Molecular and pharmacological characterization of muscarinic receptors in retinal pigment epithelium: role in light-adaptive pigment movements

Prasad V. Phatarpekar, Simon F. Durdan, Chad M. Copeland, Elizabeth L. Crittenden, James D. Neece and Dana M. García

Department of Biology, Texas State University-San Marcos, San Marcos, Texas, USA

Abstract

Muscarinic receptors are the predominant cholinergic receptors in the central and peripheral nervous systems. Recently, activation of muscarinic receptors was found to elicit pigment granule dispersion in retinal pigment epithelium isolated from bluegill fish. Pigment granule movement in retinal pigment epithelium is a light-adaptive mechanism in fish. In the present study, we used pharmacological and molecular approaches to identify the muscarinic receptor subtype and the intracellular signaling pathway involved in the pigment granule dispersion in retinal pigment epithelium. Of the muscarinic receptor subtype-specific antagonists used, only antagonists specific for M1 and M3 muscarinic receptors were found to block carbamyl choline (carbachol)-induced pigment granule dispersion. A phospholipase C inhibitor also blocked carbachol-induced pigment granule dispersion, and a similar result was obtained when retinal pigment epithelium was incubated with an inositol trisphosphate receptor inhibitor. We isolated M2 and M5 receptor genes from bluegill and studied their expression. Only M5 was found to be expressed in retinal pigment epithelium. Taken together, pharmacological and molecular evidence suggest that activation of an odd subtype of muscarinic receptor, possibly M5, on fish retinal pigment epithelium induces pigment granule dispersion.

Keywords: acetylcholine, bluegill, light-adaptation, muscarinic receptors, pigment granule movement, retinal pigment epithelium.

Molecular acetylcholine (ACh) receptors belong to the G protein-coupled receptor superfamily, members of which initiate intracellular responses by interacting with heterotrimeric G proteins and are broadly characterized by seven transmembrane segments. Molecular cloning has identified five different subtypes of muscarinic receptors (M1–5) in mammals, each encoded by a distinct gene lacking introns in the coding region (Bonner et al. 1988). The muscarinic receptors are divided into two groups, M_odd and M_even, according to their functional coupling. M_odd receptors preferentially couple to pertussis toxin-insensitive G_i11 proteins to mediate stimulation of phospholipase C (PLC). Upon activation of these subtypes, PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate, leading to the formation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol. These products act as second messengers by mobilizing Ca^{2+} from intracellular stores and activating protein kinase C respectively (Eglen and Nahorski 2000). M_even receptors preferentially couple to pertussis toxin-sensitive G_i protein to mediate inhibition of adenyl cyclase (AC), thereby decreasing cyclic AMP (cAMP) levels (Felder 1995).

The muscarinic receptors are the predominant cholinergic receptors in the central and peripheral nervous systems. They are found in cardiac and smooth muscles and in various...
exocrine glands (Caulfield 1993). In the heart, muscarinic receptors regulate the rate and force of contraction (Caulfield and Birdsall 1998; Hsieh and Liao 2002). In the CNS they are involved in motor control, temperature regulation, cardiovascular regulation and memory (Caulfield and Birdsall 1998). Dysfunction of muscarinic receptor signaling has been implicated in brain disorders such as Alzheimer’s disease, Parkinson’s disease and schizophrenia (Flynn et al. 1995; Growdon 1997; Birdsall et al. 2001).

Recently, activation of muscarinic receptors was found to elicit pigment granule dispersion in retinal pigment epithelium (RPE) isolated from bluegill (González et al. 2004). The RPE is a monolayer of cells forming a tissue located between the neural retina and the choroid (Zinn and Marmor 1979), and pigment granule movement in RPE is a light-adaptive mechanism in fish. Fish pupils have fixed diameter and adaptation to changes in light intensities is achieved in part by pigment granule movements within the RPE coupled with photoreceptor movement (Burnside and Nagle 1983). In light, cone photoreceptors contract, rod photoreceptors elongate and RPE pigment granules disperse into the cells’ long apical processes that interdigitate with the photoreceptors. The pigment granules shield the rods’ outer segments, protecting them from bleaching in bright light (Douglas 1982). In the dark, opposite photoreceptor movements occur, and RPE pigment granules aggregate into the cell bodies, increasing the exposure of rods to available light. Collectively these movements maintain the appropriate photoreceptors at their optimum light conditions (Burnside and Nagle 1983). From studies using the cholinoergic agonist carbamyl choline (carbachol), García (1998) suggested that ACh might play a role in light-adaptive pigment granule dispersion in green sunfish (Lepomis cyanellus). González et al. (2004) reported that carbachol-induced pigment granule dispersion in RPE isolated from bluegill (L. macrochirus) is mediated by a muscarinic receptor and inferred that it belonged to one of the odd-numbered subclasses. This inference was based on pharmacological evidence that antagonists specific for M1 and M3 muscarinic receptors blocked pigment granule dispersion, whereas an agonist specific for the M1 receptor activated dispersion. The agonists and antagonists specific for even-numbered muscarinic receptors (M2 and M4) failed to induce or inhibit pigment granule dispersion respectively. However, because subtype-specific pharmacological agents, which have been characterized predominantly for mammalian muscarinic receptors, are known to exhibit different affinity profiles for non-mammalian muscarinic receptors (Tietje and Nathanson 1991; Hsieh and Liao 2002), González’s inference can still be regarded as a hypothesis in need of further testing. Furthermore, the lack of agonists and antagonists with high selectivity for any particular subtype leaves the pharmacological demonstration of a functional receptor subtype rather incomplete (Caulfield and Birdsall 1998). Molecular characterization of muscarinic receptors in fish and studies of their expression and function in RPE along with pharmacological studies might help resolve the problem of identification of subtypes involved in pigment granule movement in fish RPE.

