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

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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 M_1 and M_3 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 M_2 and M_5 receptor genes from bluegill and studied their expression. Only M_5 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 M_5, on fish retinal pigment epithelium induces pigment granule dispersion.

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

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Muscarinic 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 (M_{1-5}) 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 $G_{q/11}$ 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 (IP_3) 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-inhibitory (G_i) protein to mediate inhibition of adenylyl 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

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Abbreviations used: AC, adenylyl cyclase; ACh, acetylcholine; AChE, acetylcholinesterase; 2-APB, 2-aminoethoxydiphenyl borate; BAPTA, 1,2-bis (2-aminophenoxy) ethane-N,N,N′, N′-tetraacetic acid; cAMP, cyclic AMP; 4-DAMP, 4-diphenylacetoxy-N-[2-chloroethyl] piperidine hydrochloride; DMSO, dimethylsulfoxide; FSK, forskolin; G_i, G-inhibitory protein; IC_{50}, concentration of antagonist eliciting 50% inhibition; IP_3, inositol 1,4,5-trisphosphate; LCBR, low-calcium buffered Earle's Ringer; M_x, muscarinic receptor of subtype X; NCBI, National Center for Biotechnology Information; *p*-FHHSiD, parafluorohexahydrosiladiphenidol; PI, pigment index; PLC, phospholipase C; RACE, rapid amplification of cDNA ends; RPE, retinal pigment epithelium.

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 cholinergic 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 M_1 and M_3 muscarinic receptors blocked pigment granule dispersion, whereas an agonist specific for the M_1 receptor activated dispersion. The agonists and antagonists specific for even-numbered muscarinic receptors (M_2 and M_4) 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 M_5, 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% CO_2 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 (M_1 antagonist; Tocris, Ellisville, MO, USA), methoctramine (M_2 antagonist; Sigma-Aldrich, St Louis, MO, USA), *p*-fluorohexahydrosiladiphenidol (*p*-FHHSiD, M_3 antagonist; Sigma-Aldrich), U73122 (PLC

inhibitor; Sigma-Aldrich) or 2-aminoethoxydiphenyl borate (2-APB; IP_3 receptor antagonist; Tocris) for 45 min. Drugs were prepared as 10 × 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 carbachol 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 carbachol 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 carbachol (IC_{50}) 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 pIC_{50}.

Isolation and amplification of muscarinic receptor genes

Genomic DNA of bluegill was prepared using the phenol–chloroform–isoamyl alcohol method (Hillis *et al*. 1996). To isolate and amplify M_5 muscarinic receptor gene, primers were designed using zebrafish M_5 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 M_5 muscarinic receptor gene was used as a query sequence to identify the putative fugu M_5 muscarinic receptor gene sequence from the fugu genomics project website (http://fugu.hgmp.mrc.ac.uk; release 3) using BLAST. Zebrafish and fugu M_5 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 (M5F, 5′-CACAGCCTSTGGGAGGTGATC-3′) and reverse (M5R, 5′-CACATGGGGTTGACGGT-GCTGTTGAC-3′) primers respectively. To isolate and amplify the M_2 muscarinic receptor gene, primers were designed using zebrafish M_2 muscarinic receptor gene (Hsieh and Liao 2002). The zebrafish M_2 muscarinic receptor gene was used as a query sequence to identify the putative fugu M_2 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′-AACTTCACCTWCTGGAATGCCTC-3′) and reverse (M2R, 5′-GTTCTTGTACTGGCAGAGSAG-3′) primers respectively. Genomic DNA was amplified by PCR. A 50-μL PCR reaction contained 5 μL 10 × buffer (final concentration 10 mM Tris-HCl, pH 9.0 at 4°C, 50 mM KCl, 0.1% Triton® X-100), 1 μL genomic DNA, 1 μL dNTPs (10 mM each), 3–7 μL 25 mM $MgCl_2$, 1 μL forward primer (50 pmol/μL), 1 μL reverse primer (50 pmol/μL) synthesized by Bio-synthesis Inc. (Lewisville, TX, USA) and 0.5 μL (2.5 units) *Taq* polymerase (Promega, Madison, WI, USA). The volume was increased to 50 μL with distilled water. The PCR reaction was performed under the following conditions: one cycle at 94°C for 64 s, followed by 40 cycles of 94°, 55° and 72°C for 30, 30 and 90 s respectively, followed by final extension at 72°C for 5 min. For amplification of bluegill M_2, cDNA from bluegill heart was used instead of genomic DNA.

Amplification of the fugu M_2 gene was performed by PCR on genomic fugu DNA (MRCgeneservice, Cambridge, UK). Primers were designed using the putative M_2 sequence obtained from the fugu genomic databases. The forward primer sequence was 5′-GGCCGCGTGACAATCTTCACCTC-3′, and the reverse primer sequence was 5′-ATGTGCTCATTCGTCAGTCTGAGGAC-3′. The 25-μL reactions included 0.5 μg fugu genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, thermophilic DNA polymerase buffer (Promega); 16.5 μL autoclaved, deionized H_2O 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°, 57° 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 nM $MgCl_2$), 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°, 60° 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′-AACTTCACCTWCTGGAATGCCTC-3′ (sense) and 5′-GTTCTTGTACTGGCAGAGSAG-3′ (antisense) for M_2, and 5′-CACAGCCTSTGGGAGGTGATC-3′ (sense) and 5′-CACATGGGGTTGACGGTGCTGTTGAC-3′ (antisense) for M_5; 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 M_5, total RNA was isolated from bluegill brain tissues (shown by RT–PCR to

include both M_2 and M_5 mRNA) using an RNAqueous®-4PCR 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′-TCAGAGGAGGCATAACTGTTGAAGG-3′ (antisense) and inner primer 5′-GCCAGCATAGAATAGGGGG-3′ (antisense); for the 3′ end, outer primer 5′-GTCAGCCTCATCACTATTGTGG-3′ (sense) and inner primer 5′-CACCAATGATGAGGTCAGCAG-CTG-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).

M_2 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′-TCAGAG-GAGGCATAACTGTTGAAGG-3′ (antisense) and inner primer 5′-GCCAGCATAGAATAGGGGG-3′ (antisense); for the 3′-end, outer primer 5′-GTCAGCCTCATCACTATTGTGG-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*. 1997). Many different alignments were performed with various settings for gap opening and gap extension penalties for pair-wise and multiple alignment parameters. Each resulting alignment was assessed visually. The criterion used in deciding the optimum alignment was the presence of identical and similar amino acids in the region starting from about the middle of the first transmembrane domain to the amino terminal region of the third intracytoplasmic loop (i3) and again from the carboxy terminal region of the i3 loop to the carboxy terminal region of the protein. These regions are conserved across subtypes (Bonner 1989). The other criterion was the perfect alignment of motifs conserved across all muscarinic receptors. Using this method the optimum alignment was obtained with a gap opening penalty of 52 and gap extension penalty of 1.25 for pair-wise alignment parameters, and a gap opening penalty of 22 and gap extension penalty of 0.45 for multiple alignment parameters. Protein alignment was used to obtain nucleotide alignment using the program CodonAlign (http://www.sinauer.com/hall/), which creates a DNA alignment based on alignment of the corresponding proteins. It introduces into each DNA sequence a triplet gap at the position of each gap in the aligned protein sequence (Hall 2001). The DNA alignment file was executed in PAUP* (Swofford 2002). Under the distance criterion an unrooted phylogram was obtained using both the alignments. In 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 + I (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

