MUSCARINIC RECEPTOR SUBTYPES INVOLVED IN PIGMENT GRANULE DISPERSION IN RETINAL PIGMENT EPITHELIUM

THESIS

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Master of SCIENCE

by

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San Marcos, Texas
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ABSTRACT

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May 2005

SUPERVISING PROFESSOR: DANA M. GARCIA

Previous studies have shown muscarinic receptors are involved in light driven pigment dispersion in retinal pigment epithelia (RPE). Pharmacological agents can be used to test this hypothesis and by selective inhibition, elucidate the sub-class of the receptor involved, and the intracellular downstream signaling pathway. RPE was isolated from *Lepomis macrochirus*, commonly known as bluegill, and treated with various pharmacological agents, including muscarinic receptor antagonists and agents to block second messenger pathway targets. Telenzepine and p-FHHSiD, M_1 and M_3 receptor antagonists, respectively, blocked pigment granule dispersion induced by carbachol, while methoctramine, an M_2 antagonist did not. U73122 and 2-APB, a phospholipase C inhibitor and IP_3-receptor antagonist, respectively, were also able to block carbachol-

induce dispersion. Therefore, I conclude that carbachol-induced pigment dispersion is mediated through M_{odd} receptors. I also propose that Ca^{2+} could be necessary for pigment dispersion induced by carbachol.

INTRODUCTION

I set out to use pharmacological agents to elucidate the muscarinic receptor responsible for carbachol-induced pigment granule dispersion in retinal pigment epithelium (RPE) and the downstream signaling pathway. The RPE used in my research were taken from *Lepomis macrochirus*, commonly known as bluegill.

Previous studies have shown muscarinic receptors are involved in light driven pigment dispersion in retinal pigment epithelia (RPE). Pharmacological agents can be used to test this hypothesis and by selective inhibition, elucidate the sub-class of the receptor involved and the intracellular downstream signaling pathway. Here I report the involvement of M_{odd} receptors and the possible role of calcium in pigment dispersion.

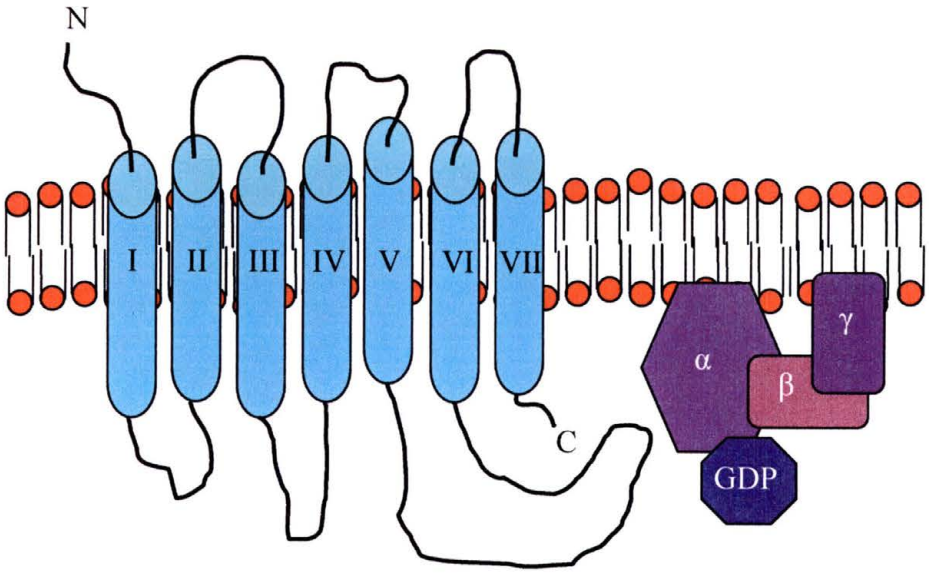
Muscarinic Receptors

Muscarinic acetylcholine receptors are of wide interest because of their possible involvement in Alzheimer's disease, Parkinson's disease, asthma, analgesia, as well as intestinal, urinary bladder and cardiac function (Caulfield and Birdsall, 1998). Muscarinic acetylcholine receptors belong to a superfamily of receptors known as the G protein-coupled receptors, or GPCRs (Figure 1). A characteristic of this superfamily is a seven transmembrane structure with the N-terminus having an extracellular location (Creason *et al.*, 2000). Acetylcholine, a neurotransmitter, binds to muscarinic acetylcholine receptors; thus, acetylcholine is an extracellular ligand. Acetylcholine can

also interact with nicotinic receptors. Nicotinic receptors are ligand-gated ion channels structurally unrelated to muscarinic receptors.