In this paper we report further pharmacological characterization of the signaling pathway involved in pigment granule dispersion in RPE as well as isolation of muscarinic receptor genes from bluegill genomic DNA and cDNA, and their expression in RPE, retina and other tissues including heart and brain. Our results obtained using both molecular and pharmacological approaches support a model in which activation of an odd subtype of muscarinic receptor, specifically M3, on fish RPE induces pigment granule dispersion.

Materials and methods

Fish maintenance

Experiments were performed using protocols approved by the Institutional Animal Care and Use Committee. Bluegill (L. macrochirus) were obtained from Johnson Lake Management (San Marcos, TX, USA). Fish were maintained in aerated 55-gallon aquaria on a 12-h light/12-h dark cycle for at least 2 weeks before use.

Pharmacological analysis of signaling pathways

Pharmacological experiments were carried out following the method of González et al. (2004). In brief, fish were dark adapted for 30 min in a light-tight box 6 h after the onset of light. Fish were killed by severing the spinal cord and double pithing. The eyes were removed and hemisected. The anterior portion was discarded and the retina was removed from the posterior eyecup. The RPE was flushed from the eyecup using a stream of low-calcium, bicarbonate buffered Earle’s Ringer (LCBR) solution, aerated with 5% CO2 and 95% air (pH 7.2). Pigment granule aggregation was induced by a 45-min incubation in 10 μM forskolin (FSK) (LC Laboratories, Woburn, MA, USA). The FSK was removed by washing three times with LCBR and the RPE was divided into samples. To determine whether ACh induced pigment granule dispersion, tissue was then treated with 100 nM ACh (Sigma, St Louis, MO, USA) or carbachol (Chemicon, Temecula, CA, USA) alone or in the presence of 100 μM huperzine-A (LC Laboratories) for 45 min before being fixed overnight using 0.5% glutaraldehyde, 0.5% paraformaldehyde and 0.8% potassium ferricyanide in phosphate-buffered saline. The cells were then examined using phase-contrast microscopy. Pigment indices (PIs; Bruenner and Burnside 1986) were recorded for 30 cells from each treatment. A minimum of three fish was used to provide tissue for replicates of each treatment group (n = number of fish). Treatment means were analyzed using ANOVA followed by Tukey’s post hoc test for multiple comparisons. Treatments were judged to be significantly different when p < 0.05.

To test the receptor and downstream signaling pathways involved in carbachol-induced pigment granule dispersion, RPE was isolated and treated with FSK as described above. Isolated tissue was then treated with 100 μM carbachol (ICN Biomedicals, Inc., Aurora, OH, USA) alone or in the presence of telenzepine (M1 antagonist; Torcis, Ellsville, MO, USA), methoctramine (M4 antagonist; Sigma-Aldrich, St Louis, MO, USA), p-fluorohexahydroisadiphenidol (p-FHHSiD, M3 antagonist; Sigma-Aldrich), U73122 (PLC

inhibitor; Sigma-Aldrich) or 2-aminoethoxydiphenyl borate (2-APB; IP3 receptor antagonist; Toceis) for 45 min. Drugs were prepared as 10 x stock solutions and were used that day or frozen (−20°C) for later use. As p-FHHSiD was prepared in ethanol as a 100-μm stock solution, a vehicle control consisting of carbahchol plus 10% ethanol was performed. Similarly, U73122 was prepared as a 1-mm stock solution in dimethylsulfoxide (DMSO), as was 2-APB, and a vehicle control was also carried out consisting of carbahchol plus 1% DMSO. Cells were then fixed and analyzed as described above. For telenzepine and p-FHHSiD, the concentration of antagonist leading to 50% inhibition of the response to carbahchol (IC50) was estimated using Excel (Microsoft, Redmond, WA, USA) along with Xlfit4 (http://www.idbs.com/xlfit4/). A Boltzmann sigmoidal curve was fitted to each data set, and the highest and lowest Y value (PI) obtained from the fitted curve was used to calculate the midpoint. The X value, or log concentration, corresponding to that Y value was then obtained, and was converted to the negative log, or pIC50.

Isolation and amplification of muscarinic receptor genes
Genomic DNA of bluegill was prepared using the phenol-chloroform-isooamyl alcohol method (Hillis et al. 1996). To isolate and amplify M2 muscarinic receptor gene, primers were designed using zebrafish M2 muscarinic receptor gene (C. F. Liao, J. Y. Hsieh and M. Y. Fang, submitted to National Center for Biotechnology Information (NCBI) in 2001, unpublished). The zebrafish M2 muscarinic receptor gene was used as a query sequence to identify the putative fugu M2 muscarinic receptor gene sequence from the fugu genomics project website (http://fgu.hgmp.mrc.ac.uk; release 3) using BLAST. Zebrafish and fugu M2 genes were aligned using the program CLUSTAL W (available on the computer program BioEdit; http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Conserved regions, at least 20 nucleotides long, were selected near 5'- and 3'-ends of the coding strands to design forward (M2F, 5'-CGGTGCTGTTGAC-3') and reverse (M2R, 5'-GTCTTTGACTGCGAGGAGSAG-3') primers respectively. To isolate and amplify the M2 muscarinic receptor gene, primers were designed using zebrafish M2 muscarinic receptor gene (Hsieh and Liao 2002). The zebrafish M2 muscarinic receptor gene was used as a query sequence to identify the putative fugu M2 muscarinic receptor gene sequence from the fugu genomics project website using BLAST. The genes were aligned as mentioned above and conserved regions, at least 20 nucleotides long, were selected near 5'- and 3'-ends of the genes to design forward (M2F, 5'-AACCTCACCCTWCTGGAGATGCCCT-3') and reverse (M2R, 5'-GTCTTTGACTGCGAGGAGSAG-3') primers respectively. Genomic DNA was amplified by PCR. A 50-μL PCR reaction contained 5 μL 10 x buffer (final concentration 10 mM Tris-HCl, pH 8.3, 50 mM KCl and 15 mM MgCl2), dNTPs (2.5 mM each), 50 pmol each primer and 5 units DNA Taq polymerase (Promega). PCR consisted of one cycle of 94°C for 64 s followed by 35 cycles, of 95°C, 60°C and 72°C for 30, 30 and 90 s respectively, followed by a final extension of 72°C for 5 min. The primers used were 5'-AACCTCACCCTWCTGGAGATGCCCT-3' (sense) and 5'-GTCTTTGACTGCGAGGAGSAG-3' (antisense) for M2, and 5'-CACAGCCTTGGAGAGGAAGGT-3' (sense) and 5'-CACATGGGAGAAGGTGCTTTGAG-3' (antisense) for M2; all were synthesized by Bio-synthesis Inc. PCR products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide. PCR products were sent for sequencing to Retrogen.