Table 1 Name, species and the NCBI accession number of muscarinic receptor proteins used in phylogenetic analyses

Name	Species	Accession no.	Name	Species	Accession no.
HsM1	*Homo sapiens*	NP_000729	RnM3	*Rattus norvegicus*	NP_036659
Mmul1	*Macaca mulatta*	AAB95157	MmM3	*Mus musculus*	NP_150372
SsM1	*Sus scrofa*	CAA28003	CpM3	*Cavia porcellus*	AAL67911
RnM1	*Rattus norvegicus*	AAB20705	GgalM3	*Gallus gallus*	AAA65961
MmM1	*Mus musculus*	NP_031724	HsM4	*Homo sapiens*	NP_000732
CpM1	*Cavia porcellus*	AAL67909	MmM4	*Mus muscles*	NP_031725
HsM2	*Homo sapiens*	NP_000730	CpM4	*Cavia porcellus*	AAL67912
SsM2	*Sus scrofa*	CAA28413	GgalM4	*Gallus gallus*	AAA48563
RnM2	*Rattus norvegicus*	NP_112278	XlM4	*Xenopus laevis*	CAA46694
MmM2	*Mus musculus*	AAG14343	HsM5	*Homo sapiens*	NP_036257
CpM2	*Cavia porcellus*	AAL67910	MmulM5	*Macaca mulatta*	AAB95159
GgalM2	*Gallus gallus*	AAB04106	RnM5	*Rattus norvegicus*	AAA40658
DrM2	*Danio rerio*	AAK93793	MmM5	*Mus musculus*	AAL26028
TrM2	*Takifugu rubripes*	AAU09270	CpM5	*Cavia porcellus*	AAL67913
LmM2	*Lepomis macrochirus*	AAY66420	GgalM5	*Gallus gallus*	AAF19027
HsM3	*Homo sapiens*	AAM18940	DrM5	*Danio rerio*	AAK93794
PpM3	*Pongo pygmaeus*	BAA94483	TrM5	*Takifugu rubripes*	NA
PtM3	*Pan troglodytes*	BAA94481	LmM5	*Lepomis macrochirus*	AAW73155
GgM3	*Gorilla gorilla*	BAA94482	DmM	*Drosophila melanogaster*	NP_523844
SsM3	*Sus scrofa*	CAA31215	CeM	*Caenorhabditis elegans*	AAD48771

M_1, M_2, M_3, M_4 and M_5 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.

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

Name	Species	Accession no.	Name	Species	Accession no.
HsM1	*Homo sapiens*	NM_000738	RnM3	*Rattus norvegicus*	NM_012527
Mmul1	*Macaca mulatta*	AF026262	MmM3	*Mus musculus*	NM_033269
SsM1	*Sus scrofa*	X04413	CpM3	*Cavia porcellus*	AY072060
RnM1	*Rattus norvegicus*	S73971	GgalM3	*Gallus gallus*	L10617
MmM1	*Mus musculus*	NM_007698	HsM4	*Homo sapiens*	NM_000741
CpM1	*Cavia porcellus*	AY072058	MmM4	*Mus musculus*	NM_007699
HsM2	*Homo sapiens*	NM_000739	CpM4	*Cavia porcellus*	AY072061
SsM2	*Sus scrofa*	X04708	GgalM4	*Gallus gallus*	J05218
RnM2	*Rattus norvegicus*	NM_031016	XlM4	*Xenopus laevis*	X65865
MmM2	*Mus musculus*	AF264049	HsM5	*Homo sapiens*	NM_012125
CpM2	*Cavia porcellus*	AY072059	MmulM5	*Macaca mulatta*	AF026264
GgalM2	*Gallus gallus*	M73217	RnM5	*Rattus norvegicus*	M22926
DrM2	*Danio rerio*	AY039653	MmM5	*Mus musculus*	AF264051
TrM2	*Takifugu rubripes*	AY693715	CpM5	*Cavia porcellus*	AY072062
LmM2	*Lepomis macrochirus*	DQ066619	GgalM5	*Gallus gallus*	AF201960
HsM3	*Homo sapiens*	AF498917	DrM5	*Danio rerio*	AY039654
PpM3	*Pongo pygmaeus*	AB041398	TrM5	*Takifugu rubripes*	NA
PtM3	*Pan troglodytes*	AB041396	LmM5	*Lepomis macrochirus*	AY834251
GgM3	*Gorilla gorilla*	AB041397	DmM	*Drosophila melanogaster*	NM_079120
SsM3	*Sus scrofa*	X12712	CeM	*Caenorhabditis elegans*	AF139093

M_1, M_2, M_3, M_4 and M_5 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.

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.

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 M_1 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 ($n = 3$). The pIC_{50} value for telenzepine was estimated to be 8.5.

RPE treated with *p*-FHHSiD, an M_3-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-

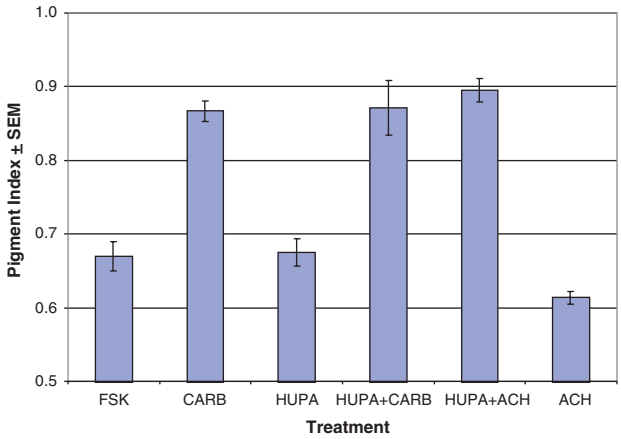
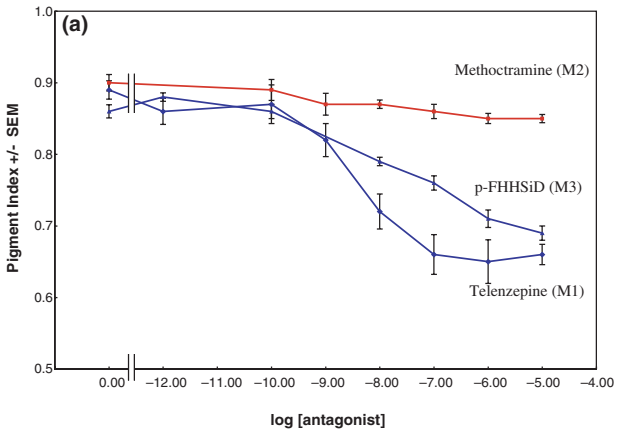


Fig. 1 Acetylcholine in the presence of an AChE inhibitor induces pigment granule dispersion in isolated RPE. RPE was isolated from bluegill and treated with 10 μM FSK to induce pigment granule aggregation. Following pigment aggregation, tissue was treated 100 nM ACh (ACH) or 0.1 μM carbachol (CARB) in the presence or absence of the AChE inhibitor huperzine-A (HUPA). For each sample, $n = 3$. Values are mean ± SEM. Although neither huperzine-A nor ACh alone had an effect on pigment position, ACh in the presence of huperzine-A induced pigment granule dispersion as robustly as did carbachol; PIs were significantly greater than those of FSK-treated tissues or tissues treated with either drug alone ($p < 0.0001$, ANOVA followed by Tukey's *post hoc* test).