GPCRs receive their name based on their ability to recruit, or couple to, heterotrimeric G-proteins, which consist of three different subunits: α, β, and γ (Gether, 2000; Hann and Chazot, 2004). In the inactive form, the α subunit is bound to GDP and is also bound tightly to the βγ complex (Figure 1). When the GPCR binds an extracellular ligand, the signal transduction begins with a conformational change by the GPCR, which initiates a conformational change in the α subunit of the G-protein. This conformational change in the α subunit causes the release of GDP due to decreased affinity for guanine nucleotides. Since the intracellular concentration of GTP is greater than GDP, GTP binds to the α subunit. This change triggers the separation of the α subunit from the βγ complex. The α subunit bound to GTP now serves as a regulator of effector proteins. The βγ complex separated from the α subunit can also regulate effector proteins, which generate intracellular signals. The signal is terminated when the α subunit, bound to GTP, hydrolyzes the bound GTP to GDP. The α subunit with GDP bound then reunites with the βγ complex (Hann and Chazot, 2004).

Fig. 1. Diagram of muscarinic receptor coupled to G-protein. Muscarinic acetylcholine receptors belong to a family of receptors known as G-protein coupled receptors (GPCRs). GPCRs are characterized by having seven transmembrane domains (TM I-VII) with an extracellular N-terminus and an intracellular C-terminus. The third intracellular loop binds with the α subunit of G-proteins once an extracellular ligand is bound, causing a conformational change in the α subunit, which in turn causes the release of GDP due to decreased affinity for guanine nucleotides. Since the intracellular concentration of GTP is greater than GDP, GTP binds to the alpha subunit. The G alpha-GTP and beta/gamma complex separate and become effectors. The effects of the activation of G-proteins depend on which muscarinic subtype is involved and to which G-protein it is coupled.


N
I
II
III
IV
V
VI
VII
C
α
β
γ
GDP

There are five known subtypes of muscarinic receptors, M_1-M_5 (Caulfield and Birdsall, 1998; Creason *et al.*, 2000). Five, intronless genes encode all of the known muscarinic acetylcholine receptors in vertebrates (Caulfield and Birdsall, 1998; Creason *et al.*, 2000; Hirota, 2001). Muscarinic receptors can be divided into two groups, M_{odd} (M_1, M_3, and M_5) and M_{even} (M_2 and M_4). One of the defining differences between the subtypes, is the divergence of the sequences of the third intracytoplasmic (i3) loop (Burstein *et al.*, 1995; Caulfield and Birdsall, 1998). The actual contact with G-proteins occurs with the i3 loop of the muscarinic receptor (Burstein *et al.*, 1998). According to Blin *et al.* (1995), there are four amino acid residues in both the i2 and i3 (C-terminus) loops that are responsible for G-protein activation. The biological functions of the M_{odd} and M_{even} groups also demonstrate the differences between the two groups.

The M_{odd} receptors preferentially couple to the pertussis toxin-insensitive G_q proteins (Creason *et al.*, 2000). M_{odd} receptors specifically couple with G_{11}, a member of the G_q family, denoted as $G_{q/11}$ (Akam, 2001). G_q proteins activate phospholipase C-β (PLC) (Broadley and Kelly, 2001). PLC-β hydrolyzes phosphoinositides such as phosphatidylinositol 4,5-biphosphate (PIP_2), which yields inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 and DAG act as second messengers. IP_3 binds to receptors located on the endoplasmic reticulum, causing the release of calcium (Ca^{2+}) (Zeng and Wess, 2000; Broadley and Kelly, 2001). Ca^{2+}, once released from the endoplasmic reticulum, alters protein kinase C (PKC), a serine- and threonine-specific protein kinase, so that it migrates from the cytosol to the intracellular side of the plasma membrane. At this location, PKC can be activated by the combination of Ca^{2+} and DAG (Farago and Nishizuka, 1990; Blumberg, 1991; Nelsestuen and Bazzi, 1991; Broadley

