CARBACHOL-INDUCED

PIGMENT GRANULE DISPERSION

THESIS

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the Requirements

For the Degree

Master of Science

by

Alfredo González III

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ABSTRACT

The retinal pigment epithelium (RPE) is a monolayer of pigmented, simple cuboidal epithelium with long apical processes that interdigitate with photoreceptors. In teleosts, the RPE aids in light adaptation through pigment granule movements. In the bright light, pigment granules are dispersed into the long apical processes thereby shielding rods from excessive light. The opposite occurs in dark conditions; pigment granules are aggregated into the cell body of the RPE allowing the maximal exposure of the rods to incoming light. Using isolated bluegill (*Lepomis macrochirus*) RPE, I examined the effects that carbachol, and acetylcholine analog, has on pigment granule dispersion in a dose-dependent manner, and the maximum effects of carbachol were observed at concentrations as low as 10 nM. Atropine was highly effective in blocking carbachol-induced dispersion. The role of specific muscarinic receptor subtypes (M1-M3) in mediating carbachol-induced dispersion was investigated. The M1 and M3 blockers pirenzepine and 4-DAMP, respectively, were effective in blocking carbachol-induced dispersion. The M2 antagonist, AFDX-116, did not block carbachol-induced dispersion. Consistent with these results, the M1 agonist, 4-chlorophenyl stimulated pigment granule dispersion, while arecaidine, an M2 agonist, did not. I conclude that carbachol-induced pigment granule dispersion is mediated via M1 and M3 but not M2 subtypes of the muscarinic receptor family.

INTRODUCTION

The visual system can adjust to a wide range of ambient light. In the vertebrate eye (Figure 1), several mechanisms are employed permit vision over a wide range of external lighting conditions. For example, two kinds of photoreceptors, rods and cones, may be used depending upon lighting conditions. Rods are adapted to function in dark conditions while cones function in bright light. In mammals, the iris determines pupil size and controls the amount of light that reaches the retina.

In teleosts, pupils have fixed diameter. Light adaptation depends on pigment granule movements within the retinal pigment epithelium (RPE) and movements of the photoreceptors (Douglas, 1982). In bright light, pigment granules within the RPE disperse into the long apical processes while cones and rods contract and elongate, respectively (Burnside *et al.* 1993). Opposite movements occur in dark conditions. These movements expose the appropriate photoreceptor outer segments to their optimal lighting conditions (Figure 2). Furthermore, *in vivo* studies suggest that retinomotor movements occur in anticipation of light onset or darkness in green sunfish (*Lepomis cyanellus*) that have been entrained on 12 hour dark/12 hour light conditions, suggesting that retinomotor movements are controlled, in part, via circadian rhythm modulators (Burnside and Ackland, 1984; Dearry and Barlow, 1987). In addition, there is evidence that dopamine plays a role in light-adaptive and circadian retinomotor movements

because agents that antagonize dopamine inhibit retinomotor movements (Dearry and Burnside, 1986).

Light and dark adaptive and circadian retinomotor movements occur in response to a paracrine messenger system. Changing lighting conditions does not trigger pigment granule movements in RPE separated from the retina (Bruenner and Burnside, 1986); however, medium conditioned by retinas maintained in constant light causes light adaptive pigment dispersion in isolated RPE (Dearry and Burnside, 1989). These findings gave impetus to elucidating the paracrine system(s) that mediate light and dark adaptation in green sunfish retina, and ensuing studies were informed by the discovery that elevating that elevating endogenous cyclic adenosine monophosphate (cAMP) induced dark-adaptive retinomotor movements (Burnside *et al.*, 1982).

Because catecholamines are known to influence cyclic nucleotide metabolism and dopamine is the predominant catecholamine in the retina (see Dearry and Burnside, 1985), Dearry and Burnside (1989) tested the hypothesis that dopamine was involved in light adaptation in green sunfish. Dopamine acting through D1 and D2 receptors causes an increase and decrease in cytoplasmic cAMP, respectively. Dopamine was found to mediate light-adaptive and circadian retinomotor movements in green sunfish via D2 receptors (Dearry and Burnside, 1986; Dearry and Burnside, 1989; Ball *et al.*, 1993). On the other hand, in goldfish (*Carassius auratus*) with chemically ablated dopaminergic cells, bright light caused pigment granule dispersion (Ball *et al.* 1993), suggesting an alternate paracrine system may also play a role in light-adaptive pigment granule movements in the pigment epithelium. In addition, García (1998) recently reported a role

for acetylcholine in light adaptive pigment dispersion in green sunfish, based on studies using the cholinergic agonist carbachol.

Acetylcholine acts through nicotinic or muscarinic receptors. Nicotinic receptors are cation channels which open upon binding acetylcholine, leading to depolarization of the cell membrane. Muscarinic receptors are metabotropic receptors and initiate a cascade of events leading to downstream biochemical reactions. Since the focus of my research revolves around muscarinic receptors, I only detail here the downstream events that occur upon stimulation of the muscarinic receptor.

Muscarinic receptors are members of the G-protein-coupled receptor family, which have a hallmark characteristic of seven transmembrane α-helical domains (Figure 3). Muscarinic receptors can be further subdivided into five subtypes designated as M1 - M5. Each subtype is characterized by differences in their amino acid sequence within the third inner loop as well as their respective downstream actions (Caulfield and Birdsall, 1998) (Figure 4). Stimulation of the M_{even} (M2 and M4) muscarinic receptors has downstream effects that decrease cAMP levels by way of adenylyl cyclase inhibition. Cyclic AMP binds to and activates cAMP-dependent protein kinases. Activated protein kinases in turn phosphorylate proteins, affecting their function. Reducing cAMP levels decreases the activity of these protein kinases.

Activation of the M_{odd} muscarinic receptor subtypes have the downstream effect of increasing cytosolic calcium ion levels (Caulfield and Birdsall, 1998). Calcium ions play the role of the second messenger. Calcium ions may then interact with calmodulin, a Ca^{+2} binding protein required for activation of many Ca^{+2}-dependent kinases.

Analysis of muscarinic receptors may be accomplished by the use of agonists, agents that mimic the *in vivo* ligand, and antagonists, agents the block the natural ligand from binding to the receptor. In the study presented here, I employ pharmacological agents to characterize the muscarinic receptor pathway involved in carbachol-induced pigment granule dispersion. I test the hypothesis that carbachol-induced pigment granule dispersion is mediated via M_{even} muscarinic receptors, since calcium does not affect pigment granule migrations in RPE isolated from green sunfish (King-Smith *et al.* 1996) and M-odd muscarinic receptors have downstream effects of increasing cytosolic calcium levels (Caulfield and Birdsall, 1998). I present evidence that carbachol-induced pigment granule dispersion in the RPE of bluegill (*Lepomis macrochirus*) is dose-dependant and is mediated via muscarinic receptors. Furthermore, I provide evidence that carbachol-induced pigment granule migration is mediated by odd-numbered muscarinic receptor subclasses since both pirenzepine (M1 antagonist) and 4-DAMP (M3 antagonist) inhibit carbachol-induced pigment granule dispersion, while the M2 antagonist, AF-DX 116, did not. Consistently, the M1 agonist, 4-chlorophenyl (4-[N-(4-chlorophenyl) carbamoyloxy]-4-pent-2-ammonium iodide), hereafter referred to as 4-chlorophenyl, caused pigment granule dispersion, while the M2 agonist, arecaidine but-2-ynyl ester tosylate (arecaidine), did not.