Amplification of the fugu M2 gene was performed by PCR on genomic fugu DNA (MRGeneservice, Cambridge, UK). Primers were designed using the putative M2 sequence obtained from the fugu genomic databases. The forward primer sequence was 5'-GGCCCGAGTCACATCTCACCT-3', and the reverse primer sequence was 5'-ATGTGCTCATTCGAGTCAGTCTGAGGAGC-3'. The 25-μL reactions included 0.5 μg fugu genomic DNA, 1.5 mM MgCl2, 0.2 mM dNTPs, thermophilic DNA polymerase buffer (Promega); 16.5 μL autoclaved, deionized H2O and 0.25 μL Taq polymerase at 5 units/μL (Promega). A MyCycler (Bio-Rad, Hercules, CA, USA) was used as follows: 94°C for 1 min, 30 cycles of 94°C, 57°C and 72°C for 30, 30 and 60 s respectively, followed by 72°C for 5 min.

All PCR products were subjected to electrophoresis on a 1% agarose gel at 120 V for 30 min, stained with ethidium bromide and then viewed under UV light to verify the presence of amplified product.

DNA sequencing and analysis
All PCR products were sent to Retrogen (San Diego, CA, USA) for sequencing. The DNA sequences and deduced amino acid sequences were analyzed for similarity to known sequences using BLAST programs available on NCBI website. Sequences were also analyzed phylogenetically (see below).

Isolation of mRNA from bluegill tissues, generation of cDNA and subsequent PCR
Total RNA was extracted from approximately 10 mg samples of heart, RPE, retina, muscle or brain tissue of bluegill fish (L. macrochirus) using guanidinium thiocyanate and passage through a silica-based filter (RNAqueous-4PCR kit; Ambion, Austin, TX, USA), following the manufacturer’s instructions. After extraction, 1–2 μg total RNA from each tissue was then used to generate cDNA, using the RETROscript® kit (Ambion). The two-step protocol was employed (see manufacturer’s instructions), with the total RNA and oligo(dT) being heat denatured at 82°C for 3 min before the addition of the remaining RT solutions, including RT buffer, and subsequent RT.

The cDNA transcripts were amplified in a volume of 50 μL by PCR. These reactions comprised 5 μL each cDNA synthesis solution, PCR buffer (final concentration 10 mM Tris-HCl, pH 8.3, 50 mM KCl and 15 mM MgCl2), dNTPs (2.5 mM each), 50 pmol each primer and 5 units DNA Taq polymerase (Promega). PCR consisted of one cycle of 94°C for 64 s followed by 35 cycles of 95°C, 60°C and 72°C for 30, 30 and 90 s respectively, followed by a final extension of 72°C for 5 min. The primers used were 5'-AACCTCACCCTWCTGGAGATGCCCT-3' (sense) and 5'-GTCTTTGACTGCGAGGAGSAG-3' (antisense) for M2, and 5'-CACAGCCTTGGAGAGGAAGGT-3' (sense) and 5'-CACATGGGAGAAGGTGCTTTGAG-3' (antisense) for M2; all were synthesized by Bio-synthesis Inc. PCR products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide. PCR products were sent for sequencing to Retrogen.

Identification of full-length coding region of bluegill muscarinic receptor genes by rapid amplification of cDNA ends (RACE)
In order to obtain the full-length coding region of bluegill M2, total RNA was isolated from bluegill brain tissues (shown by RT-PCR to
include both M2 and M5 mRNA) using an RNAqueous®-PCR kit (Ambion). Then, 16 μL of this RNA was used together with a RACE kit (FirstChoice® RLM-RACE kit; Ambion) to obtain both 5'- and 3'-ends, following the kit protocol. Gene-specific primers were used in combination with the primers for the 5'- and 3'-end linkers for nested PCR as follows: for the 5' end, outer primer 5'-TCAGAGGAGGCATAACTGTGAAGG-3' (antisense) and inner primer 5'-GCCAGCATAAGATAAGG-3' (antisense); for the 3' end, outer primer 5'-GTCAAGCTCATCCTATGTG-3' (sense) and inner primer 5'-CAACAATGATAGGCTAGAGCTG-3' (sense) (synthesized by Bio-synthesis Inc.). PCR products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide. PCR products were sent for sequencing (Retrogen).

M2 5'- and 3'-ends were identified in a similar way, again using the total RNA isolated from bluegill brain tissues. However, the gene-specific primers used were for the 5'-end, outer primer 5'-TCAGAGGAGGCATAACTGTGAAGG-3' (antisense); for the 3'-end, outer primer 5'-GTCAAGCTCATCCTATGTG-3' (antisense) and inner primer 5'-CCACCTCCACAGTCTTGTAG-3' (antisense) (synthesized by Bio-synthesis Inc.).