and Kelly, 2001). Ca^{2+} also can activate calmodulin (CaM). Some of the various functions of CaM are regulation of growth, proliferation and movement. CaM activates Ca^{2+}/calmodulin-dependent kinases (CaM-kinases; Chin and Means, 2000); calcineurin, a protein phosphatase (Klee *et al.*, 1998); various adenylyl cyclase (AC) isoforms and some phosphodiesterases (PDE) (Chin and Means, 2000).

The M_{even} receptors preferentially couple to the pertussis toxin-sensitive G_i family of G-proteins (Creason *et al.*, 2000). The G_i protein family inhibits adenylyl cyclase (see Hann and Chazot, 2004). The inhibition of adenylyl cyclase results in the decrease of cyclic adenosine monophosphate (cAMP) production from ATP. Cyclic-AMP activates cAMP-dependent protein kinase (PKA). PKA phosphorylates many substrates. Residual cAMP is then degraded by PDEs, which results in decreases in cAMP-dependent intracellular events (Broadley and Kelly, 2001).

Retinal Pigment Epithelium

RPE is a single layer of tissue that functions in the physiological support of the retina (see García, 1998). In fish, which have fixed pupil diameters, the RPE assists in adapting the eye to changing amounts of light. In the presence of light, pigment granules disperse into apical processes to help protect photoreceptors against photobleaching; the pigment granules aggregate to the cell bodies of the RPE when light is dim (Figures 2 and 3). This aggregation enables rods to capture the maximum amount of light available (see King-Smith *et al.*, 1996; García, 1998).

Fig. 2. Diagram of an eye. Fish and other lower vertebrates have fixed pupils. As light enters, the retinal pigment epithelial cells protect photoreceptors from photobleaching. Cones (blue) and rods (red) interdigitate with the apical processes of the RPE cells (excerpt).