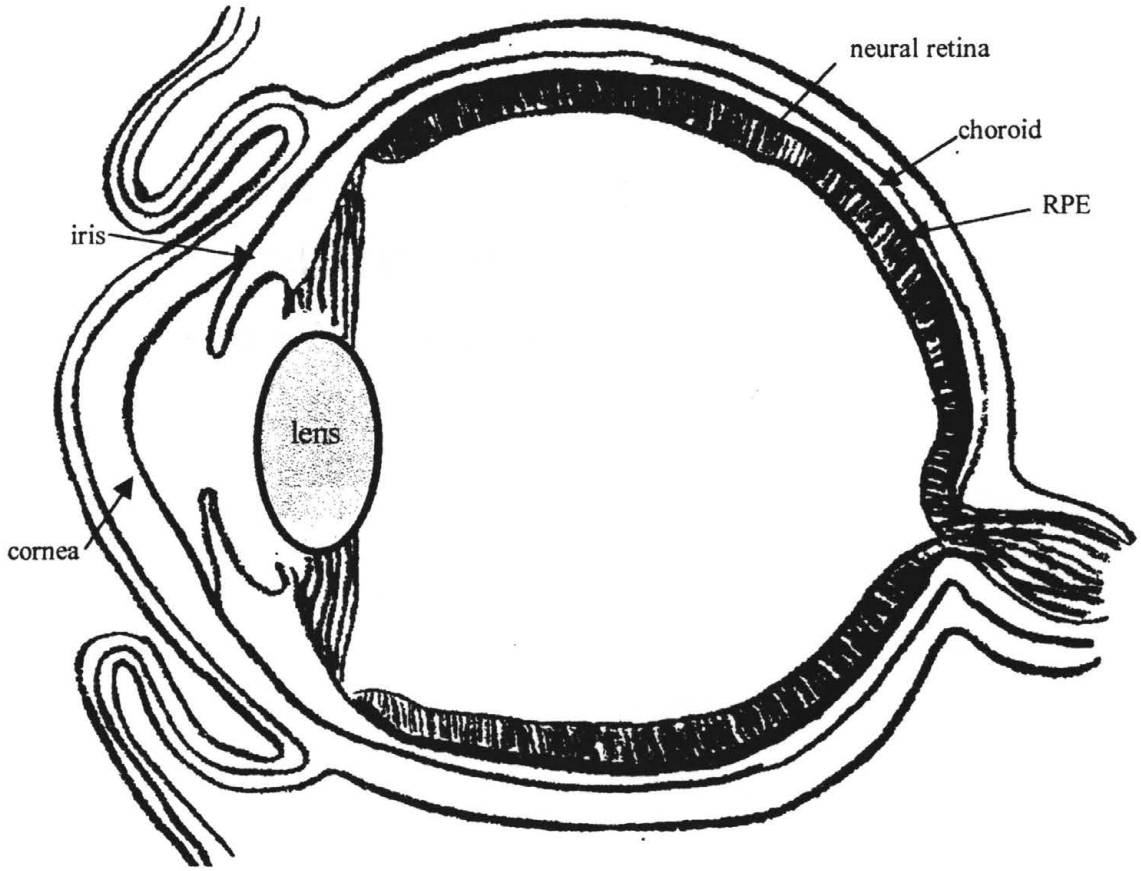
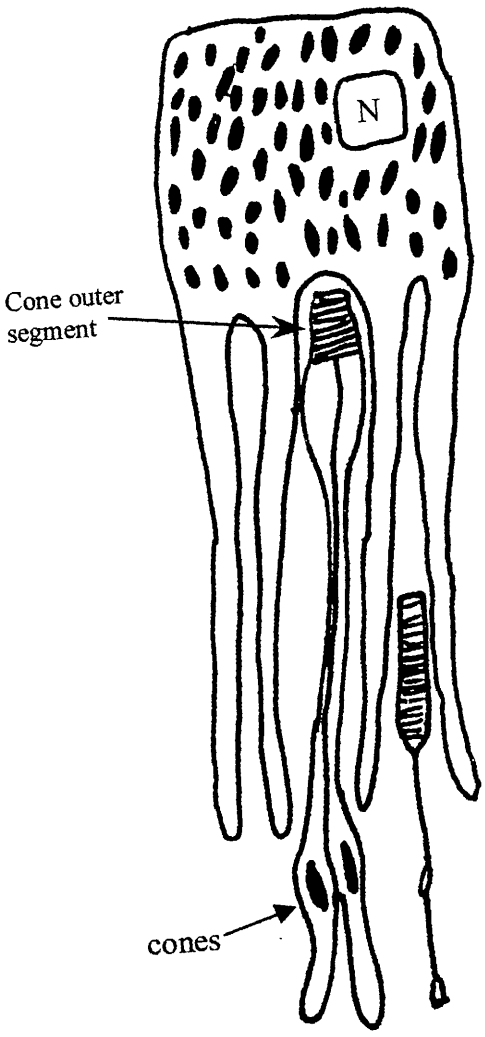


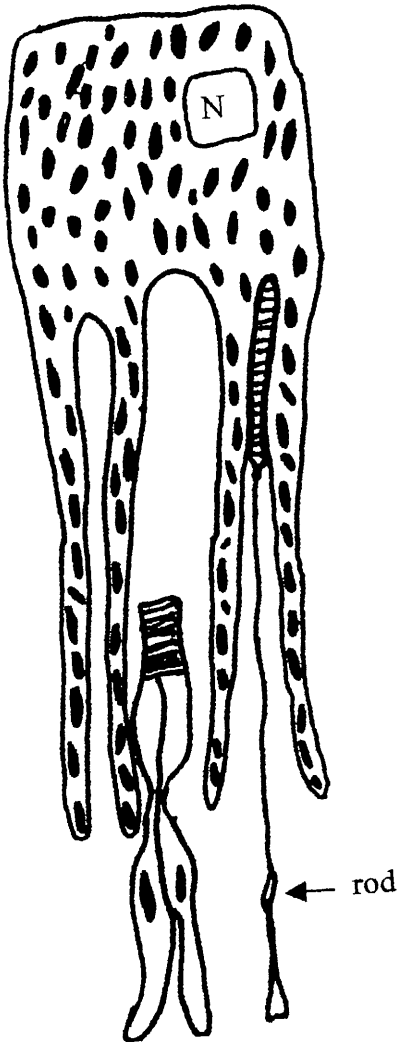
Figure 1. Diagram of a saggital vertebrate eye showing the location of the retinal pigment epithelium (RPE). The RPE lies anterior to the choroid and posterior to the retina.

Figure 2

Diagram illustrating the positions that rods, cones, and pigment granules assume in different lighting conditions. In dark conditions, rods shorten, cones elongate, and pigment granules aggregate into the cell body. Opposite movements are seen in bright conditions. These movements expose the appropriate photoreceptor outer segment to incoming light. Rod outer segments are exposed in dim light, and cone outer segments are exposed in bright light.



Dark-Adapted



Light-Adapted

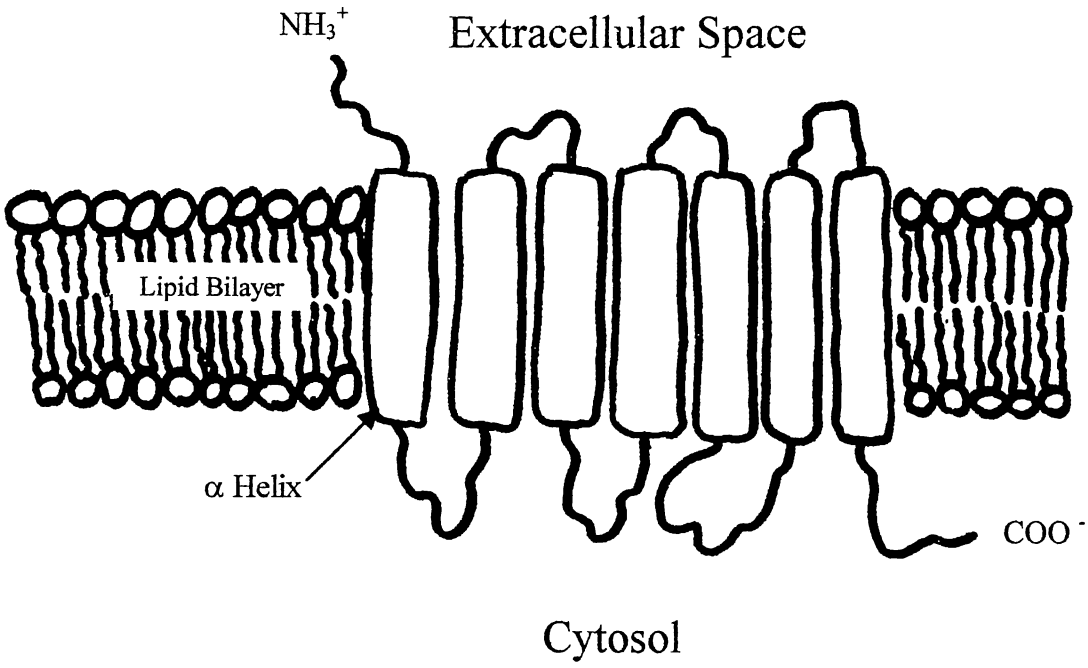
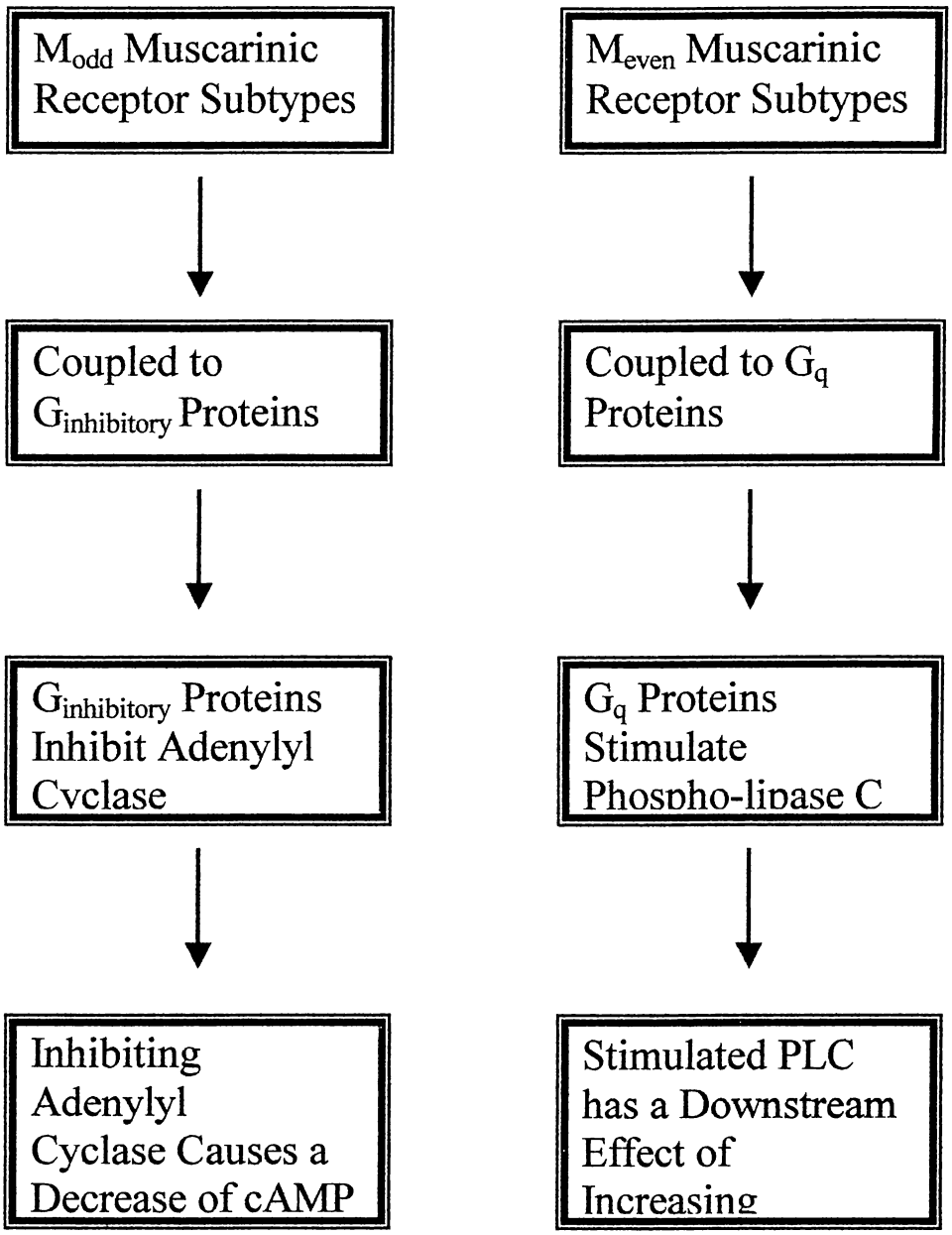


Figure 3. Diagram of a muscarinic receptor showing the seven α-helical domains that span the lipid bilayer.