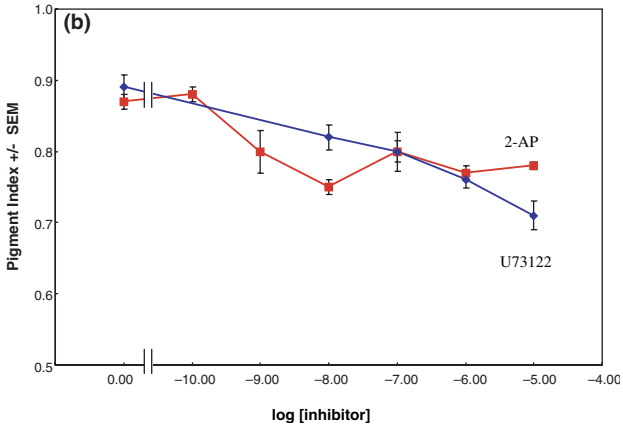


Fig. 2 Inhibitors of the M_{odd} muscarinic receptor signaling pathway inhibit carbachol-induced pigment granule dispersion. (a) RPE was isolated from bluegill and treated with 10 μM FSK to induce pigment granule aggregation. Following pigment aggregation, tissue was treated 0.1 μM carbachol and increasing concentrations of M_1 antagonist telenzepine, the M_2 antagonist methoctramine or the M_3 antagonist *p*-FHHSiD. For each sample, $n = 3$. Values are mean ± SEM. The M_1 and M_3 antagonists blocked pigment granule dispersion, but the M_2 antagonist did not. (b) Following treatment with FSK, isolated RPE was treated with 0.1 μM carbachol and increasing concentrations of the PLC inhibitor U73122 or the IP_3 receptor antagonist 2-APB. Both of these agents, which block downstream effectors in the M_{odd} muscarinic receptor signaling pathway, blocked carbachol-induced pigment granule dispersion. For each sample, $n = 3$. Values are mean ± SEM.

treated cells ($p = 1.0$). The pIC_{50} for *p*-FHHSiD was estimated to be 7.2.

Methoctramine, a muscarinic receptor antagonist selective for M_2, did not block carbachol-induced pigment granule dispersion at any concentration examined (up to 10 μM; Fig. 2a). There were no statistically significant differences between the PIs of RPE treated with carbachol alone and that treated with methoctramine.

As M_{odd} 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 (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 μM 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 (PI =0.87 ± 0.01; $n = 3$).

Isolation of muscarinic receptor genes from bluegill genomic DNA

To isolate the M_5 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_5 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 M_5 muscarinic receptor gene is shown in Fig. 3(a). The sequence showed greatest identity with M_5 muscarinic receptor genes. The deduced amino acid sequence encoded by the bluegill M_5 muscarinic receptor gene was 527 amino acids long. The bluegill M_5 muscarinic receptor shared 65.3% amino acid identity with human M_5, whereas it shared only 46.7, 41.9, 53.1 and 41.5% amino acid identity with human M_1, M_2, M_3 and M_4 respectively. Comparison with other vertebrate muscarinic receptors showed that bluegill M_5 muscarinic receptor shared a high degree of identity with M_5 muscarinic receptors (Fig. 3b). The deduced amino acid sequence showed greater identity with the M_5 receptor proteins in fish than with other vertebrate M_5 receptors. The bluegill M_5 muscarinic receptor had 88.4,