Phylogenetic analysis
Amino acid sequences of different muscarinic receptor subtypes from all available taxa were downloaded from the protein database available at the NCBI website (Table 1). Sequences of coding strands (nucleotide) of muscarinic receptors were downloaded in FASTA format from NCBI GenBank (Table 2). Amino acid sequence alignment was performed with Clustal X (Thompson et al. 2005). The resulting trees all the muscarinic receptors formed a monophyletic network except Drosophila melanogaster and Caenorhabditis elegans muscarinic receptors, which formed a separate monophyletic group. Modeltest 3.06 (Posada and Crandall 1998) was used to select the model of evolution for Bayesian analysis using DNA alignment. The general time reversible + 1 (invariant sites) + G (gamma distribution) model was selected for having the highest log likelihood. The Bayesian analysis was performed using MrBayes 3.0 (Huelsenbeck and Ronquist 2001). C. elegans muscarinic

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M1, M2, M3, M4 and M5 refer to the muscarinic receptor subtypes 1, 2, 3, 4 and 5 respectively. NA, not available in NCBI database. The sequence denoted as NA was isolated and identified in the present study.
receptor was selected as an outgroup for the analysis. The initial setting included Markov Chain Monte Carlo search, which was set to run 1000 generations with a sample frequency of 100. Based on the time required to run 1000 generations, another run was set up to run for 15 min. The runs were repeated with increasing number of generations until the sum of the log likelihoods of trees converged to a stable value. Based on the number of generations taken to stabilize the log likelihood value, a final run was set up in which the number of generations was 20 times the number of generations taken to stabilize the sum of log likelihood values of the trees. The final setting included a Markov Chain Monte Carlo search set to run 400 000 generations with a sample frequency of 100, and burnin, the number of trees that would be ignored while the consensus tree was created, was set to 0.1 times the number of trees (400). The tree file produced in MrBayes was opened in PAUP*, and a majority consensus tree was constructed.

Table 2 Name, species and the GenBank accession number of muscarinic receptor genes (coding strands) used in phylogenetic analyses

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M1, M2, M3, M4 and M5 refer to the muscarinic receptor subtypes 1, 2, 3, 4 and 5 respectively. NA, not available in NCBI database. Sequences denoted as NA were isolated and identified in the present study.

Results

Pharmacological studies

To address the question of whether the native ligand ACh induces pigment granule dispersion, isolated RPE was subjected to a dose–response analysis. Initial results indicated that ACh at concentrations as high as 100 nM had no effect on pigment granule position (mean ± SEM; PI = 0.61 ± 0.02; n = 3) relative to that in FSK-treated cells (PI = 0.67 ± 0.02; n = 3). However, when 100 nM ACh was used in conjunction with the acetylcholinesterase (AChE) inhibitor huperzine-A (100 μM), pigment granule dispersion was as robust (PI = 0.90 ± 0.03; n = 3) as that induced by 100 nM carbachol in the absence (PI = 0.87 ± 0.03; n = 3) or presence (PI = 0.87 ± 0.07; n = 3) of huperzine-A (Fig. 1). These three treatments caused statistically significant pigment granule dispersion relative to that in FSK-treated cells (p < 0.05). The PI of cells treated with 100 μM huperzine-A indicates that, by itself, it has no effect on pigment granule position (PI = 0.67 ± 0.04; n = 3) compared with FSK-treated cells (PI = 0.68 ± 0.04; n = 3).

Telenzepine, an M1 muscarinic receptor antagonist, was tested for its ability to block carbachol-induced pigment granule dispersion (Fig. 2a). The mean PI of FSK-treated cells (PI = 0.69 ± 0.01; n = 4) was significantly different from that of carbachol-treated cells (PI = 0.89 ± 0.01; n = 4) (p < 0.001). At concentrations as low as 1 nM, telenzepine significantly (p < 0.001) inhibited pigment granule dispersion relative to that in carbachol-treated cells; telenzepine-treated cells had a mean PI of 0.82 ± 0.02 (n = 3) as that in FSK-treated cells (PI = 0.86 ± 0.02; n = 3). The pIC50 value for telenzepine was estimated to be 8.5.

RPE treated with p-FHHSiD, an M3-selective antagonist, was also inhibited from dispersing pigment granules. At concentrations as low as 10 nM, cells treated with p-FHHSiD were significantly (p < 0.001) less dispersed than cells treated with carbachol alone, the former having a mean PI of 0.79 ± 0.01 (n = 3) (Fig. 2a). As p-FHHSiD was prepared in ethanol, a vehicle control was tested with 10% ethanol and carbachol (PI = 0.86 ± 0.02; n = 3). The mean PI was not significantly different from that of carbachol-
treated cells \((p = 1.0)\). The pIC\(_{50}\) for \(p\)-FHHSiD was estimated to be 7.2.

Methoctramine, a muscarinic receptor antagonist selective for M2, did not block carbachol-induced pigment granule dispersion at any concentration examined (up to 10 \(\mu M\); Fig. 2a). There were no statistically significant differences between the PIs of RPE treated with carbachol alone and that treated with methoctramine.

As Modd receptors appeared to be involved in pigment granule dispersion, U73122, a PLC inhibitor, was used to test whether carbachol activated PLC. RPE cells treated with U73122 at concentrations as low as 100 nM were significantly \((p < 0.001)\) less dispersed than carbachol-treated controls, the former having a mean PI of 0.80 ± 0.05 \((n = 3)\) (Fig. 2b). The observation that the PLC inhibitor blocked carbachol-induced dispersion suggested that PLC activity is involved in mediating carbachol-induced dispersion. As PLC activity may result in the release of intracellular Ca\(^{2+}\) via IP\(_3\) receptor activation, we tested whether the IP\(_3\) receptor antagonist 2-APB could block carbachol-induced dispersion (Fig. 2b). At concentrations as low as 1 nM \((\text{PI} = 0.80 ± 0.06; \ n = 3)\), 2-APB was effective at blocking pigment granule dispersion caused by carbachol. In contrast, for vehicle controls containing 0.1 \(\mu M\) carbachol and 0.1% DMSO, the PI was 0.87 ± 0.02 \((n = 3)\) which was not statistically different from that for cells treated with carbachol alone \((\text{PI} = 0.87 ± 0.01; \ n = 3)\).