Figure 4

Flowchart illustrating the different downstream pathways in M_{even} and M_{odd} muscarinic receptors. M_{even} (i.e., M2 and M4) muscarinic receptors are coupled to $G_{inhibitory}$ proteins that have a downstream effect of decreasing cAMP. M_{odd} (i.e., M1, M3, and M5) muscarinic receptors are coupled to phospholipase C that has a downstream effect of increasing intracellular Ca^{+2}.


M_{odd} Muscarinic Receptor Subtypes
M_{even} Muscarinic Receptor Subtypes
Coupled to $G_{inhibitory}$ Proteins
Coupled to G_q Proteins
$G_{inhibitory}$ Proteins Inhibit Adenylyl Cyclase
G_q Proteins Stimulate Phospho-lipase C
Inhibiting Adenylyl Cyclase Causes a Decrease of cAMP
Stimulated PLC has a Downstream Effect of Increasing

MATERIALS AND METHODS

Fish Maintenance

Experiments were performed using protocols approved by the SWT Institutional Animal Care and Use Committee (protocol approval #99-09). Bluegill (*Lepomis macrochirus*) were purchased from Johnson Lake Management, San Marcos, TX. Fish were kept in aerated 55-gallon aquaria on a 12 hour light/12 hour dark cycle room for at least two weeks prior to use.

Isolation of RPE and Drug Treatment

All experiments were carried out in dim, incandescent light (2 lux). In order to facilitate isolation of RPE, fish were dark-adapted for thirty minutes, in a light-tight box, prior to dissection during subjective midday (i.e., ca. 6 hours after light onset). In total darkness, the fish were killed by severing the spine followed by double pithing. Eyeballs were removed and hemisected and the cornea, lens, vitreous humor, and the retina were discarded. RPE sheets were flushed out of the eyecup by applying a steady stream of modified Ringer's solution (isolation buffer). The isolation buffer contained 24 mM $NaHCO_3$, 3 mM HEPES (free acid), 116 mM NaCl, 5 mM KCl, 1 mM $NaH_2PO_4 \cdot H_2O$, 26 mM dextrose, 1 mM ascorbic acid, 0.8 mM $MgSO_4$, 1 mM EGTA, and 0.9 mM $CaCl_2$. Free Ca^{2+} concentrations were estimated to be 10^{-5} M (García and Burnside 1994). The isolation buffer was gassed with a mixture of 95% air and 5% CO_2 for at least 15 minutes prior to and throughout the dissection to maintain the pH at 7.2. The RPE sheets were

divided into 6 samples and incubated in 24 well plates in a humidified chamber according to the regimens described below.

Treatment of RPE with Forskolin Prior to Treatments with Cholinergic Agents

Isolated RPE undergo pigment dispersion (García and Burnside, 1994); therefore, pigment granule aggregation in all samples was induced by a 45-minute treatment with the adenylate cyclase stimulator, forskolin (Calbiochem, La Jolla, CA). Forskolin was washed out 3 times using isolation buffer prior to further treatment with agonists and antagonists (Figure 5).

Treatment of RPE with Agonists

The effectiveness of various cholinergic agonists was evaluated by dose response analysis (see below). Agonists (see Table 1) were prepared in isolation buffer and were applied following wash out of forskolin (see above). In order to maintain the pH (7.2) constant throughout the experiment, the tissue was incubated in agonist for 45 minutes in a humidified chamber gassed with a mixture of 95% air and 5% CO_2 on a gyratory shaker (50 rpm). The cells were fixed by adding a 2X stock solution to the isolation buffer to achieve a final concentration of 0.5% glutaraldehyde, 0.5% paraformaldehyde, and 0.8% potassium ferricyanide.

Treatment of RPE with Antagonists

Separate experiments were conducted using antagonists, also prepared in isolation buffer, as a 2X stock solution. After washing out the forskolin with isolation buffer, antagonists (see Table 1) were applied, immediately followed by application of carbachol (100nM) in equal parts. After a 45-minute incubation, the RPE was fixed (see above).

Preparation of Tissue for Measurement and Statistical Analysis

After fixing the tissue overnight, individual RPE cells were dissociated by chopping the RPE sheets on a glass slide using a No. 1 coverslip. RPE cells were then mounted on the slide using the fixative as the mounting medium and were viewed under a phase contrast microscope. The determination of pigment granule position was done by calculating pigment indices (PI) (Bruenner and Burnside, 1986). The pigment index is a ratio of the length of the cell occupied by pigment to the total length of the cell (see below) (Figure 6). Using an ocular micrometer, thirty cells per treatment per fish were measured and the mean PI was calculated.

$$PI = \frac{Length\ of\ the\ cell\ occupied\ by\ pigment\ granules}{Total\ length\ of\ the\ cell}$$

Dose response curves were plotted to determine if the agonist or antagonist treatment affected the pigment index. The pigment indices plotted are the average of the mean PI calculated (see above). The error bars represent the standard error of the mean. The n values represent 4 fish unless otherwise noted. To determine if the treatment means were significantly different, I used one-way analysis of variance (one-way ANOVA) followed by Tukey's multiple comparison test. Linear regression analysis using SPSS statistical software (Chicago, IL) was performed to determine R^2 values, which indicate how much variability among treatment means could be attributed to the dose of the agent. To test if carbachol induced dispersion in control samples, treatment means between the forskolin sample and the control sample were analyzed using a Student's t-test. All statistical analyses were utilized assuming that the pigment indices

were normally distributed and variances between means were equal. Statistical significance was reported when $p < 0.05$.

Figure 5

Diagram that illustrates the experimental design. Bluegills are dark-adapted for 30 minutes prior to dissection. Eyeballs are removed and hemisected. Cornea, lens, vitrous humor, and neural retina are discarded. RPE sheets are flushed out and treated with forskolin for 45 minutes. After washing out forskolin, RPE are treated with agonist for an additional 45 minutes. *When testing antagonists, the antagonist is added prior to 100 nM carbachol.

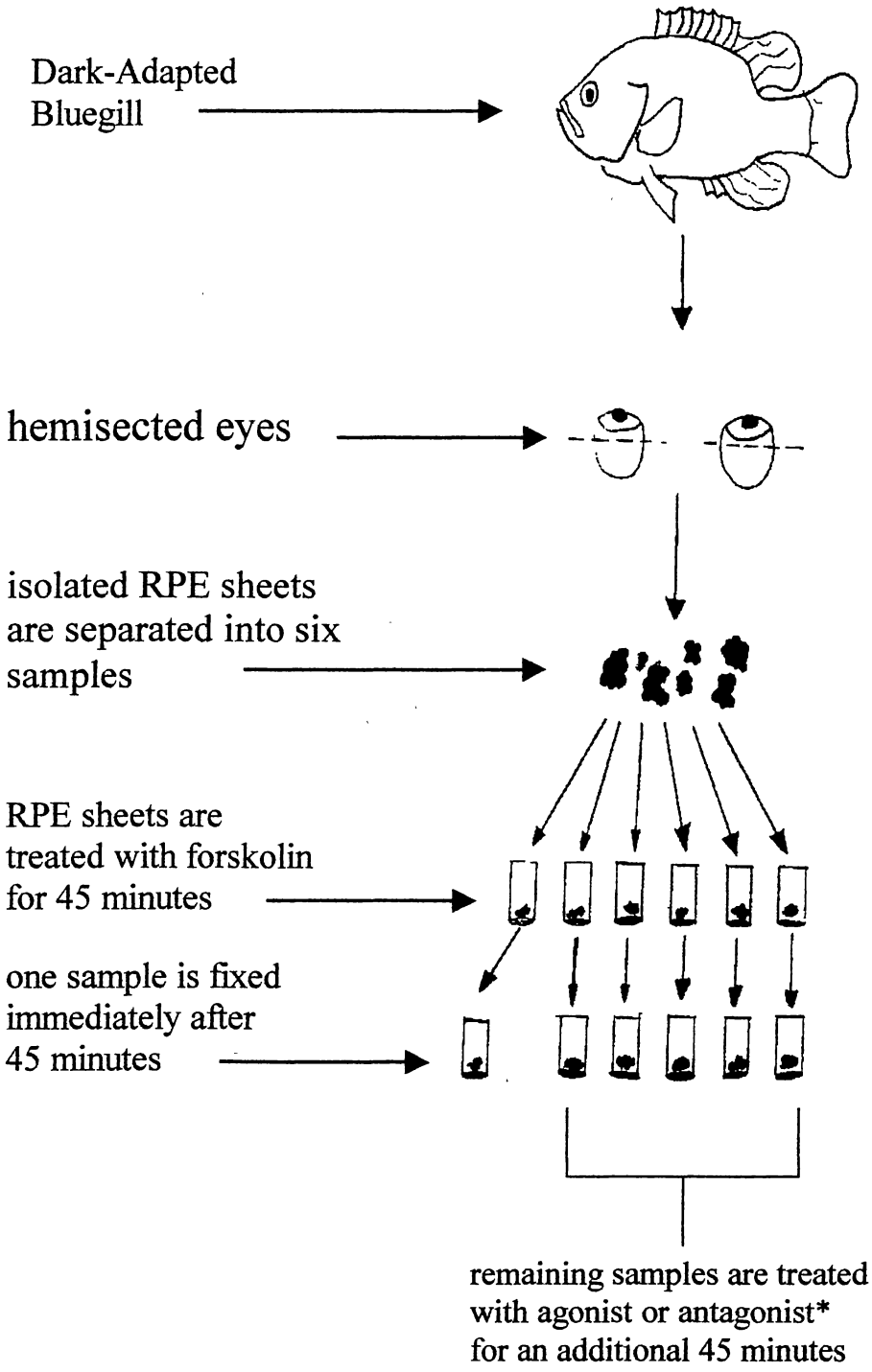

Dark-Adapted
Bluegill
hemisected eyes
isolated RPE sheets
are separated into six
samples
RPE sheets are
treated with forskolin
for 45 minutes
one sample is fixed
immediately after
45 minutes
remaining samples are treated
with agonist or antagonist*
for an additional 45 minutes