(a)

```
ATGGAAGGAGAGAACATACTGAACTCCACGCTAAATGCCAGTACCATGGACAGCCACCTAGTCCCTCACAGT
 M  E  G  E  N  I  L  N  S  T  L  N  A  S  T  M  D  S  H  L  V  P  H  S
CTCTGGGAGGTGATCACCATTGCGACTGTGTCAGCTATAGTCAGCCTCATCACTATTGTGGGGAATGTCCTG
 L  W  E  V  I  T  I  A  T  V  S  A  I  V  S  L  I  T  I  V  G  N  V  L
GTGATGCTCTCCTTTAAAGTCAACAGCCAGCTAAAGACAGTGAATAATTACTACCTGCTGAGTCTGGCAGCT
 V  M  L  S  F  K  V  N  S  Q  L  K  T  V  N  N  Y  Y  L  L  S  L  A  A
GCTGACCTCATCATAGGTGTTTTCTCCATGAATCTGTATACCTCTTACATACTGATGGGCTACTGGGCCTTA
 A  D  L  I  I  G  V  F  S  M  N  L  Y  T  S  Y  I  L  M  G  Y  W  A  L
GGAAACCTCGCCTGCGATCTGTGGTTGGCGGTGGACTATGTAGCCAGTAACGCCTCAGTCATGAACCTGTTG
 G  N  L  A  C  D  L  W  L  A  V  D  Y  V  A  S  N  A  S  V  M  N  L  L
GTAATCAGTTTTGATAGATATTTTTCCATCACCAGACCTCTGACCTACAGGGCCAAACGGACTCCCAAACGA
 V  I  S  F  D  R  Y  F  S  I  T  R  P  L  T  Y  R  A  K  R  T  P  K  R
GCTGGGATCATGATAGGTTTGGCCTGGCTGGTTTCACTTATCCTTTGGGCGCCCCCTATTCTATGCTGGCAA
 A  G  I  M  I  G  L  A  W  L  V  S  L  I  L  W  A  P  P  I  L  C  W  Q
TACTTCGTAGGAAAAAGGACTGTCCCTGAGAGGCAATGCCAGATCCAGTTTTTCTCTGAGCCTGTGATAACA
 Y  F  V  G  K  R  T  V  P  E  R  Q  C  Q  I  Q  F  F  S  E  P  V  I  T
TTTGGGACAGCGATTGCTGCCTTTTATATCCCTGTATCTGTCATGACAATCCTATACTGTCGAATCTACAAG
 F  G  T  A  I  A  A  F  Y  I  P  V  S  V  M  T  I  L  Y  C  R  I  Y  K
GAGACAGAGAAGAGGACCAAAGATCTGGCGGAGCTGCAGGGGATTAACTATCCCACAGAACCTGGGGTCACC
 E  T  E  K  R  T  K  D  L  A  E  L  Q  G  I  N  Y  P  T  E  P  G  V  T
CAGCCTCAGAAGACCATTATCAGATCTTGTTTTAGCTGTAAGTTAAGGTCAGCTTCAAATGACAGGAATCAA
 Q  P  Q  K  T  I  I  R  S  C  F  S  C  K  L  R  S  A  S  N  D  R  N  Q
GCCTCTTGGTCTTCCTCCAGCAGAAGCAATGCTGCCAAATCAGCAGCCACCACCAATGACGAGTGGTCCAAA
 A  S  W  S  S  S  S  R  S  N  A  A  K  S  A  A  T  T  N  D  E  W  S  K
GCTAGTCAGCTGACCACCTTCAACAGTTATGCCTCCTCTGAGGATGAGGACAGGCCTGTGTCTCCAGGGGGA
 A  S  Q  L  T  T  F  N  S  Y  A  S  S  E  D  E  D  R  P  V  S  P  G  G
TTCCAGGTGCCCTCTTTCAGGAACCAGGCTTGTGAGACCATGAAGACTGGGGTGGGCAGTGAGAACGAGCAG
 F  Q  V  P  S  F  R  N  Q  A  C  E  T  M  K  T  G  V  G  S  E  N  E  Q
CTCAGCAGCTATGAAGAGGATAGCTTCTTCCAGACACCACCCAAAAGTAACTCTCAGAGGAGCAACAAGTGT
 L  S  S  Y  E  E  D  S  F  F  Q  T  P  P  K  S  N  S  Q  R  S  N  K  C
GTGTCCTACAAGTTCAAGCCTGTGGCCAAGGACACGCACGTGGAGCACCACAGCAAAAACGGAGACACCAAA
 V  S  Y  K  F  K  P  V  A  K  D  T  H  V  E  H  H  S  K  N  G  D  T  K
ATGGCTTCGTCCACGTTCTCCTCGGCTGAGTCCATGAGCGTTCCATCCACCTCGTCAACATCTAAGCCCATA
 M  A  S  S  T  F  S  S  A  E  S  M  S  V  P  S  T  S  S  T  S  K  P  I
GACGCCACGCTGAAGAACCAGATCACCAAGAGGAAGCGGATGGTGCTGATCAAGGAGAGGAAGGCAGCTCAG
 D  A  T  L  K  N  Q  I  T  K  R  K  R  M  V  L  I  K  E  R  K  A  A  Q
ACTCTCAGTGCTATCTTGCTGGCCTTCATCCTAACATGGACGCCTTATAACATCATGGTGCTTATTTCCACC
 T  L  S  A  I  L  L  A  F  I  L  T  W  T  P  Y  N  I  M  V  L  I  S  T
TTCTGCTCAGACTGCATTCCCCTCTCGCTCTGGCATTTGGGCTACTGGCTGTGCTACGTCAACAGCACCGTC
 F  C  S  D  C  I  P  L  S  L  W  H  L  G  Y  W  L  C  Y  V  N  S  T  V
AACCCCATGTGCTACGCGCTTTGTAACAAGACTTTCCAGAAGACCTTCCGTATGCTCTTACTTTGCCAGTGG
 N  P  M  C  Y  A  L  C  N  K  T  F  Q  K  T  F  R  M  L  L  L  C  Q  W
AGGAAGAAAAGGATTGAGGAGAAATTATACTGGTATGGACAAAATCCAGTGGTCAGCTCTAAACTGACATGA
 R  K  K  R  I  E  E  K  L  Y  W  Y  G  Q  N  P  V  V  S  S  K  L  T  .
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Fig. 3 (a) Nucleotide and deduced amino acid sequences of the bluegill M_5 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 M_5 receptor with known vertebrate M_5 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), Rn (*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 M_5 receptor and bluegill M_5 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 M_5 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.

(b)

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HsM5      MEGDSYHNATTVNGTPVNHQPLERHRLWEVITIAAVTAVVSLITIVGNVLVMISFKVNSQLKTVNNYY  [68]
MmulM5    ................Y.............S.................................... [68]
MmM5      ...E....E...........A....G................M..................... [68]
RnM5      ...E..N-ES..........A....G........V.......M..................... [67]
CpM5      ...EA.P......P..G......Y.QV...V.....S..........................  [68]
GgalM5    ..VNLFS.S.VT.SSSI..KQ..G.S.....................................  [68]
DrM5      -----MGVEKLTLRANITEVQ.VT.S.....A..T.S.I..F...I..I...L..........  [63]
TrM5      --MEEASAVNSTSN.SSADTH.VT.S........T.S.I............L..........  [66]
LmM5      --MEGENILNSTLNASTMDSH.VP.S........T.S.I............L..........  [66]
                                          ------------TM1--------      ---

HsM5      LLSLACADLIIGIFSMNLYTTYILMGRWALGSLACDLWLALDYVASNASVMNLLVISFDRYFSITRPL  [136]
MmulM5    ..........................................................................  [136]
MmM5      ..........................V.....................................  [136]
RnM5      ..........................V.....................................  [135]
CpM5      .F.........V..............................................  [136]
GgalM5    ..................S...I.H.S.....................................  [136]
DrM5      .....F......V.......S.....Y.....................................  [131]
TrM5      .....A......V.......S.....Y....N.S..............................  [134]
LmM5      .....A......V.......S.....Y....N........V.......................  [134]
          ---------TM2--------                    ------------TM3--------

HsM5      TYRAKRTPKRAGIMIGLAWLISFILWAPAILCWQYLVGKRTVPLDECQIQFLSEPTITFGTAIAAFYI  [204]
MmulM5    ............V.....................................................  [204]
MmM5      ...................V.....................P.......................  [204]
RnM5      ...................V.....................P.......................  [203]
CpM5      ............L......V.....................P.....................V  [204]
GgalM5    ..........................V......F..E....PE.......Y..............  [204]
DrM5      .........I..........V.......P......F.......ERQ.....F...V...........F  [199]
TrM5      ....................V.LV....P......F.......ERQ.....F...V.....  [202]
LmM5      ....................V.L.....P......F.......ERQ.....F...V.....  [202]
                    ------------TM4--------                  ---------TM5

HsM5      PVSVMTILYCRIYRETEKRTKDLADLQGSDSVTKAEKRKPAHRALFRSCLRCPRPTLAQRERNQASWS  [272]
MmulM5    ................................E..............................T...  [272]
MmM5      ................................AEVK.......T.L..FFS....S.....  [272]
RnM5      ................................AE.K..E..Q.T.L..FFS....S.....  [271]
CpM5      ........................E.......AE.A..R..P...L...FS..H...V....S.....  [272]
GgalM5    ............K..........E....E..AEF.TI..RK-.FLK..FS.KQQN.VK...C.....  [271]
DrM5      ..........L..K...R......E...IN.S.NSSG--D.QPQKI...FG.KHVSN-----------  [254]
TrM5      ............K..........E...INYT.DSVVP-QPQKTII...FN.QVNLAS-.DT..T...  [268]
LmM5      ............K..........E...INYP.EPGVT-QPQKTII...FS.KLRSAS-ND.......  [268]
          -----------

HsM5      SSRRSTSTTGKPSQATGPSANWAKAEQLTTCSSYPSSEDEDKPATDPVLQVVYKSQGKESPGEEFSAE  [340]
MmulM5    .....A..S.......D..T.Q.................................R..........S.  [340]
MmM5      ............T...DL..D.E....V.N...C......A.AT....F.....NEA.....K..NTQ  [340]
RnM5      ...........TT...DL..D.E....V............A..T....F.M....EA.....K.SNTQ  [339]
CpM5      ...K....S.T......H..H...VG..A.G.R..VP...AQ.PA...F.A..N..A....R..L..K  [340]
GgalM5    ..S....A.V.A...ASTCTE....D........A...E...L.....F..A.R.PS.-GKE...NER  [338]
DrM5      ----TSR.QA----LSHTN.AKTLDD...NFN..A...E..RSGN-----------------------  [291]
TrM5      ..S..NAAKS----VACANDD.S..D....FN..A......R.GSPGGF.S---------SFRKQ.C.  [323]
LmM5      ..S..NAAKS----.ATTNDE.S..S....FN..A......R.VSPGGF..P--------SFRNQAC.  [324]

HsM5      ETEETFVKAETEKSDYDTPNYLLSPAAAHRPKSQKCVAYKFRLVVKADGNQETNNGCHKVKIMPCPFP  [408]
MmulM5    DA.......Q...H.S.....F.............................T..N............S..  [408]
MmM5      ..K....SPR..NN.....K.F...G....L.................T.......R.......S..  [408]
RnM5      ..K..V.NTR..N......K.F........L.................T.......R.......S..  [407]
CpM5      .AK.KA...QR..T...I.K.F........L..........Q.......T.......R.......S..  [408]
GgalM5    .SQDIV..EQP.EN.FE.QQ.F...--......K..............T..A....R....T..SAA  [404]
DrM5      -FQ.SCRHQ.NKSES.EEE.FFPT.VK.SPT.TK...S...KPKDVS----PLK.TNGDA.PGASS.S  [354]
TrM5      ASKSRVGSESEQL.S.EEDS.FQT.PKPQSQ..G...S...KSAA.DGAHA.RQSKNGDT.MASSV.S  [391]
LmM5      TMKTGVGSENEQL.S.EEDSFFQT.PKSNSQR.N...S...KP.A.D-THV.HHSKNGDT.MASST.S  [391]

HsM5      VAK-------EPSTKGLNPNPSHQMTKRKRVVLVKERKAAQTLSAILLAFIITWTPYNIMVLVSTFCD  [469]
MmulM5    ...-------.....................M.....................................  [469]
MmM5      .S.-------D......D.HL.........M.....................................  [469]
RnM5      .S.-------D.....PD..L.........M.....................................  [468]
CpM5      .S.-------GTPS...E..L.........M.....................L..............  [469]
GgalM5    LS.-------D..I.SMD..INN.T.....M..I...............................S  [465]
DrM5      S.ESVNAPSSSS.S.PIDGTLKC.I.....M..I..K..............L.........I....S  [422]
TrM5      S.ESVNAPSTSSTS.PSDATLKN.I.....M..I.................L.........I....S  [459]
LmM5      S.ESMSVPSTSSTS.PIDATLKN.I.....M..I.................L.........I....S  [459]
                                                  ---------TM6-----------

HsM5      KCVPVTLWHLGYWLCYVNSTVNPICYALCNRTFRKTFKMLLLCRWKKKKVEEKLYWQGNSKLP-----  [532]
MmulM5    ..........................................................................-----  [532]
MmM5      ....................I.................L.....................-----  [532]
RnM5      ....................I.................L.....................-----  [531]
CpM5      T.I.....................................--V.EK...A.....-----  [530]
GgalM5    D.I.L.W.................M......K..........F.Q..............TRM.-----  [528]
DrM5      D.I.LS.................M......K..Q...R.....Q.R.QRA......C.QNPAVGSKLT  [490]
TrM5      D.I.LS.................M......K..Q...R.....Q....RI......Y.QNPAVTSKLT  [527]
LmM5      D.I.LS.................M......K..Q...R.....Q.R..RI......Y.QNPVVSSKLT  [527]
               ----------TM7----------
```