**Isolation of muscarinic receptor genes from bluegill genomic DNA**

To isolate the M\(_3\) muscarinic ACh receptor gene, bluegill genomic DNA was subjected to PCR using primers based on the homologous regions in the transmembrane domain I and transmembrane domain VII of zebrafish and fugu M\(_3\) muscarinic receptor genes. Agarose gel electrophoresis demonstrated the presence of an \(~1400\)-bp fragment. Upon sequencing a 1385-bp sequence was generated. The 5’- and 3’-ends of the coding sequence were obtained using RACE. The entire
sequence corresponding to the coding region of the M₅ muscarinic receptor gene is shown in Fig. 3(a). The sequence showed greatest identity with M₅ muscarinic receptor genes. The deduced amino acid sequence encoded by the bluegill M₅ muscarinic receptor gene was 527 amino acids long. The bluegill M₅ muscarinic receptor shared 65.3% amino acid identity with human M₅, whereas it shared only 46.7, 41.9, 53.1 and 41.5% amino acid identity with human M₁, M₂, M₃ and M₄ respectively. Comparison with other vertebrate muscarinic receptors showed that bluegill M₅ muscarinic receptor shared a high degree of identity with M₅ muscarinic receptors (Fig. 3b). The deduced amino acid sequence showed greater identity with the M₅ receptor proteins in fish than with other vertebrate M₅ receptors. The bluegill M₅ muscarinic receptor had 88.4, 88.4,

**Fig. 3** (a) Nucleotide and deduced amino acid sequences of the bluegill M₅ muscarinic receptor gene. Nucleotide residues are numbered in the 5' to 3' direction. The predicted amino acid sequence is shown below the nucleotide sequence. (b) Alignment of amino acid sequence of bluegill M₅ receptor with known vertebrate M₅ receptors using the program Clustal X (Thompson et al. 1997). The names of the sequences are prefixed with Hs (Homo sapiens, human), Mmul (Macaca mulatta, rhesus monkey), Mm (Mus musculus, mouse), Fm (Rattus norvegicus, rat), Cp (Cavia porcellus, guinea pig), Ggal (Gallus gallus, chick), Dr (Danio rerio, zebrafish), Tr (Takifugu rubripes, fugu), and Lm (Lepomis macrochirus, bluegill). TrM5 and LmM5 represent fugu putative M₅ receptor and bluegill M₅ receptor respectively. Residues identical to those of human M₅ are indicated by dots. Dashes in the alignment represent gaps inserted. Transmembrane domains (TMs) of bluegill M₅ receptor are delineated by dashes below the sequences. Amino acid residues critical for either ligand binding or G protein coupling are shown in bold. For details see text.

Muscarinic receptors in retinal pigment epithelium

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75.5, 67.5 and 65.4% amino acid identity with fugu, zebrafish, chicken and rat $M_3$ respectively.

$M_2$ muscarinic ACh receptor gene was isolated by subjecting cDNA from bluegill heart to PCR using primers based on the homologous regions in the N- and C-terminal domains of zebrafish and fugu $M_2$ muscarinic receptor genes. Agarose gel electrophoresis demonstrated the presence of an $\sim$1500-bp fragment. Upon sequencing a 1400-bp sequence was generated. The 5'- and 3'-ends of the coding sequence were obtained using RACE. The entire coding region sequence corresponding to the $M_2$ muscarinic receptor gene is shown in Fig. 4(a). The sequence showed greatest identity with $M_2$ muscarinic receptor genes. The deduced amino acid sequence encoded by the bluegill $M_2$ muscarinic receptor gene was 502 amino acids long. The bluegill $M_2$ muscarinic receptor shared 68.7% amino acid identity with human $M_2$, but only 41, 44.4, 58.4 and 45.6% amino acid identity with human $M_1$, $M_3$, $M_4$ and $M_5$ respectively. Comparison with other vertebrate muscarinic

(a)  

![Figure 4(a)](image-url)
Muscarinic receptors in retinal pigment epithelium

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Fig. 4 Continued.
receptors showed that bluegill M2 muscarinic receptor shared a high degree of identity with other M2 muscarinic receptors (Fig. 4b). The deduced amino acid sequence showed greater identity with the M2 receptors proteins in fish than with other vertebrate M2 receptors, with bluegill M2 muscarinic receptor having 92, 83, 71.5 and 68.3% amino acid identity with fugu, zebrafish, chicken and rat M2 respectively.

The fugu M2 muscarinic receptor gene was amplified from commercially obtained fugu genomic DNA. A 1500-nucleotide sequence was obtained from the 1500-bp product. The deduced amino acid sequence shown in Fig. 5 was 500 amino acids long. The fugu M2 muscarinic receptor showed 76% identity with the zebrafish M2 receptor, and 65 and 40% identity with the chick and human M2 receptors respectively.

Expression of bluegill M2 and M5 muscarinic receptors was studied in RPE and retina along with brain and heart by RT–PCR. Brain and retina were found to express both M2 and M5, whereas heart and RPE expressed only M2 and M5 respectively (Fig. 6).