Figure 6

Diagram that illustrates the method used in measuring pigment granule position. Pigment position is measured using a pigment index (PI = A/B), which is a ratio of the length of cell occupied by pigment (A) to the total length of the cell (B). The length of the cell occupied by pigment is determined by measuring the most dispersed pigment granule from the base of the cell. The total length of the cell is measured from the base of the cell to the tip of the longest apical process.

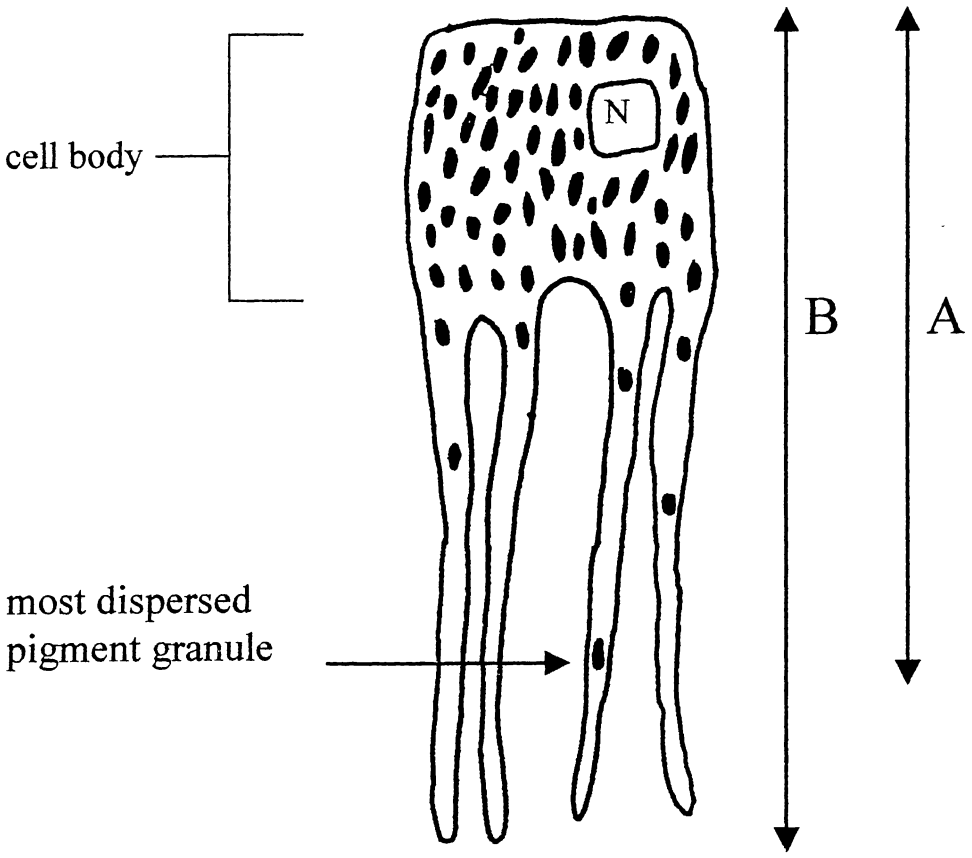

cell body
N
B
A
most dispersed
pigment granule

Table 1: List of agonists and antagonists used and their receptor targets.

	Receptor Target	Source	References
Agonists			
Carbachol (carbamylcholine chloride)	M1-M5	ICN Biomedicals, Aurora, OH	Howe *et al.*, 1986
Arecaidine but-2-ynyl ester tosylate	M2	Tocris, Ballwin, MO	Moser *et al.* 1989
4-chlorophenyl (4-[N-(4-chlorophenyl) carbamoyloxy]-4-pent-2-ammonium iodide)	M1	Tocris, Ballwin, MO	Nilsson, B.M. *et al.* 1992
Antagonists			
Atropine	M1-M5	Sigma, St. Louis, MO	Hulme *et al.*, 1978
Pirenzepine	M1	ICN Biomedicals Aurora, OH	Hammer *et al.* 1980
AF-DX 116	M2	Tocris, Ballwin, MO	Barlow and Shepard 1986; Hammer *et al.* 1986
4-DAMP	M3	Tocris, Ballwin, MO	Anton *et al.* 1989

RESULTS

Effects of Forskolin on Pigment Granule Position

Forskolin induced dark adaptation in isolated RPE in a dose-dependent manner (Figure 7). Cells incubated in 10 μM forskolin were significantly aggregated compared to control samples incubated in isolation buffer alone ($p < 0.05$). Therefore, in subsequent experiments I induced pigment aggregation by culturing tissue in 10 μM forskolin prior to treatment with muscarinic agents.

Effects of Carbachol on Pigment Granule Position

The application of carbachol to RPE that had been treated with forskolin caused pigment granule dispersion in a dose-dependent manner (Figure 8). Cells treated with 10 nM carbachol had significantly higher pigment indices ($PI = 0.84 \pm 0.04$; $n = 6$) than control treated cells ($PI = 0.73 \pm 0.03$; $n = 6$) ($p < 0.05$), however, the latter underwent slight, statistically significant dispersion, as well. Cells treated with concentrations greater than 10 nM did not disperse significantly further. Even though the maximal effect of carbachol was seen with the 10 nM treatment, to ensure receptor saturation, I used 100 nM carbachol in subsequent experiments testing antagonist effects.

Effects of Atropine

To examine if muscarinic receptors were involved in mediating carbachol-induced dispersion, I tested the ability of atropine, a muscarinic antagonist that

blocks M_1-M_5 receptors, to inhibit carbachol-induced dispersion. I found that atropine is a highly effective blocker of carbachol-induced dispersion (Figure 9). At concentrations as low as 10 pM, atropine completely inhibited carbachol-induced dispersion. The mean pigment indices of cells treated in carbachol alone (PI = 0.83 ± 0.02) were significantly different from cells treated with 10 pM atropine and 100 nM carbachol (PI = 0.69 ± 0.03) ($p < 0.05$).

Examining the Effects of Specific Muscarinic Receptor Antagonists and Agonists

After discovering that carbachol-induced dispersion could be inhibited by atropine, I proceeded to investigate which muscarinic receptor subtype might be responsible for mediating carbachol-induced pigment granule dispersion. I began by testing the effects of pirenzepine, an M1 blocker. Pirenzepine was effective at blocking carbachol-induced dispersion (Figure 10). The 10 nM pirenzepine treatment (PI = 0.67 ± 0.02) was significantly more aggregated than the control (PI = 0.81 ± 0.01) but was not significantly different from the higher concentration treatments of pirenzepine.

After finding that the M1 blocker, pirenzepine, was effective in inhibiting carbachol-induced dispersion, I examined the efficacy of the M1 agonist, 4-chlorophenyl (see methods), in inducing pigment granule dispersion. I found that 4-chlorophenyl is a potent stimulator of pigment granule dispersion (Figure 11). The pigment granule position (PI = 0.80 ± 0.01) in the 10 nM treatment was more dispersed than cells treated in buffer alone (PI = 0.70 ± 0.01) ($p < 0.05$). Further, 10 nM 4-chlorophenyl induced the maximal response ($p < 0.05$), since greater concentrations did not disperse pigment granules significantly further.

The ability of the M3 antagonist, 4-DAMP, to block carbachol-induced dispersion was examined, and 4-DAMP was found to be an efficacious blocker of carbachol-induced dispersion (Figure 12). Maximal inhibition was seen in the 10nM treatment (PI = 0.65 ± 0.02), and cells incubated in this treatment were significantly more aggregated than control treated cells (PI = 0.75 ± 0.00) ($p<0.05$).

I examined the ability of the M2 blocker, AF-DX 116, to inhibit carbachol-induced pigment dispersion. I determined that AF-DX 116 was not effective in blocking carbachol-induced dispersion (Figure 13). The control treated cells (PI = 0.87 ± 0.01) were more dispersed than cells treated in forskolin alone (PI = 0.68 ± 0.01) ($p < 0.05$). However, the pigment indices of RPE treated in AF-DX 116 were not significantly different from pigment indices of the control treatment.

I tested the ability of the M2 agonist, arecaidine, to induce pigment granule dispersion in aggregated RPE. Neither the pigment indices of the control treated cells nor the pigment indices of cells treated in arecaidine were significantly different from cells treated in forskolin alone (Figure 14). Therefore, arecaidine did not induce pigment granule dispersion.

Figure 7

Forskolin-induced pigment granule aggregation. RPE sheets were isolated, and the initial pigment index was 0.98 ± 0.00 ($n = 6$). RPE sheets were then incubated for 45 minutes. All forskolin treated cells had pigment indices that were significantly more aggregated than cells treated in buffer alone ($p < 0.05$). The pigment index in cells treated with 10 μM forskolin was not significantly different from the cells treated with 100 μM. The n values are in parenthesis and represent the number of fish used in each treatment.