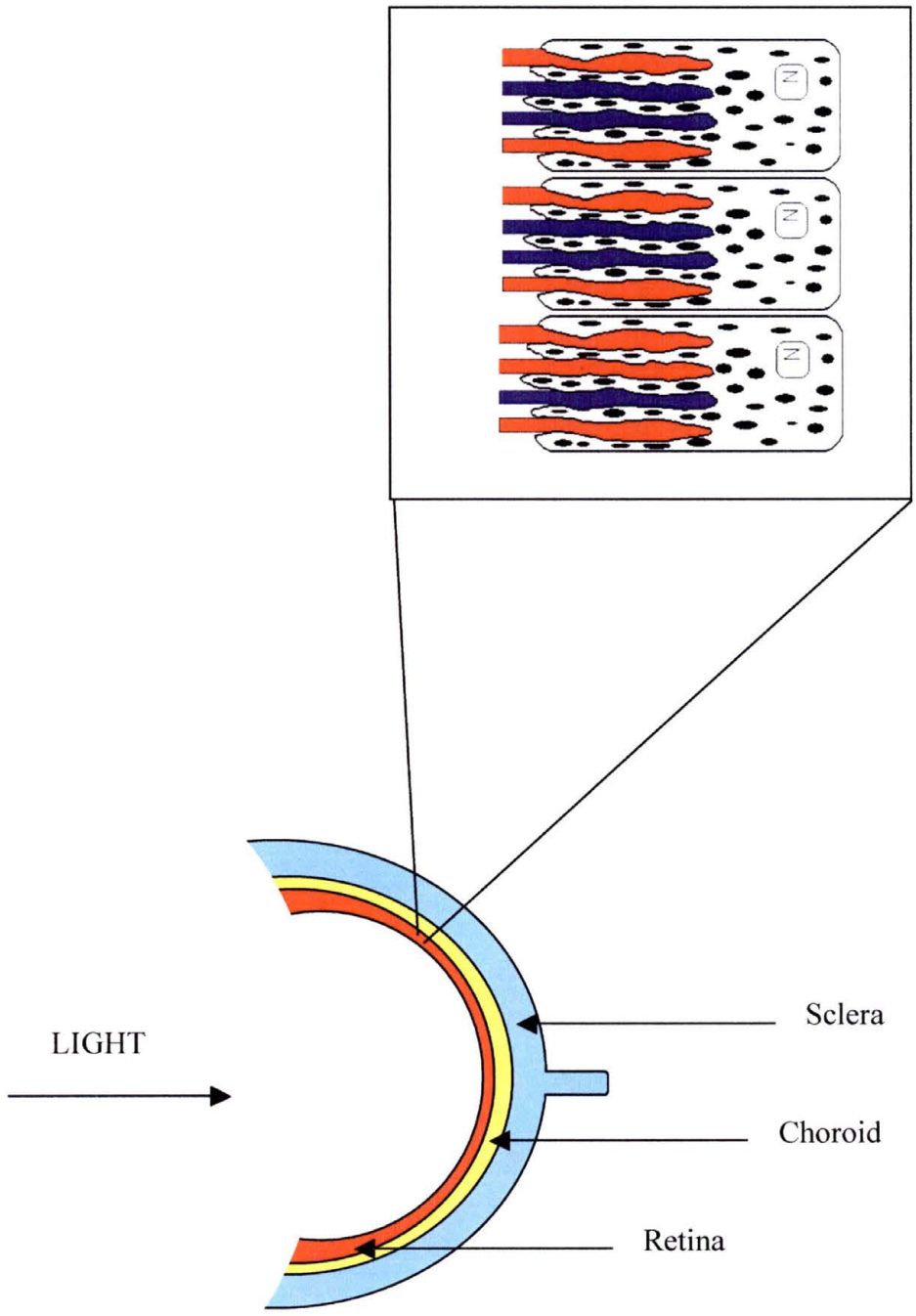
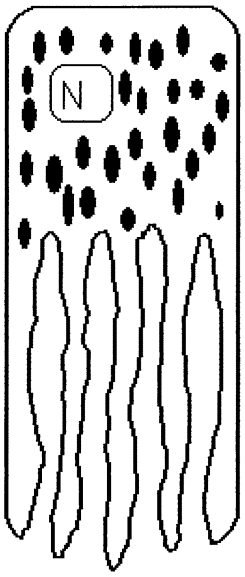
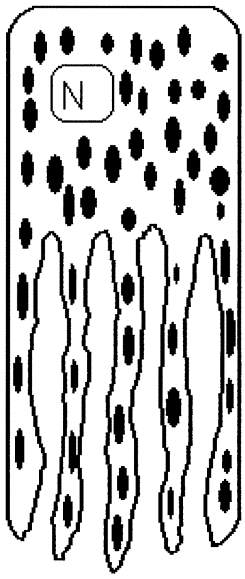

LIGHT
Sclera
Choroid
Retina

Fig. 3. Diagram of RPE with pigment granules aggregated and dispersed. Two isolated RPE cells with melanin pigments are shown. The "N" labels the nucleus of each cell. The cell on the left illustrates an aggregated RPE cell. The pigment granules aggregate into the cell body to allow photoreceptors to capture light maximally. The cell on the right illustrates a dispersed RPE cell. The pigment granules in this diagram are dispersed, which protects the photoreceptors from photobleaching. Figure adapted from García and Koke (1996).