![Nucleotide and deduced amino acid sequences of the fugu M2 muscarinic receptor gene. Nucleotide residues are numbered in the 5' to 3' direction. The predicted amino acid sequence is shown below the nucleotide sequence.](image-url)
Phylogenetic analysis
Phylogenetic analysis was performed to verify subtype identity of the muscarinic receptor genes isolated in the present study. A phylogenetic tree was obtained using nucleotide alignment and employing Bayesian analysis. Vertebrate muscarinic receptors formed one ingroup. Within the ingroup, two monophyletic groups were observed, one formed by odd-numbered muscarinic receptors and another by even-numbered muscarinic receptors. Within these groups, receptors belonging to the same subtype formed monophyletic clades. The bluegill M5 receptor formed a monophyletic unit with other M4 receptors, within which it formed a terminal clade with fugu and zebrafish M5 receptors. This terminal clade formed a sister group to the other vertebrate M4 receptors, which were grouped together. A similar arrangement of clades was observed for bluegill M2 receptor (Fig. 7). High Bayesian support (>70) was observed for all the monophyletic groups described above.

Discussion
In this paper we showed that ACh is effective in inducing pigment granule dispersion in RPE isolated from the retina of bluegill fish, that it is likely to act through an M2 receptor, and that RPE expresses the M2 receptor, which we isolated and sequenced along with the M2 receptor gene from bluegill generated by RT–PCR. DNase 1-treated total RNA isolated from the various tissues was used as template for RT–PCR reactions. Equal aliquots of cDNA were amplified with oligonucleotides for either M2 (1479 bp) or M5 (1385 bp) receptors. Thirty microliters of each RT–PCR product was loaded on a 1.5% gel and stained with ethidium bromide. Each PCR product was sequenced to confirm its identity.

Virtually all evidence from previous studies in the literature suggests that dopamine had been established by Darry and Burnside (1985, 1988, 1989) as an important light and circadian signal for inducing light-adaptive retinomotor movements in green sunfish and by Dearry et al. (1990) in bullfrog. In both fish and frog, dopamine was effective at nanomolar concentrations in inducing light-adaptive pigment granule dispersion. However, work by others (Douglas et al. 1992; Ball et al. 1993) raised the possibility that other neurochemicals might be involved in regulating light adaptation in fishes because treatments designed to deplete retinal dopamine levels failed to prevent light-induced or circadian retinomotor movements. We propose that ACh may fulfill that role.

In the present study we extended earlier pharmacological studies by employing additional subtype-specific antagonists not tested by González et al. (2004) for M1, M2 and M3 receptors. M3-selective agents were not available. The M2 antagonist used in the present study failed to block carbachol-induced pigment granule dispersion whereas antagonists specific for M1 or M3 receptors blocked the dispersion. These findings extend earlier results in which González et al. (2004) used the general muscarinic antagonist atropine to...
show carbachol at nanomolar concentrations operates through muscarinic receptors to induce pigment granule dispersion in bluegill RPE. Furthermore, González et al. (2004) observed that antagonists specific for M2 and M4 receptors failed to block carbachol-induced pigment granule dispersion, and an agonist specific for M2 receptors failed to induce pigment granule dispersion. In contrast, antagonists specific for M1 and M3 receptors blocked carbachol-induced pigment granule dispersion, and an agonist specific for M1 receptors activated dispersion. These observations led us to hypothesize that cholinergic activation of pigment granule dispersion is mediated through an M_odd receptor. Our current findings corroborate those of González et al. (2004), adding support for a model for M_odd-mediated pigment granule dispersion.

Earlier pharmacological studies of human, rat and chick RPE also indicated that they express muscarinic receptors. The earliest evidence for the presence of muscarinic receptors in RPE was provided by Friedman et al. (1988) in cultured human RPE cells, who employed binding studies with [3H]quinuclidinyl benzilate, a muscarinic receptor antagonist. Rat and chick RPE cells have also been shown to express muscarinic receptors (Salceda 1994; Fischer et al. 1998). Pharmacological studies by Feldman et al. (1991), Osborne et al. (1991) and Crook et al. (1992) demonstrated that muscarinic receptors in human RPE cells mediate phosphoinositide hydrolysis, which is further coupled to intracellular Ca2+ flux; this receptor-mediated phosphoinositide hydrolysis is pertussis toxin-insensitive. Feldman et al. (1991) and Crook et al. (1992) further suggested that the M3 receptor was involved in phosphoinositide hydrolysis, based on the efficiency of subtype-specific antagonists in blocking the action of carbachol.

Immunological evidence has demonstrated the presence of odd-numbered muscarinic receptor subtypes (M1 and M3) in cultured human RPE cells (Narayan et al. 2003). Friedman et al. (1988) observed that muscarinic agonists had no effect on intracellular cAMP levels in human RPE cells, nor did they alter the isoproterenol-induced stimulation of AC, indicating the absence of even-numbered muscarinic receptor subtypes (M2 and M4). Interestingly, chick RPE has been shown immunohistochemically to express M2 and M4 along with M3 muscarinic receptors (Fischer et al. 1998). Although pharmacological results obtained in the present study indicate the expression of only odd-numbered muscarinic receptor subtypes in bluegill RPE cells, it is important to note that in both chick (Tietje and Nathanson 1991) and zebrafish (Hsieh and Liao 2002) M2 receptors show a high affinity for pirenzepine, which in mammals has been characterized as a relatively selective M1 antagonist (Eglen et al. 2001).