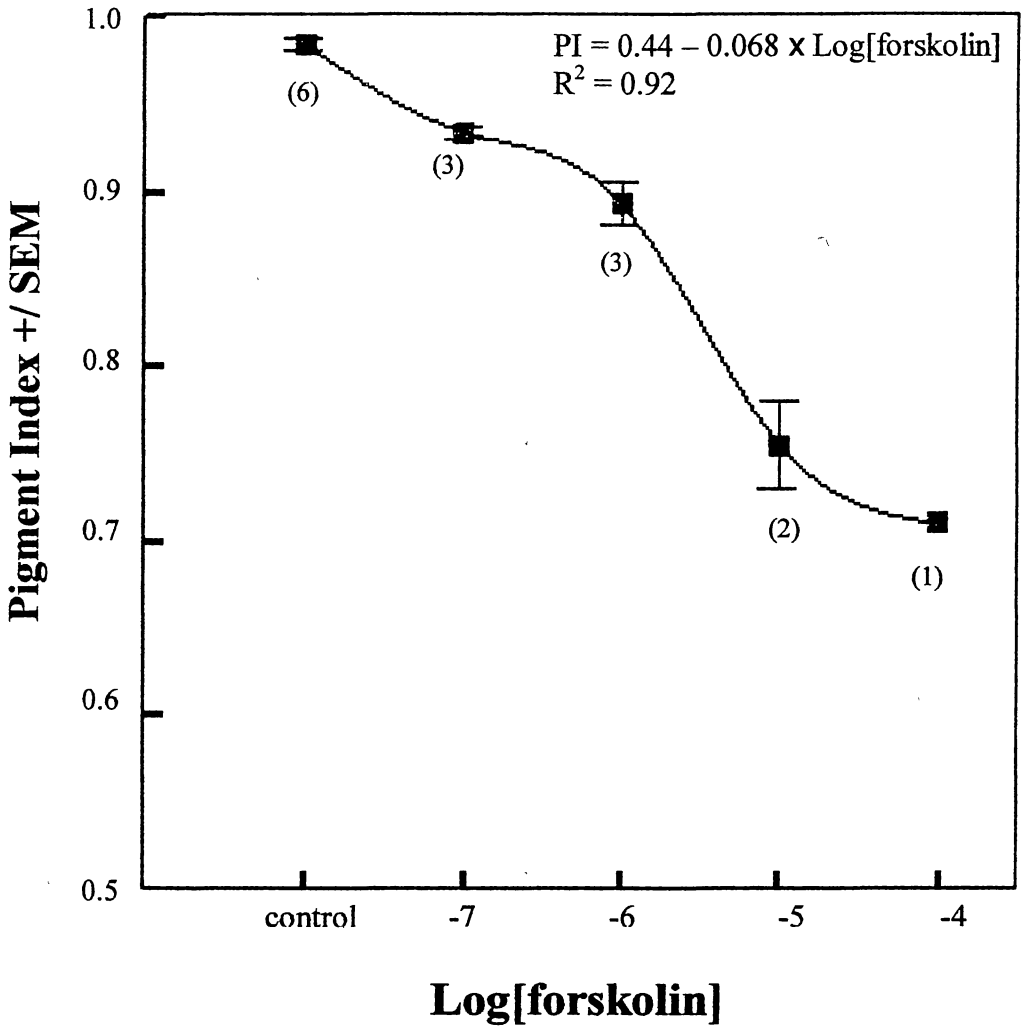

PI = 0.44 – 0.068 x Log[forskolin]
R^2 = 0.92
(6)
(3)
(3)
(2)
(1)
1.0
0.9
0.8
0.7
0.6
0.5
Pigment Index +/ SEM
control
-7
-6
-5
-4
Log[forskolin]

Figure 8

Carbachol-induced pigment granule dispersion. RPE sheets were isolated and induced to aggregate with 10 μM forskolin. After 45 minutes, forskolin-treated cells had a mean pigment index of 0.68 ± 0.02 ($n = 6$). RPE sheets were treated with carbachol for an additional 45 minutes. The pigment indices of RPE treated with carbachol ($\geq$ 10 nM) were significantly more dispersed than cells treated in buffer alone ($p < 0.05$). There were no significant differences between the average pigment indices of cells treated with 10 nM or greater.

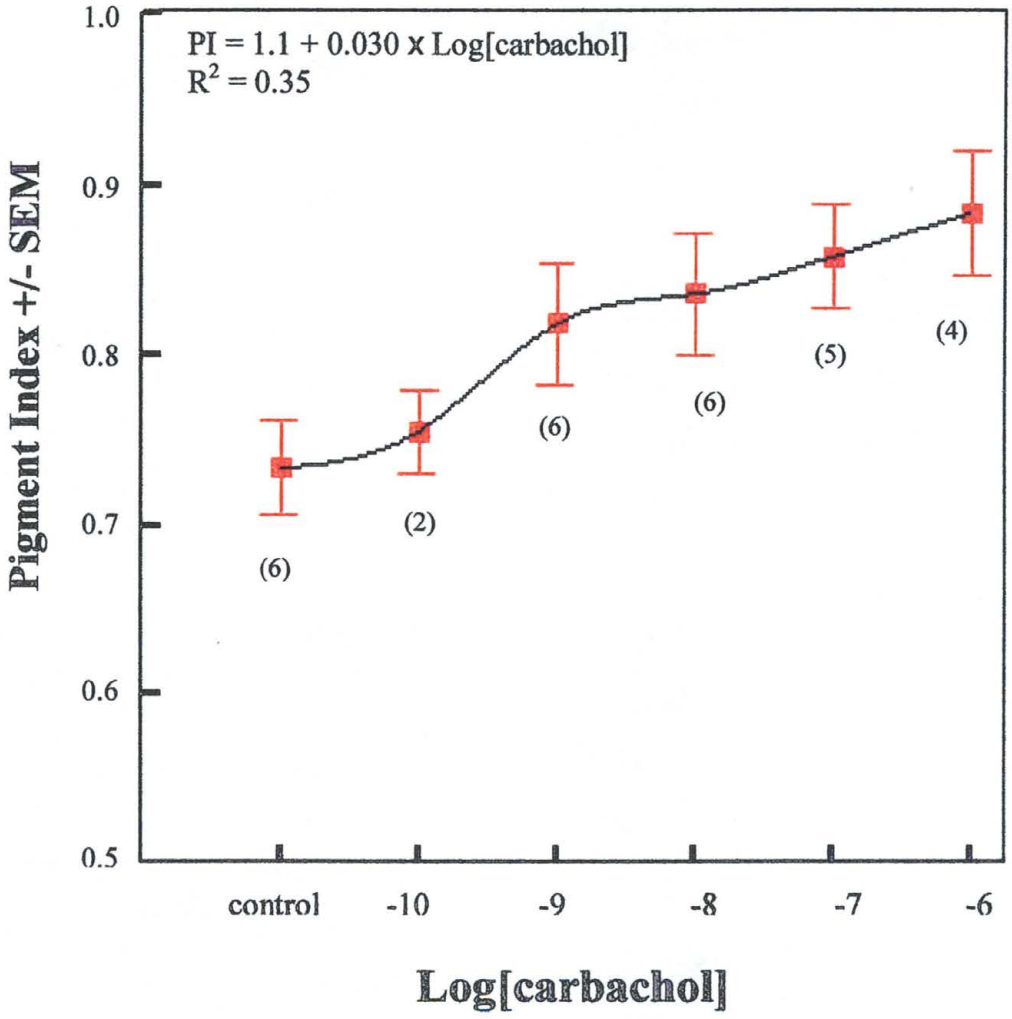

PI = 1.1 + 0.030 x Log[carbachol]
R^2 = 0.35
Pigment Index +/- SEM
1.0
0.9
0.8
0.7
0.6
0.5
(6)
(2)
(6)
(6)
(5)
(4)
control
-10
-9
-8
-7
-6
Log[carbachol]

Figure 9

Atropine blocked carbachol-induced dispersion. RPE sheets were isolated and induced to aggregate as described in the legend to Figure 8. Forskolin-treated cells had a mean pigment index of 0.68 ± 0.03. RPE sheets were then treated with atropine in the presence of 100 nM carbachol for an additional 45 minutes. Statistically significant difference was observed between control cells ($PI = 0.83 \pm 0.02$; $n = 4$) and cells treated with atropine ($p < 0.05$). There were no significant differences among cells treated in 10 pM or greater concentrations of atropine.