Aggregated



Dispersed



The History of Studies of Retinomotor Movements

Retinomotor movement is a collective term for the movement of cones, rods, and pigment granules located within RPE cells. Initially, retinomotor pigment migration in blue striped grunt (*Haemulon sciurus*) was examined by Dr. Beth Burnside of the University of California at Berkeley. Burnside and Basinger (1983) reported that increases in cAMP induced dark-adaptive movement, where pigment granules aggregated into the cell body, allowing the photoreceptors to capture more light. Following this study, Burnside and Ackland (1984) examined the effects of circadian phase on retinomotor movements in green sunfish (*Lepomis cyanellus*). Burnside and Ackland (1984) compared the effects of exogenous cAMP analogs on the movement of rods, cone, and RPE pigment. Dearry and Burnside (1985) later reported that dopamine inhibited dark-adaptive pigment movement induced by forskolin, an adenylyl cyclase activator, and 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor. Dearry and Burnside (1988, 1989) further reported the regulation of retinomotor movements by dopamine in green sunfish. Dopamine is the most abundant catecholamine in vertebrate retinas (Dearry, 1991). Since dopamine can act through D1 or D2 receptors, which increase or decrease cAMP, respectively, it was conjectured that dopamine must have been binding D2 receptors, inhibiting adenylyl cyclase and decreasing cAMP. Sulpiride, a D2 antagonist, blocked dopamine-induced dispersion, but D1 and α-adrenergic antagonists were unsuccessful. Furthermore, LY 171555, a D2 agonist caused pigment dispersion. In contrast, SKF 38393, a D1 agonist, did not induce pigment granule dispersion (Dearry and Burnside, 1988; Dearry and Burnside, 1989).

García and Burnside (1994) then set out to discover the signaling system responsible for inducing dark-adaptive pigment granule aggregation. A battery of candidate agents was tested, but only exogenously applied cAMP induced pigment granule aggregation in green sunfish RPE. García and Burnside found that exogenous cAMP could be introduced into the cytoplasm via organic anion transporters, and hypothesized that cAMP secreted by the retina entered the RPE and activated PKA. King-Smith *et al.* (1996) were unable to show a role for Ca^{2+} in the regulation of pigment granule movement; they showed that the depletion of intra- and extracellular Ca^{2+} had no effect on pigment granule dispersion or aggregation. King-Smith *et al.* (1996) reported that Ca^{2+} changes were neither necessary nor sufficient to induce pigment movement.

García (1998) tested the effects of carbachol, an acetylcholine analog, on pigment granule movement. The work was initiated prior to the publication of King-Smith *et al.* (1996) to examine the role of Ca^{2+} in pigment granule movement. García (1998) isolated RPE from green sunfish and treated it with forskolin, which previously had been demonstrated to cause pigment granule aggregation, presumably by increasing cAMP (Dearry and Burnside, 1985). The forskolin was removed and then the RPE was treated with carbachol and pigment granule dispersion was seen. This paper showed for the first time a cholinergic mechanism to be involved in pigment granule movement (García, 1998).

With an interest in cholinergic mechanisms, the García laboratory wanted to look at agonists and antagonists of muscarinic receptors to illuminate the pathways through which carbachol-induced dispersion. As mentioned earlier, acetylcholine works through two types of receptors, muscarinic and nicotinic. González *et al.* (2004) tested the ability

of atropine, a muscarinic receptor antagonist, to inhibit carbachol-induced pigment granule dispersion and found that at concentrations in the Pico molar range, atropine completely inhibits such dispersion. González *et al.* (2004) reported that pigment granule dispersion is mediated by muscarinic receptors and that the pharmacology was consistent with M_{odd} receptors. Table 1 shows the cholinergic agonists and antagonists used in the González *et al.* (2004) study.

Table 1. Pharmacological agents used by González *et al.* (2004). RPE was isolated from dark-adapted bluegill and treated with 10 μM forskolin. RPE was then washed of forskolin and treated with 0.1 μM carbachol and increasing concentrations of the drugs listed. This table was adapted from González *et al.* (2004). I analyzed González's raw data by recreating dose response curves. pIC_{50} values were estimated using Microsoft Excel with XLfit4. See Methods for details.