The implication from the present study and the work of González et al. (2004) that M_odd receptors activate pigment granule dispersion was somewhat unexpected as King-Smith et al. (1996) had shown that pigment granule movements were insensitive to changes in cytosolic calcium levels. In fact, the demonstration that pigment movement seemed most sensitive to cAMP levels, aggregating when cAMP levels were raised and dispersing when they were lowered (García and Burnside 1994; King-Smith et al. 1996), and the observation by Deary and Burnside (1988) that dopamine acts on D2 receptors to inhibit AC and induce light-adaptive pigment granule dispersion, led García (1998) to hypothesize that carbachol-induced pigment granule dispersion involved M_even receptors which, like D2 receptors (see Robinson and Caron 1997; Watts et al. 2001), preferentially couple to G_i proteins to inhibit AC, thereby decreasing cAMP levels. However, decreased intracellular cAMP might come about through at least two cooperating mechanisms: decreased AC activity combined with phosphodiesterase activity. This decrease might be mediated through M_odd receptors (M1, M3, M5 or some combination). These receptors preferentially couple to pertussis toxin-insensitive Gi11α to activate PLC which catalyzes hydrolysis of phosphotidylinositol (4,5) bisphosphate to IP3 and diacylglycerol. IP3 liberates calcium stored in the endoplasmic reticulum by binding to the IP3 receptor, an IP3-sensitive calcium channel (Eglen and Nahorski 2000). A rise in cytosolic free calcium has been shown in some systems to inhibit cAMP accumulation by activating calmodulin, which modulates the activities of a number of enzymes including phosphodiesterase I, leading to degradation of cAMP (Beavo 1995). Muscarinic agonists have been shown to activate calmodulin-dependent phosphodiesterases to lower cAMP levels in a number of systems, including fibroblasts, thyroid cells and astrocytoma cells (Nemecek and Honeyman 1982; Van Erneux et al. 1985; Tanner et al. 1986). The calcium–calmodulin complex also activates calcineurin, a calcium-sensitive phosphatase that inhibits type 9 AC, thereby decreasing cAMP generation (Wera and Hemmings 1995; Antoni et al. 1995; Paterson et al. 1995). Decreases in intracellular cAMP might therefore be mediated through M_odd receptors by either of these pathways. Even though calcium–calmodulin also activates AC1, AC8 and AC3, intracellular calcium from IP3-sensitive stores is unable to affect these calcium-sensitive AC isoforms (Sunahara and Taussig 2002).

If muscarinic receptor activation does lead to an increase in intracellular calcium, which then leads to pigment granule dispersion, why did treatment with ionomycin fail to induce pigment granule dispersion in RPE isolated from green sunfish (King-Smith et al. 1996)? King-Smith et al. (1996) induced pigment granule aggregation by treating cells with 1 mM cAMP, shown by García and Burnside (1994) to enter cells through organic anion transporters, and in the continued presence of cAMP challenged RPE to disperse pigment by treatment with ionomycin. Therefore, even if Ca2+ stimulated increased phosphodiesterase activity (Nemecek and Honeyman 1982; Van Erneux et al. 1985; Tanner et al. 1986; Beavo 1995) or decreased AC activity (Wera and Hemmings

mediated through Modd receptor subtypes activating PLC induced pigment granule dispersion in bluegill RPE is an inhibitor. Taken together, our results indicate that carbachol-IP3-sensitive calcium channel. Muscarinic receptors in retinal pigment epithelium do not rule out a model in which Ca2+ is normally required for pigment granule dispersion. King-Smith et al. (1996) also showed that pigment granule dispersion could be induced even when intracellular Ca2+ levels were prevented from rising either by removing Ca2+ from the medium or by chelating Ca2+ with 2-bis (2-amino-phenoxo) ethane-N, N', N'-tetraacetic acid (BAPTA). In these experiments, dispersion was induced by washing out cAMP. In the absence of extracellular cAMP, two mechanisms could lead to lowering of intracellular cAMP levels either independently or in combination: efflux of cAMP via organic anion transporters (Sampath et al. 2002) and phosphodiesterase activity (Beavo 1995). Therefore, although these experiments suggest that Ca2+ is not required for pigment granule dispersion as long as cAMP levels are lowered, they do not rule out a model in which Ca2+ is normally required for dispersion induced by muscarinic receptor activation. We are currently undertaking experiments to further test whether Ca2+ is required for carbachol-induced pigment granule dispersion.

In this study we observed that incubation of RPE isolated from bluegill with a PLC inhibitor blocked carbachol-induced pigment granule dispersion, and a similar result was obtained when RPE was incubated with an IP3 receptor inhibitor. Taken together, our results indicate that carbachol-induced pigment granule dispersion in bluegill RPE is mediated through Modd receptor subtypes activating PLC and thereby increasing intracellular calcium through an IP3-sensitive calcium channel. Muscarinic receptors in human RPE cells have been shown to mediate phosphoinositide hydrolysis (Feldman et al. 1991; Osborne et al. 1991; Crook et al. 1992) which has been shown to be coupled to intracellular calcium flux (Feldman et al. 1991). The receptor-mediated phosphoinositide hydrolysis has been shown to be pertussis toxin insensitive (Osborne et al. 1991), suggesting that the muscarinic receptors involved are of odd-numbered subtype, most likely M5 (Feldman et al. 1991; Crook et al. 1992).

Although both the pharmacological ranking of antagonists and the studies using inhibitors of the effectors of Modd receptors were consistent with the interpretation of Modd, and particularly M5 involvement, in carbachol-induced pigment granule dispersion, only a molecular characterization could be considered definitive. In order to characterize muscarinic receptors expressed in bluegill RPE at the molecular level, we first isolated muscarinic receptor genes from bluegill genomic DNA. In preliminary studies, the isolation of muscarinic receptor genes from genomic DNA was initiated by applying degenerate primers, whose selection was based on the amino acid sequences conserved across all five muscarinic subtype receptors from a variety of species. Interestingly, only two sequence fragments were returned; one showed homology to M5 muscarinic receptors and the other to M5 muscarinic receptors. However, the quality of these sequences was low, and they were not used in the analysis of gene expression presented here. Hsieh and Liao (2002) also isolated only M2 and M5 gene fragments using degenerate primers based on conserved amino acid sequences on zebrafish genomic DNA. Although work in our laboratory is in progress to further explore the bluegill genome for the presence of other muscarinic receptors, our preliminary results using degenerate primers only indicate the presence of M2 and M5 in the bluegill genome. Further probing using non-degenerate primers based on known zebrafish and putative fugu gene sequences on genomic and cDNA and employing RACE yielded full sequences of M2 and M5 genes. Phylogenetic analyses using nucleotide alignment showed that M2 and M5 were grouped with their respective subtypes.