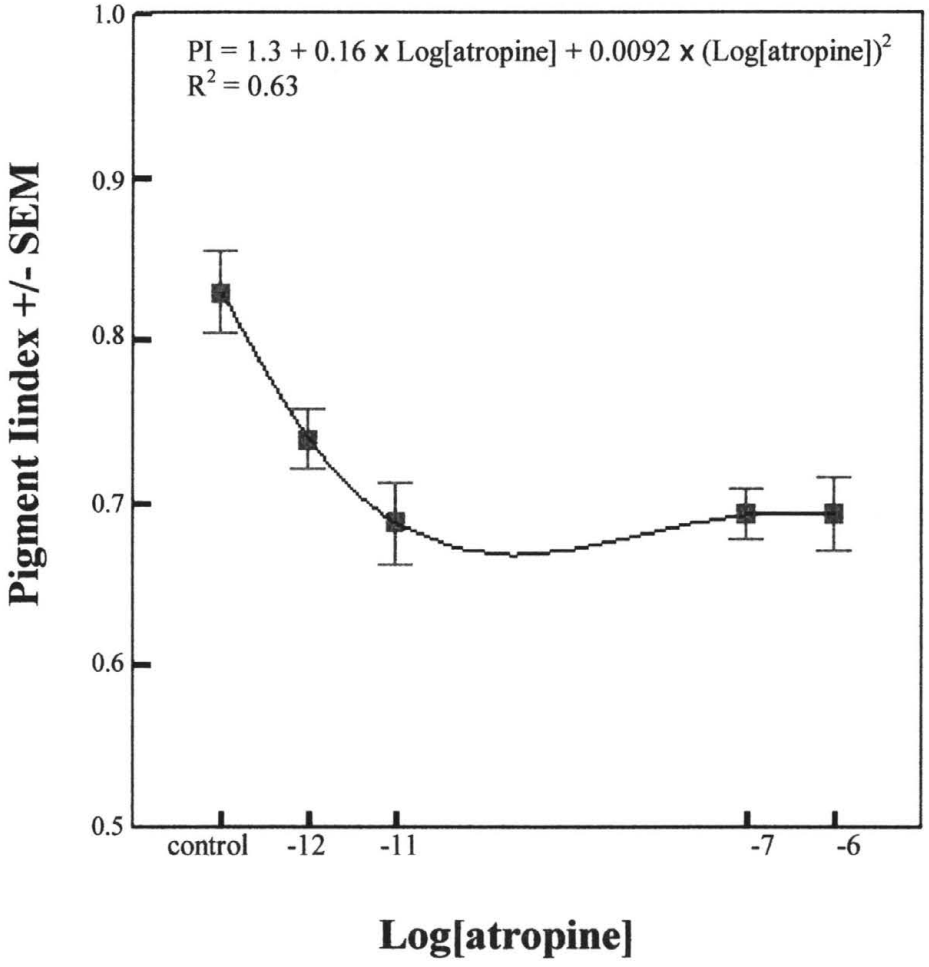

PI = 1.3 + 0.16 x Log[atropine] + 0.0092 x (Log[atropine])^2
R^2 = 0.63
Pigment Iindex +/- SEM
1.0
0.9
0.8
0.7
0.6
0.5
control
-12
-11
-7
-6
Log[atropine]

Figure 10

Pirenzepine blocks carbachol-induced dispersion. RPE sheets were isolated and induced to aggregate as described in the legend to Figure 8. Forskolin-treated cells a mean pigment index of PI = 0.58 ± 0.03. RPE sheets were then treated with pirenzepine for an additional 45 minutes in the presence of 100 nM carbachol. A statistically significant difference was observed between control cells (PI = 0.79 ± 0.02) and cells treated with ≥ 100 pM ($p < 0.05$). There were no statistically significant differences between cells treated in concentrations of 10 nM or greater.

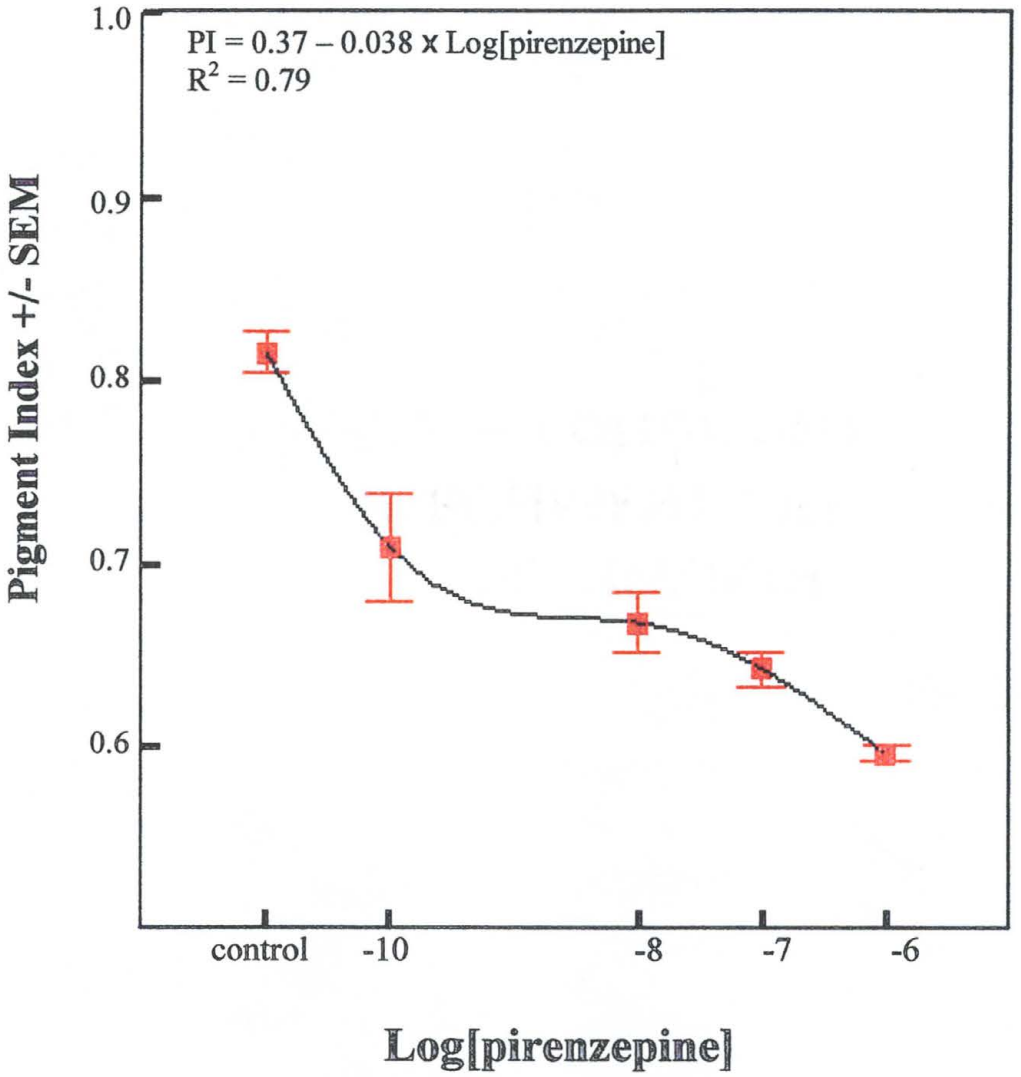

PI = 0.37 – 0.038 x Log[pirenzepine]
R^2 = 0.79
Pigment Index +/- SEM
1.0
0.9
0.8
0.7
0.6
control
-10
-8
-7
-6
Log[pirenzepine]

Figure 11

4-Chlorophenyl-induced pigment granule dispersion. RPE sheets were isolated and induced to aggregate as described in the legend to Figure 8. Forskolin-treated cells had a mean pigment index of 0.63 ± 0.01. RPE sheets were then treated with 4-chlorophenyl. RPE sheets treated with 10 nM 4-chlorophenyl ($PI = 0.80 \pm 0.01$) dispersed significantly more than control cells ($PI = 0.70 \pm 0.02$) ($p < 0.05$). There were no statistically significant differences between pigment indices of cells treated with ≥ 10 nM concentrations of 4-chlorophenyl.

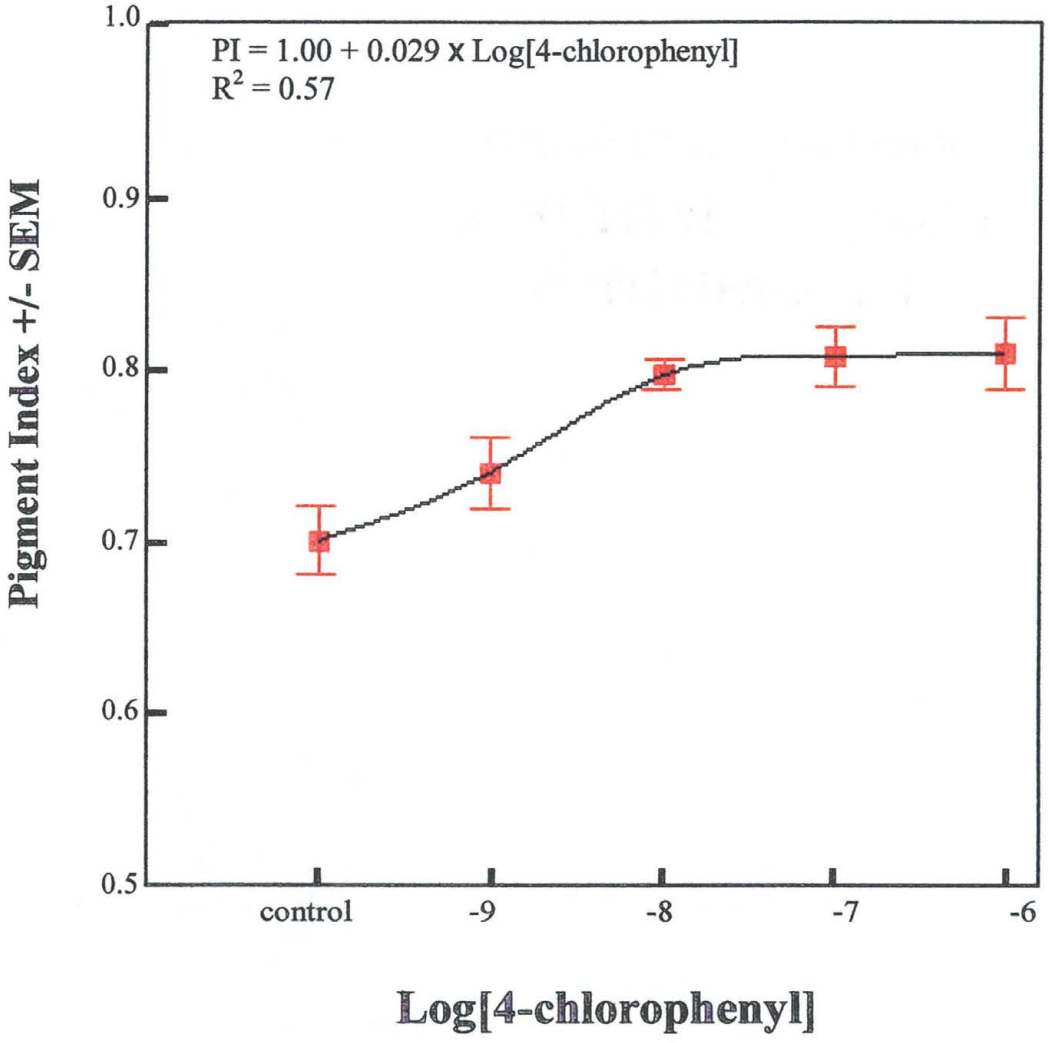

PI = 1.00 + 0.029 x Log[4-chlorophenyl]
R^2 = 0.57
Pigment Index +/- SEM
1.0
0.9
0.8
0.7
0.6
0.5
control
-9
-8
-7
-6
Log[4-chlorophenyl]

