—expressing cells in the ciliary muscle also express β-adrenergic receptors.

In conclusion, the m3 muscarinic receptor is the predominant receptor in the iris–ciliary body of the human eye. The m2 and m4 subtypes are also present at lower levels in all the tissues. The m1 and m5 subtypes appear
Acknowledgments

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References