Fig. 3 Continued.

75.5, 67.5 and 65.4% amino acid identity with fugu, zebrafish, chicken and rat M_5 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 ~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)

```
ATGGATGTATTCAATTATACCTACTGGAATGCCTCTGAAGGCAATGACACAGAAGTTGTGGAAGAGAGCGAA
 M  D  V  F  N  Y  T  Y  W  N  A  S  E  G  N  D  T  E  V  V  E  E  S  E
AGCGCCTACAAGACTGTGGAGGTGGTGTTCATCGTGTTGGTGGCTGGTTCCCTAAGTTTGGTTACAGTTATT
 S  A  Y  K  T  V  E  V  V  F  I  V  L  V  A  G  S  L  S  L  V  T  V  I
GGAAATATCCTTGTCATGCTTTCCATCAAAGTTAATCGGAACTTACAGACTGTCAACAACTATTTTTTATTT
 G  N  I  L  V  M  L  S  I  K  V  N  R  N  L  Q  T  V  N  N  Y  F  L  F
AGCCTTGCATGTGCTGACCTAATAATCGGACTGTGCTCTATGAACTTGTACACAGTCTACATAGTAATAGGG
 S  L  A  C  A  D  L  I  I  G  L  C  S  M  N  L  Y  T  V  Y  I  V  I  G
TACTGGCCACTGGGGCCGGTGGTGTGTGACCTGTGGTTAGCCTTGGACTATGTTGTCAGCAATGCATCTGTT
 Y  W  P  L  G  P  V  V  C  D  L  W  L  A  L  D  Y  V  V  S  N  A  S  V
ATGAATCTTCTCATCATAAGCTTTGACAGATATTTCTGTGTGACCAAGCCGCTCAGCTACCCCGTCAAAAGG
 M  N  L  L  I  I  S  F  D  R  Y  F  C  V  T  K  P  L  S  Y  P  V  K  R
ACCACCAAGATGGCAGGAATGATGATTGCTGCAGCCTGGGTCCTGTCTTTCATTCTCTGGGCACCAGCCATT
 T  T  K  M  A  G  M  M  I  A  A  A  W  V  L  S  F  I  L  W  A  P  A  I
CTCTTCTGGCAGTTCATTGTTGGTGGGCGGACAGTACCTGAAAAGGAGTGCTACATCCAGTTCTTCTCTAAC
 L  F  W  Q  F  I  V  G  G  R  T  V  P  E  K  E  C  Y  I  Q  F  F  S  N
GCTGCAGTCACTTTTGGCACTGCCATTGCCGCCTTTTACCTGCCTGTCATCATCATGATTCAGCTCTACTGG
 A  A  V  T  F  G  T  A  I  A  A  F  Y  L  P  V  I  I  M  I  Q  L  Y  W
CAGATCTCCAGAGCGAGCAAGAGCCGTGTAAAGAAGGATAACCGCAAGCCATCAGCAGCCAATCCAGAACCC
 Q  I  S  R  A  S  K  S  R  V  K  K  D  N  R  K  P  S  A  A  N  P  E  P
CTGTCGCCTGGCCAGAGGAGGAACAACACACCAAAACCCAACAATAACAACGTACCAGGGGAAGACACCGTG
 L  S  P  G  Q  R  R  N  N  T  P  K  P  N  N  N  N  V  P  G  E  D  T  V
CCTTCCCAGAGCCTGAATGCCGATGATGGAGCTAACCAGCATGATGGAAAGTTGCAAAATGGCAAAGGACCT
 P  S  Q  S  L  N  A  D  D  G  A  N  Q  H  D  G  K  L  Q  N  G  K  G  P
TCCTCGACCACTGCTGAGGGAGAAACTGAATGTGATGAGGTGGCGAGGGAGAACTGCACTCCTGGAGAAGAA
 S  S  T  T  A  E  G  E  T  E  C  D  E  V  A  R  E  N  C  T  P  G  E  E
AAGGAGAGCTCCAATGATTCAACATCAGGCAGCGTGGCTGCGTCCAATCAGAAGGATGAGGAGGCGGCTCCC
 K  E  S  S  N  D  S  T  S  G  S  V  A  A  S  N  Q  K  D  E  E  A  A  P
TCTGCCGTCAACTCTGGTGCAGAGACAAGCCAACCGCTTCCAAGGCAGCGAGCCAAGGCAGGGGGCTCCAAG
 S  A  V  N  S  G  A  E  T  S  Q  P  L  P  R  Q  R  A  K  A  G  G  S  K
CTGACCTGCATCAAGATCAAGACAAAATCACCCAAGGGTGACTGCTACACACCATCCAACGCCACCGTTGAG
 L  T  C  I  K  I  K  T  K  S  P  K  G  D  C  Y  T  P  S  N  A  T  V  E
ATCGTCCCTGCCTCAGACCGGCAGAATCACGTGGCACGGAAGATTGTGAAGATGACAAAGCAACCTCCCAAC
 I  V  P  A  S  D  R  Q  N  H  V  A  R  K  I  V  K  M  T  K  Q  P  P  N
AAGAAGAAGAAAGCACCACCATCACGGGAGAAAAAAGTTACCCGCACCATAATGGCCATCCTGGTAGCTTTT
 K  K  K  K  A  P  P  S  R  E  K  K  V  T  R  T  I  M  A  I  L  V  A  F
GTAGCCACCTGGACTCCTTATAATGTGATGGTGCTTATTAACACCTTCTGCTCCAGCTGCATCCCTAATACA
 V  A  T  W  T  P  Y  N  V  M  V  L  I  N  T  F  C  S  S  C  I  P  N  T
GTGTGGACTATTGGCTACTGGCTGTGCTACATCAACAGCACCATCAACCCAGCCTGCTACGCTTTGTGCAAT
 V  W  T  I  G  Y  W  L  C  Y  I  N  S  T  I  N  P  A  C  Y  A  L  C  N
GCCACTTTTAAAAAGACATTCAAACATCTTCTCCTCTGCCAGTATAAAAATATAAGGTCAGCCAGATAA
 A  T  F  K  K  T  F  K  H  L  L  L  C  Q  Y  K  N  I  R  S  A  R  .
```