Certain amino acids and amino acid motifs are conserved among all the muscarinic receptors, and are known to be critical for receptor–ligand interactions and receptor-G protein coupling. Four aspartic acid residues, one each in the second transmembrane, first extracellular loop, proximal end of the third transmembrane domain and at the interface of third transmembrane domain and second intracytoplasmic loop, are conserved across known muscarinic receptors. Site-directed mutagenesis of these residues has suggested that an aspartic acid residue in the second transmembrane domain and one at the interface of the third transmembrane domain and second intracytoplasmic loop are critical for normal receptor–G protein interaction, whereas aspartic acid residues at the proximal end of the third transmembrane domain and first extracellular loop are likely sites of ligand binding (Fraser et al. 1989). The aspartic acid residue at the proximal
end of the third transmembrane domain is predicted to make ionic interactions with the positively charged amino group present in virtually all muscarinic receptor ligands (Wess 1993). This residue is conserved among all the receptors that bind biogenic amine ligands. Both bluegill M2 and M5 receptors have all four aspartic acid residues at the appropriate positions.

The ligand specificity of muscarinic receptors is determined by additional interactions between the hydroxyl groups of a series of conserved serine, threonine and tyrosine residues in the transmembrane domains with the electron-rich moieties in biogenic amine ligands (Wess 1993). Of these conserved amino acids, most of which do not occur in other G protein-coupled receptors, tyrosine residues in the third, sixth and seventh transmembrane domains, and threonine residues in the fifth transmembrane domain, have been found to be critical for agonist binding; a conserved serine residue in the second transmembrane domain has been found to influence antagonist binding affinities (Wess et al. 1991). All these serine, threonine and tyrosine residues are present at the appropriate positions in the transmembrane domains of bluegill M2 and M5 receptors. Site-directed mutagenesis studies have implicated a threonine residue in the sixth transmembrane domain for the high affinity of muscarinic receptors for pirenzepine (Ellis and Seidenberg 2000). All the muscarinic receptor subtypes described so far have the threonine residue at the corresponding location, except mammalian M2. However, chick and zebrafish M2 receptors carry this residue at the corresponding position and show high affinity for pirenzepine (Tietje and Nathanson 1991; Hsieh and Liao 2002). The deduced amino acid sequences of bluegill M2 and M5 genes indicate the presence of a threonine residue at the corresponding location in the sixth transmembrane domain. Thus, the present finding of potent inhibition of carbachol-induced dispersion by pirenzepine (González et al. 2004) and the closely related drug telenzepine does not exclude the possibility of M2 involvement.

By employing random saturation mutagenesis, Burstein et al. (1995) and Hill-Eubanks et al. (1996) identified the critical amino acids for selectivity of G protein coupling in the C-terminal (C-i3) and N-terminal (N-i3) regions of the third intracytoplasmic loop of human M5 receptor respectively. The motifs isoleucine-tyrosine-threonine-arginine at N-i3 and lysine-alanine-alanine at C-i3 were identified as functionally important in M5od receptors. These conserved residues and motifs are present at the corresponding position in the deduced amino acid sequence of the M5 gene isolated from bluegill in the present study.

Wess et al. (1997) have shown that the ability of M2 receptor to interact with G protein specifically depends on the presence of a four-amino acid motif, valine-threonine-isoleucine-leucine (VTIL), located at the third intracytoplasmic loop and sixth transmembrane domain junction. The bluegill M2 receptor has these residues at the corresponding position, except for leucine which is replaced by methionine. However, point mutation studies of the residues in the VTIL motif have shown that valine, threonine and isoleucine are engaged in specific interaction with G protein and contribute to the specificity and efficiency of receptor/G protein coupling, whereas leucine is not critical for determining the specificity of the interaction (Wess et al. 1997). Thus replacement of leucine with methionine may not be of any consequence as far as G protein coupling is concerned.

We studied expression of both the muscarinic receptors in retina and RPE along with brain and heart. Both the receptors were found to be expressed in brain and retina. M2 and M5 are known to have a wide distribution in brain (Caulfield 1993; Eglen and Nahorski 2000; Bymaster et al. 2003) and M2 is expressed in embryonic chick retina (McKinnon and Nathanson 1995; McKinnon et al. 1998), but this is the first report of M5 expression in retina. Heart was found to express M2 but not M5. This expression pattern is consistent with the previously reported results in zebrafish heart (Hsieh and Liao 2002).

RT–PCR showed that RPE expresses M5 but not M2. Although the presence of muscarinic receptors in RPE has been demonstrated pharmacologically and immunologically (Friedman et al. 1988; Feldman et al. 1991; Osborne et al. 1991; Crook et al. 1992; Salceda 1994; Fischer et al. 1998; Narayan et al. 2003), this is the first molecular evidence for the presence of a muscarinic receptor subtype in RPE.

The pharmacological profiling along with the molecular evidence of M5 expression in bluegill RPE converge to suggest that the M5 subtype is the most likely mediator of ACh-induced pigment granule dispersion in bluegill RPE. The availability of M5-specific pharmacological agents or RNA interference studies, along with further exploration of the bluegill genome to determine whether other muscarinic receptor genes are present, is necessary to confirm this suggestion unequivocally. These studies are currently in progress.

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