Figure 12

4-DAMP blocked carbachol-induced pigment granule dispersion. RPE sheets were isolated and induced to aggregate as described in the legend to Figure 8. Forskolin-treated cells had a mean pigment index of 0.62 ± 0.02. RPE sheets were then incubated in 4-DAMP in the presence of 100 nM carbachol. Statistically significant differences were observed between control cells ($PI = 0.75 \pm 0.00$) and cells treated with 10 nM or greater concentrations of 4-DAMP ($p < 0.05$). There were no statistically significant differences between pigment indices of cells treated with $\geq$ 10 nM concentrations of 4-DAMP.

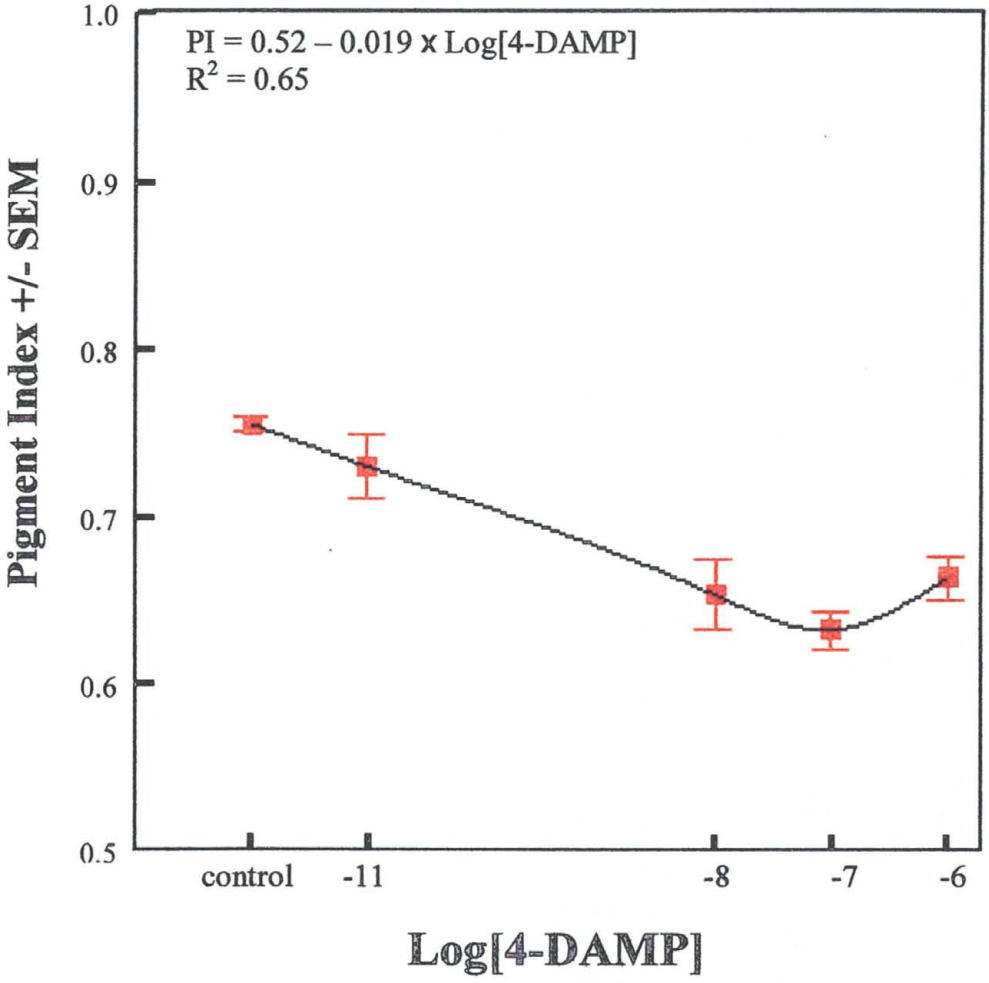

PI = 0.52 – 0.019 x Log[4-DAMP]
R^2 = 0.65
Pigment Index +/- SEM
1.0
0.9
0.8
0.7
0.6
0.5
control
-11
-8
-7
-6
Log[4-DAMP]

Figure 13

AF-DX 116 does not block carbachol-induced pigment granule dispersion. Isolated RPE sheets were induced to aggregate with a 45 minute, 10 μM forskolin treatment (PI = 0.68 ± 0.01). RPE sheets were incubated in AF-DX 116 in the presence of 100 nM carbachol for an additional 45 minutes. There were no statistically significant differences between control cells and cells treated with AF-DX 116.

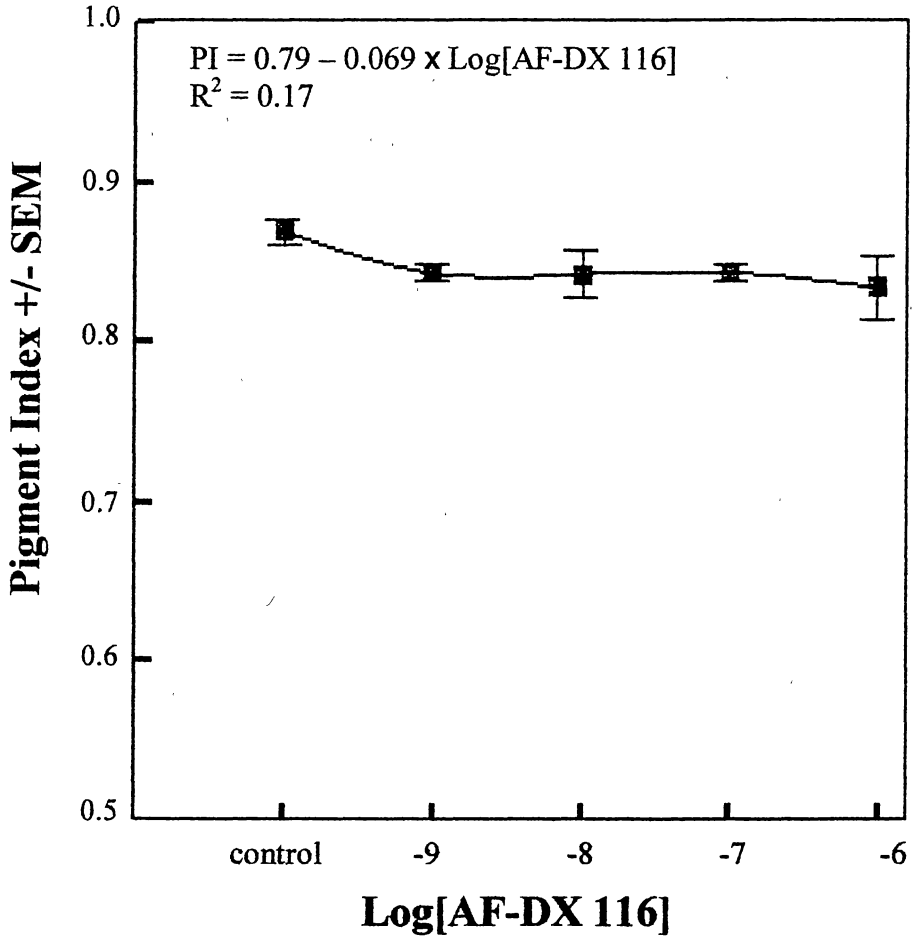
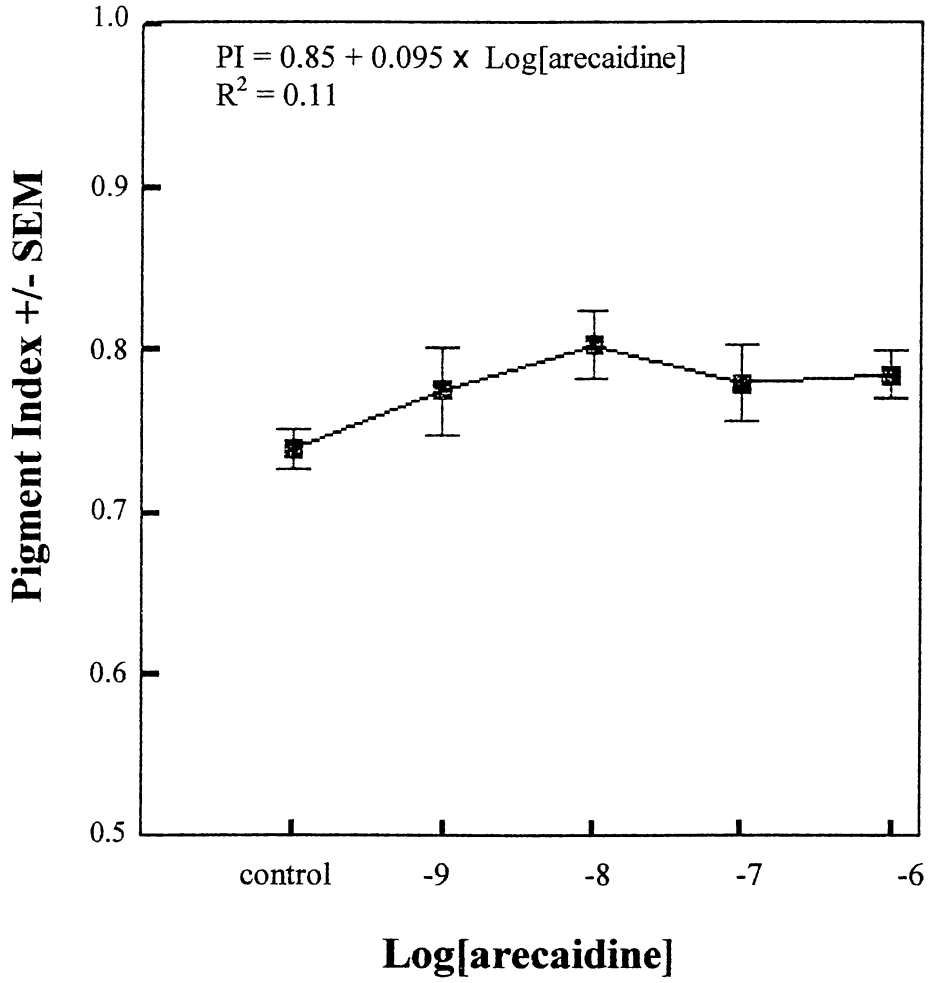