Fig. 4 (a) Nucleotide and deduced amino acid sequences of the bluegill M_2 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 M_2 receptor with known vertebrate M_5 receptors using the program Clustal X (Thompson *et al.* 1997). The names of the sequences are prefixed with Hs (*Homo sapiens*, human), Ss (*Sus scrofa*, pig), Mm (*Mus musculus*, mouse), Rn (*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). TrM2 and LmM2 represent fugu M_2 receptor and bluegill M_2 receptor respectively. Residues identical to those of human M_2 are indicated by dots. Dashes in the alignment represent gaps inserted. Transmembrane domains (TMs) of bluegill M_2 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.

(b)

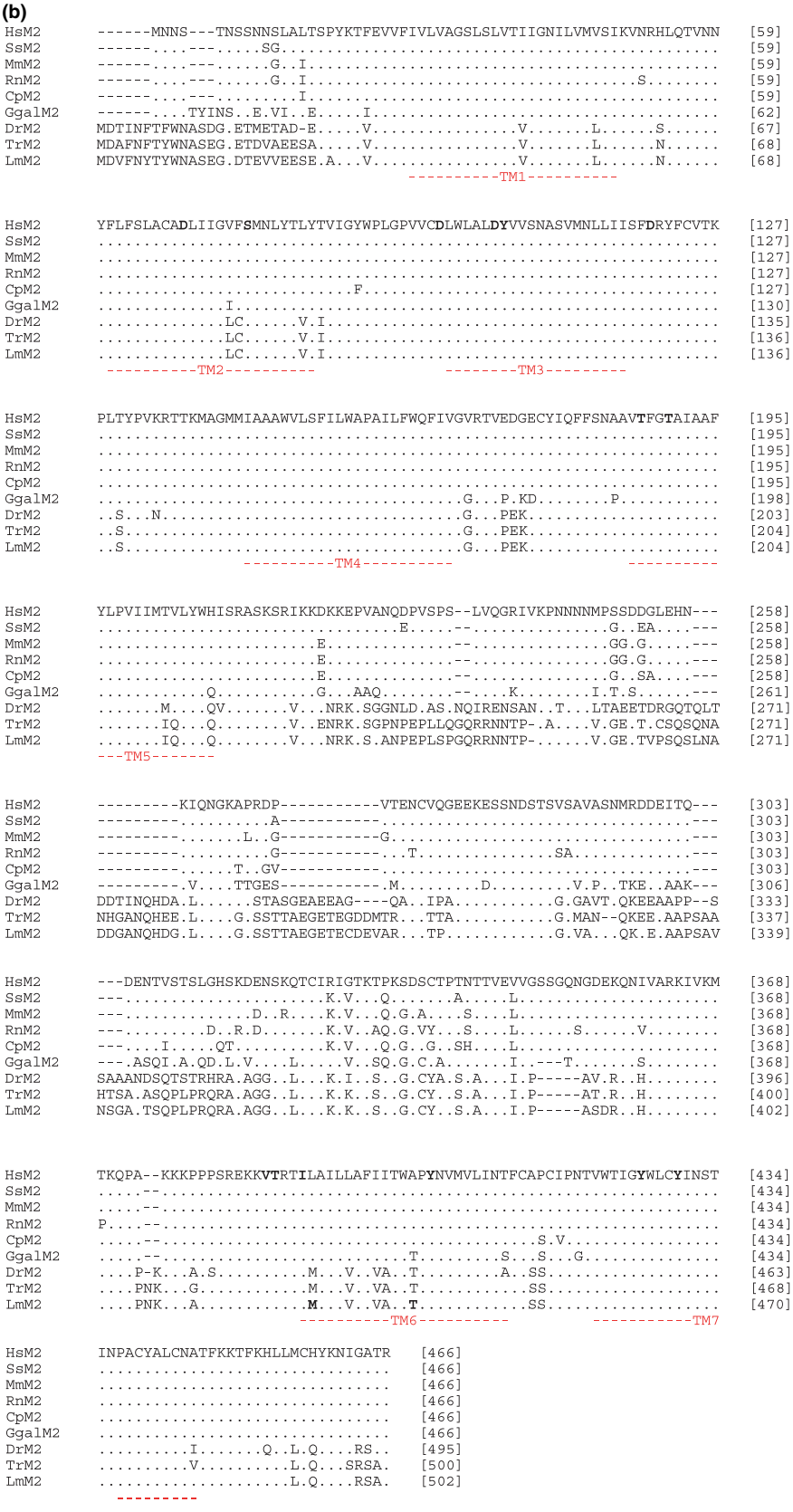

Fig. 4 Continued.

receptors showed that bluegill M_2 muscarinic receptor shared a high degree of identity with other M_2 muscarinic receptors (Fig. 4b). The deduced amino acid sequence showed greater identity with the M_2 receptor proteins in fish than with other vertebrate M_2 receptors, with bluegill M_2 muscarinic receptor having 92, 83, 71.5 and 68.3% amino acid identity with fugu, zebrafish, chicken and rat M_2 respectively.

The fugu M_2 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 M_2 muscarinic receptor showed 76% identity with the zebrafish M_2 receptor, and 65 and 40% identity with the chick and human M_2 receptors respectively.