PI = 0.79 – 0.069 x Log[AF-DX 116]
R^2 = 0.17
Pigment Index +/- SEM
1.0
0.9
0.8
0.7
0.6
0.5
control
-9
-8
-7
-6
Log[AF-DX 116]

Figure 14

Arecaidine did not cause pigment granule dispersion. RPE sheets were isolated and induced to aggregate as described in the legend to Figure 8. Forskolin-treated cells had a mean pigment index of 0.72 ± 0.02. Cells were then treated in arecaidine for an additional 45 minutes. There were no statistically significant differences between the pigment indices of cells treated with arecaidine and control cells.


PI = 0.85 + 0.095 x Log[arecaidine]
R^2 = 0.11
Pigment Index +/- SEM
1.0
0.9
0.8
0.7
0.6
0.5
control
-9
-8
-7
-6
Log[arecaidine]

DISCUSSION

In this study, I have shown that carbachol induces pigment granule dispersion and the maximal effect of carbachol was seen at concentrations as low as 1nM. This result is consistent with García's (1998) earlier study in which carbachol induced pigment granule dispersion in isolated RPE of green sunfish. However, unlike the study in green sunfish, I have shown that carbachol-induced pigment dispersion is dose-dependent and saturable, suggesting the involvement of receptor/binding sites. Atropine, a general muscarinic antagonist blocked dispersion. This result suggests muscarinic receptors mediate carbachol-induced dispersion.

The hypothesis that carbachol-induced dispersion is mediated via M_{even} muscarinic receptors was based on several assumptions and observations. Firstly, I assumed that green sunfish and bluegill were very similar in their biochemistry and cell biology, since both are members of the genus *Lepomis* in the Centrachidae family and RPE from both fishes aggregate pigment granules in response to forskolin, an activator of adenylyl cyclase (Dearry and Burnside, 1989; García and Burnside 1994; present study). Therefore, it seemed likely that other aspects of downstream signaling involved in pigment granule movements would be similar between the two species. Secondly, in the RPE of green sunfish, decreasing levels of endogenous cAMP, either by stimulating receptors that decrease cAMP levels (Burnside and Basinger 1983; Dearry and Burnside, 1989) or by washing out agents that increase intracellular cAMP (García and Burnside

1994; King-Smith *et al.* 1996), induces pigment granule dispersion and the M_{even} muscarinic receptor subtypes have downstream effects of decreasing cAMP levels (see Caulfield and Birdsall, 1998). Thirdly, no role for calcium in modulating pigment granule migration could be demonstrated in RPE isolated from green sunfish (King-Smith *et al.* 1994). Since stimulation of the odd-numbered muscarinic receptors are not coupled to adenylyl cyclase but rather increases levels of calcium (see Caulfield and Birdsall, 1998), it would seem likely that carbachol-induced pigment granule dispersion in bluegill RPE is mediated via M_{even} receptors and not M_{odd} muscarinic receptors. Surprisingly, however, this idea was not supported by my results. Carbachol-induced pigment granule dispersion in bluegill RPE is mediated via M_{odd} and not M_{even} muscarinic receptors. Pirenzepine (M1 blocker) and 4-DAMP (M3 blocker) both were effective in inhibiting carbachol-induced dispersion, while AF-DX 116 (M2 blocker) was not. Consistent with these results, the M1 agonist, 4-chlorophenyl, mimicked carbachol's effect and arecaidine (M2 agonist) did not (Table 3).

Although, there is a remote possibility that these pharmacological agents do not discriminate or even bind to muscarinic receptors in bluegill since these agents have been characterized mainly in mammalian species, other studies have used these agents in fish. Jones and King (1995) used radioactively labeled pirenzepine to elucidate the muscarinic receptor subtype in trout atria (M1) and brain (M2) tissue. Hayashi and Fujii (1994) used AF-DX 116 and 4-DAMP to identify muscarinic receptor subtypes in the melanophores of catfish. The consistency of my results in showing that an M1 agonist induced dispersion and M1 antagonist blocked dispersion argues strongly that these agents are interacting with their predicted targets.

Table 2: The effects of muscarinic receptor agonists and antagonists on carbachol-induced pigment granule dispersion.

Agonist	**Site of Action**	**Induced Pigment Granule Dispersion**
Carbachol	M1-M5	Yes
4-Chlorophenyl	M1	Yes
Arecaidine	M2	No

Antagonist*	**Site of Action**	**Blocked Pigment Dispersion**
Atropine	M1-M5	Yes
Pirenzepine	M1	Yes
AF-DX 116	M2	No
4-DAMP	M3	Yes

Earlier research has demonstrated the presence of muscarinic receptors in the RPE of different species. Human RPE possess M3 muscarinic receptors (Feldman *et al.*, 1991), rat RPE M1 and M3 receptors (Salceda, 1994; Anton *et al.*, 1989), and chick RPE express M2, M3, and M4 muscarinic receptors (Fischer *et al.*, 1998). I found that bluegill RPE contain M1 and M3 muscarinic receptors.

My results indicate that carbachol-induced pigment dispersion is mediated via M1 and/or M3 receptors. These M_{odd} receptors are typically linked to G_q proteins that are ultimately associated with calcium mobilization (see Felder, 1995). For example, stimulation of M3 receptors in smooth muscle of the small intestine has been shown to increase cytosolic calcium causing muscle contraction (see Eglen *et al.*, 1994) and activation of the M5 receptor by carbachol in human melanoma cells caused an increase in intracellular calcium that was attenuated by a prior treatment with atropine (Kohn *et al.*, 1996). Furthermore, in human RPE cells carbachol acts through M3 receptors to induce phophoinositide hydrolysis, causing cytosolic calcium levels to increase (Feldman *et al.*, 1991; Crook *et al.*, 1992). Although a role for calcium in pigment movement in the RPE of fish has not been demonstrated, calcium does play a role in causing pigment granule movements in melanophores (pigmented cells of the dermis) of cichlids. Thaler and Haimo (1992) demonstrated that changing levels of calcium as well as cAMP caused pigment granules to aggregate in cichlid melanophores.

Although I did not examine directly the role of cAMP in causing pigment movements, forskolin caused pigment granule aggregation and forskolin is known to stimulate adenylyl cyclase. Therefore, it seems possible that carbachol-induced pigment granule dispersion in bluegill RPE is mediated via downstream mechanisms that lead to

decreased cAMP levels. Although previous studies in RPE isolated from green sunfish seem to indicate that calcium does not mediate pigment migrations (King-Smith *et al.*, 1996), there can be several physiological interactions between calcium and cAMP pathways that may have been circumvented in those studies. For example, the activation of calmodulin by calcium can activate cAMP-dependent phosphodiesterases, which would lead to a breakdown of intracellular cAMP levels. The cloning of several isoforms of adenylyl cyclase adds to the complexity of delineating a possible pathway that leads to decreased levels of cAMP. Calcium can directly or indirectly inhibit some adenylyl cyclases and calcium acting via protein kinase C or calmodulin could inhibit yet other isoforms of adenylyl cyclase. For instance, human adenylyl cyclase IX is inhibited directly by Ca^{2+} (Paterson, 2000).

In conclusion, carbachol-induced pigment granule dispersion is mediated via M1 and/or M3 muscarinic receptors and not M2 receptors. Follow-up studies to delineate and confirm muscarinic mediation in carbachol-induced pigment granule dispersion in bluegill RPE may include the use of several agents that mimic or block downstream mediators of muscarinic receptors. Although decreasing cAMP levels intracellularly has been known for many years to lead to pigment granule dispersion in green sunfish RPE, the complete pathway in which this event takes place has not been delineated. A model that would elucidate a biochemical pathway that leads to pigment granule dispersion in aggregated RPE may include but is not limited to the following testable possibilities. If carbachol is acting through a G_q protein rather than a G_i protein, then treating the RPE with pertussis toxin, a blocker of G_i proteins (Cronin *et al.*, 1983), prior to carbachol treatment will not block pigment granule dispersion. Similarly to confirm that carbachol

is acting via G_q proteins, which stimulate phospholipase C, incubating cells with phorbol esters (activators of protein kinase C) (Fisone *et al.*, 1995) will cause pigment granule dispersion that is similar to that seen in carbachol-induced dispersion. Incubating cells with $G_{\beta\gamma}$ scavengers as suggested by Olianas and coworkers (1998) prior to carbachol treatment will rule out any possibility that adenylyl cyclases are regulated by $\beta\gamma$ subunits.

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