Expression of bluegill M_2 and M_5 muscarinic receptors was studied in RPE and retina along with brain and heart by RT–PCR. Brain and retina were found to express both M_2 and M_5, whereas heart and RPE expressed only M_2 and M_5 respectively (Fig. 6).

```
ATGGACGCGTTCAACTTCACCTACTGGAATGCCTCCGAAGGCAACGAGACGGATGTCGCGGAA 63
 M  D  A  F  N  F  T  Y  W  N  A  S  E  G  N  E  T  D  V  A  E  21
GAGAGCGCGAGCCCCTACAAGACGGTGGAGGTGGTGTTCATCGTGCTGGTGGCCGGGTCCCTC 126
 E  S  A  S  P  Y  K  T  V  E  V  V  F  I  V  L  V  A  G  S  L  42
AGCTTGGTCACCGTCATCGGGAACATCCTGGTCATGCTCTCCATCAAAGTCAACAGGAACCTG 189
 S  L  V  T  V  I  G  N  I  L  V  M  L  S  I  K  V  N  R  N  L  63
CAGACGGTCAACAACTATTTTTTGTTCAGCCTGGCGTGTGCTGACCTCATCATCGGACTCTGC 252
 Q  T  V  N  N  Y  F  L  F  S  L  A  C  A  D  L  I  I  G  L  C  84
TCCATGAACTTGTACACGGTCTACATTGTGATCGGCTACTGGCCTCTGGGCCCGGTGGTGTGC 315
 S  M  N  L  Y  T  V  Y  I  V  I  G  Y  W  P  L  G  P  V  V  C  105
GACCTCTGGTTGGCGTTGGACTATGTTGTCAGCAACGCGTCCGTCATGAACCTCCTCATCATC 378
 D  L  W  L  A  L  D  Y  V  V  S  N  A  S  V  M  N  L  L  I  I  126
AGCTTTGACAGATATTTTTGCGTCACCAAGCCCCTCAGCTACCCTGTCAAGAGGACCACCAAG 441
 S  F  D  R  Y  F  C  V  T  K  P  L  S  Y  P  V  K  R  T  T  K  147
ATGGCGGGAATGATGATCGCGGCGGCCTGGGTCCTTTCCTTCATCCTCTGGGCTCCAGCGATT 504
 M  A  G  M  M  I  A  A  A  W  V  L  S  F  I  L  W  A  P  A  I  168
CTCTTCTGGCAGTTCATCGTTGGTGGGAGGACGGTGCCGGAGAAGGAGTGCTACATCCAGTTT 567
 L  F  W  Q  F  I  V  G  G  R  T  V  P  E  K  E  C  Y  I  Q  F  189
TTCTCAAATGCCGCGGTGACTTTCGGCACCGCCATCGCCGCCTTTTACTTGCCTGTCATCATC 630
 F  S  N  A  A  V  T  F  G  T  A  I  A  A  F  Y  L  P  V  I  I  210
ATGATTCAGCTCTACTGGCAGATCTCCCGAGCGAGCAAGAGCCGCGTGAAGAAGGAGAACCGC 693
 M  I  Q  L  Y  W  Q  I  S  R  A  S  K  S  R  V  K  K  E  N  R  231
AAACCGTCGGGCCCCAATCCAGAGCCCCTGTTACAAGGCCAGAGGAGGAACAACACGCCAAAA 756
 K  P  S  G  P  N  P  E  P  L  L  Q  G  Q  R  R  N  N  T  P  K  252
GCCAACAATAACAACGTACCGGGGGAAGATACAGGATGTTCTCAGAGCCAGAACGCCAACCAC 819
 A  N  N  N  N  V  P  G  E  D  T  G  C  S  Q  S  Q  N  A  N  H  273
GGCGCCAACCAGCACGAGGAAAAACTGCAGAACGGCAAGGGACCGTCCTCCACCACCGCCGAG 882
 G  A  N  Q  H  E  E  K  L  Q  N  G  K  G  P  S  S  T  T  A  E  294
GGAGAAACTGAAGGAGACGACATGACGAGGGAGAACTGCACCACCGCAGAGGAGAAAGAGAGC 945
 G  E  T  E  G  D  D  M  T  R  E  N  C  T  T  A  E  E  K  E  S  315
TCCAACGATTCCACATCGGGCAGCATGGCCAACCAGAAGGAGGAGGAGGCGGCGCCCTCCGCC 1008
 S  N  D  S  T  S  G  S  M  A  N  Q  K  E  E  E  A  A  P  S  A  336
GCCCACACCAGTGCAGAGGCGAGCCAGCCGCTCCCACGCCAGCGGGCGAAGGCGGGCGGTTCG 1071
 A  H  T  S  A  E  A  S  Q  P  L  P  R  Q  R  A  K  A  G  G  S  357
AAGCTGACCTGCATCAAGATCAAGACTAAATCACCCAAGGGGGACTGCTACACGCCCTCCAAC 1134
 K  L  T  C  I  K  I  K  T  K  S  P  K  G  D  C  Y  T  P  S  N  378
GCCACCGTGGAGATCGTCCCGGCCACCGAGCGGCAGAACCACGTGGCGCGGAAGATCGTGAAG 1197
 A  T  V  E  I  V  P  A  T  E  R  Q  N  H  V  A  R  K  I  V  K  399
ATGACGAAGCAGCCGCCCAACAAGAAGAAAAAAGGGCCGCCGTCGCGGGAGAAGAAGGTGACC 1260
 M  T  K  Q  P  P  N  K  K  K  K  G  P  P  S  R  E  K  K  V  T  420
CGCACCATCATGGCCATCCTGGTGGCCTTCGTGGCCACCTGGACTCCGTACAACGTGATGGTG 1323
 R  T  I  M  A  I  L  V  A  F  V  A  T  W  T  P  Y  N  V  M  V  441
CTCATCAACACCTTCTGCTCCAGCTGCATCCCCAACACCGTCTGGACTATCGGCTACTGGCTG 1386
 L  I  N  T  F  C  S  S  C  I  P  N  T  V  W  T  I  G  Y  W  L  462
TGCTACATCAACAGCACCATCAACCCGGCCTGCTACGCCCTCTGCAACGTCACCTTCAAGAAG 1449
 C  Y  I  N  S  T  I  N  P  A  C  Y  A  L  C  N  V  T  F  K  K  483
ACCTTCAAGCACCTCCTCCTCTGCCAGTACAAGAACAGCCGCTCCGCCAGATAG          1503
 T  F  K  H  L  L  L  C  Q  Y  K  N  S  R  S  A  R stop         500
```

Fig. 5 Nucleotide and deduced amino acid sequences of the fugu M_2 muscarinic receptor gene. Nucleotide residues are numbered in the 5′ to 3′ direction. The predicted amino acid sequence is shown below the nucleotide sequence.

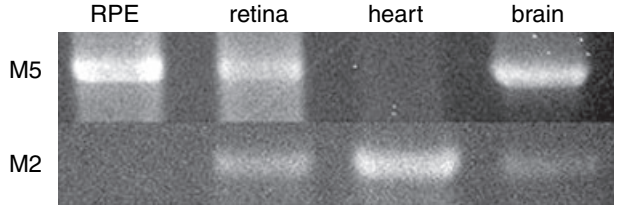

Fig. 6 The M_5 muscarinic receptor is expressed in RPE, but the M_2 receptor is not. (Top) M_5 muscarinic receptor and (bottom) M_2 muscarinic receptor cDNA from RPE, retina, heart and brain of bluegill generated by RT–PCR. DNase 1-treated total RNA isolated from the various tissues was used as template for RT–PCR reactions. Equal aliquots of cDNA were amplified with oligonucleotides for either M_2 (1479 bp) or M_5 (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.

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 M_5 receptor formed a monophyletic unit with other M_5 receptors, within which it formed a terminal clade with fugu and zebrafish M_5 receptors. This terminal clade formed a sister group to the other vertebrate M_5 receptors, which were grouped together. A similar arrangement of clades was observed for bluegill M_2 receptor (Fig. 7). High Bayesian support (> 70) was observed for all the monophyletic groups described above.

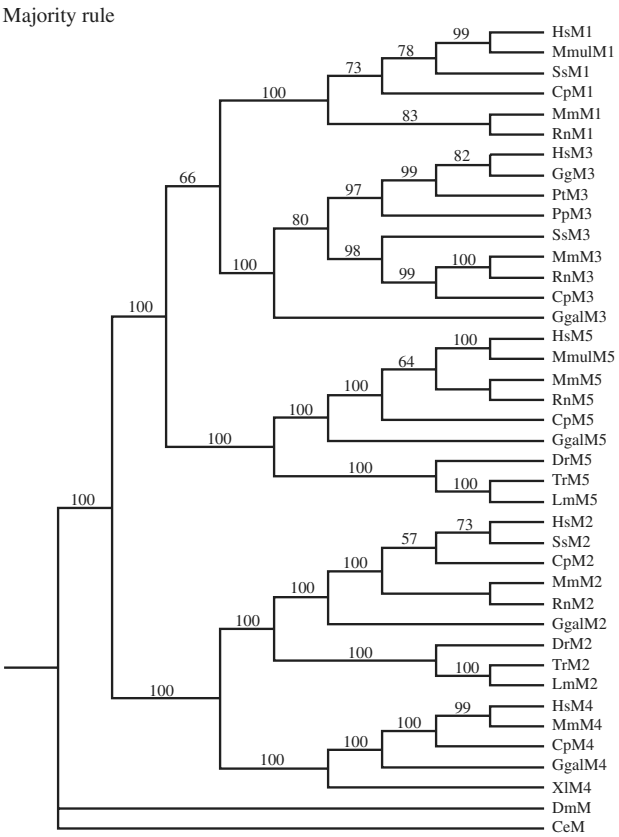

Fig. 7 Phylogenetic analysis confirms the identity of the bluegill M_2 (LmM2) and M_5 (LmM5) genes as well as the fugu M_2 (TrM2) and M_5 (TrM5) genes. The tree was obtained using nucleotide alignment employing Bayesian analysis. The numbers at each node represent support values.

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 M_{odd} receptor, and that RPE expresses the M_5 receptor, which we isolated and sequenced along with the M_2 receptor gene from bluegill. This is the first molecular demonstration of any muscarinic receptors in RPE and the first demonstration that the native ligand ACh induces pigment granule movement in RPE. The finding that ACh induces pigment dispersion adds to the repertoire of functions previously observed for ACh in the retina. These functions include motion detection (Masland *et al*. 1984) and edge detection (Jardon *et al*. 1992).

The possibility that ACh could act as a light signal was first raised by García (1998) when she discovered that the cholinergic agonist carbachol induced pigment granule dispersion in RPE isolated from green sunfish. Before that, dopamine had been established by Dearry 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 M_1, M_2 and M_3 receptors. M_5-selective agents were not available. The M_2 antagonist used in the present study failed to block carbachol-induced pigment granule dispersion whereas antagonists specific for M_1 and M_3 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 M_2 and M_4 receptors failed to block carbachol-induced pigment granule dispersion, and an agonist specific for M_2 receptors failed to induce pigment granule dispersion. In contrast, antagonists specific for M_1 and M_3 receptors blocked carbachol-induced pigment granule dispersion, and an agonist specific for M_1 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 [^{3}H]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 Ca^{2+} flux; this receptor-mediated phosphoinositide hydrolysis is pertussis toxin-insensitive. Feldman *et al*. (1991) and Crook *et al*. (1992) further suggested that the M_3 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 (M_1 and M_3) 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 (M_2 and M_4). Interestingly, chick RPE has been shown immunohistochemically to express M_2 and M_4 along with M_3 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) M_2 receptors show a high affinity for pirenzipine, which in mammals has been characterized as a relatively selective M_1 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 Dearry 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 (M_1, M_3, M_5 or some combination). These receptors preferentially couple to pertussis toxin-insensitive $G_{q/11}$ to activate PLC which catalyzes hydrolysis of phosphotidylinositol (4,5) bisphosphate to IP_3 and diacylglycerol. IP_3 liberates calcium stored in the endoplasmic reticulum by binding to the IP_3 receptor, an IP_3-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 IP_3-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 Ca^{2+} 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

1995; Antoni *et al*. 1995; Paterson *et al*. 1995), cAMP may have been maintained at levels sufficient to keep pigment aggregated as cAMP remained available for influx into the cell. In other words, Ca^{2+} by itself is not a sufficient stimulus if cAMP levels are maintained.

King-Smith *et al*. (1996) also showed that pigment granule dispersion could be induced even when intracellular Ca^{2+} levels were prevented from rising either by removing Ca^{2+} from the medium or by chelating Ca^{2+} with 2-bis (2-aminophenoxy) ethane-N, 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 Ca^{2+} is not required for pigment granule dispersion as long as cAMP levels are lowered, they do not rule out a model in which Ca^{2+} is normally required for dispersion induced by muscarinic receptor activation. We are currently undertaking experiments to further test whether Ca^{2+} 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 IP_3 receptor inhibitor. Taken together, our results indicate that carbachol-induced pigment granule dispersion in bluegill RPE is mediated through M_{odd} receptor subtypes activating PLC and thereby increasing intracellular calcium through an IP_3-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 M_3 (Feldman *et al*. 1991; Crook *et al*. 1992).

Even though earlier pharmacological studies demonstrated muscarinic receptor-mediated phosphoinositide hydrolysis in RPE, to our knowledge this is only the second study in which muscarinic receptor activation in RPE has been linked to a physiological phenomenon, specifically pigment granule dispersion. The only other functional implication so far reported for muscarinic receptor activation in RPE is phagocytosis of rod outer segments, the rate of which is altered when rat RPE is treated with carbachol (Heth *et al.* 1995; Hall *et al*. 1996).

Among the muscarinic receptor antagonists used in the present and previous studies in our laboratory (González *et al*. 2004), 4-diphenylacetoxy-N-[2-chloroethyl]piperidine hydrochloride (4-DAMP) was found to be the most potent inhibitor of carbachol-activated pigment granule dispersion in bluegill RPE, followed by pirenzepine, telenzepine and *p*-F-HHSiD, in that order. Looking at the affinities of these antagonists for different muscarinic receptor subtypes (Eglen *et al*. 2001), such a rank order of antagonist potency (4-DAMP > pirenzepine > telenzepine > *p*-F-HHSiD) fits the M_5 receptor subtype, although M_1 receptor cannot be ruled out.

Although both the pharmacological ranking of antagonists and the studies using inhibitors of the effectors of M_{odd} receptors were consistent with the interpretation of M_{odd}, and particularly M_5 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 M_2 muscarinic receptors and the other to M_5 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 M_2 and M_5 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 M_2 and M_5 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 M_2 and M_5 genes. Phylogenetic analyses using nucleotide alignment showed that M_2 and M_5 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 M_2 and M_5 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 M_2 and M_5 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 M_2. However, chick and zebrafish M_2 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 M_2 and M_5 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 pirenzipine (González *et al*. 2004) and the closely related drug telenzepine does not exclude the possibility of M_2 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 M_5 receptor respectively. The motifs isoleucine-tyrosine-threonine-arginine at N-i3 and lysine-alanine-alanine at C-i3 were identified as functionally important in M_{odd} receptors. These conserved residues and motifs are present at the corresponding position in the deduced amino acid sequence of the M_5 gene isolated from bluegill in the present study.

Wess *et al*. (1997) have shown that the ability of M_2 receptor to interact with G_i 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 M_2 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_i 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. M_2 and M_5 are known to have a wide distribution in brain (Caulfield 1993; Eglen and Nahorski 2000; Bymaster *et al*. 2003) and M_2 is expressed in embryonic chick retina (McKinnon and Nathanson 1995; McKinnon *et al*. 1998), but this is the first report of M_5 expression in retina. Heart was found to express M_2 but not M_5. This expression pattern is consistent with the previously reported results in zebrafish heart (Hsieh and Liao 2002).

RT–PCR showed that RPE expresses M_5 but not M_2. 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 M_5 expression in bluegill RPE converge to suggest that the M_5 subtype is the most likely mediator of ACh-induced pigment granule dispersion in bluegill RPE. The availability of M_5-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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