Agonist	Target	Induced Pigment Granule Dispersion
Carbachol	M1-M5	Yes
4-Chlorophenyl	M1	Yes
Arecaidine but-2-ynyl ester tosylate	M2	No

Antagonist	Target	Estimated pIC_{50}	Blocked Pigment Granule Dispersion
Atropine	M1-M5	12.0	Yes
Pirenzepine	M1	9.6	Yes
AF-DX 116	M2	NA	No
4-DAMP	M3	10.3	Yes
Tropicamide	M4	NA	No

Molecular evidence of the presence of M_{odd} receptor

After the pharmacology study performed in the García laboratory by Alfredo González III and Elizabeth Crittenden, Prasad Phatarpekar worked on the isolation and sequencing of muscarinic receptor genes from fish. He was able to isolate and with the assistance of James Neece, sequence bluegill M_5 (Phatarpekar *et al.*, 2004) and fugu M_2 (Neece *et al.*, 2004). Dr. Simon Durdan and Mr. Phatarpekar jointly performed an expression study of M_2 and M_5 mRNA testing for expression in RPE, retina, heart and brain of bluegill fish by RT-PCR. They showed that M_5 was expressed in RPE, retina and brain; whereas, M_2 was not found in RPE, but was expressed in retina, heart and brain (Phatarpekar *et al.*, 2004; García, personal communication).

The molecular studies and previous pharmacology from our laboratory currently supports the idea that pigment granule dispersion acts through M_{odd} receptors, more specifically, M_5 in bluegill.

Thesis Statement

The previous studies summarized above have shown that the M_{odd} receptors are likely involved in pigment dispersion in RPE. Therefore, inhibition of various downstream effectors activated by $G_{q/11}$ should block pigment granule dispersion induced by carbachol. To corroborate earlier results suggesting the involvement of M_{odd} receptors and to extend them by testing second messenger pathways likely to be involved, pharmacological agents, including muscarinic receptor antagonists and agents to block second messenger pathway targets, were tested. Telenzepine and p-FHHSiD, M_1 and M_3 receptor antagonists, respectively, blocked pigment granule dispersion induced by

carbachol, while methoctramine, an M_2 antagonist did not. U73122 and 2-APB, a phospholipase C inhibitor and IP_3-receptor antagonist, respectively, were also able to block carbachol-induced dispersion.

MATERIALS AND METHODS

Fish Maintenance

Bluegill (*Lepomis macrochirus*) were purchased from Johnson Lake Management, San Marcos, TX and maintained in 55-gallon aerated tanks. Bluegill were subjected to a 12 hour light/12 hour dark cycle for at least two weeks prior to dissection. The Institutional Animal Care and Use Committee (IACUC) of Texas State University-San Marcos (protocol # 03E3AB42BD_03) approved all of the protocols used.

Tissue Isolation

Bluegill were dark-adapted midway through the light cycle (6 hours into the light cycle). Dark adaptation was accomplished by placing the bluegill into a light-tight box where they were maintained in an aerated tank. Once the fish had dark adapted for 30-60 minutes, they were killed by severing the spinal cord followed by double pithing, all of which occurred in dim light ($\leq$ 2 lux). Light was measured using a Lutron LX-101 lux meter (Lutron, Coopersburg, PA). The eyeballs were then removed and hemisected to separate the anterior portion of the eye from the posterior eye cup (Figure 4). RPE was removed from the eye cup by flushing with a modified Ringer's solution (buffer). The solution consisted of the following: 24 mM $NaHCO_3$, 3 mM HEPES (free acid), 116 mM NaCl, 5 mM KCl, 1 mM $NaH_2PO_4*H_2O$, 26 mM dextrose, 1 mM ascorbic acid, 0.8 mM $MgSO_4*7H_2O$, 1 mM EGTA, 0.9 mM $CaCl_2$. All of the chemicals were purchased from Sigma-Aldrich, St. Louis, MO. The Ringer's solution was titrated with 1 M NaOH to a

pH of 7.4. The buffer was gassed prior to dissection with 95% air/5% CO_2 to maintain a pH of 7.2.

Drug Treatment

After the RPE was removed, it was treated with forskolin (LC Laboratories, Woburn, MA) as described previously (García, 1998; González *et al.*, 2004). The RPE pieces were then distributed evenly among several 1.7 ml microcentrifuge tubes. A sample of RPE was fixed as described below immediately after treatment with forskolin. RPE in the other tubes were washed free of forskolin, then treated with 100 nM carbachol diluted from a 10X stock plus increasing concentrations of the pharmacological agents to be tested (see Table 2 for range of concentrations used). Again, tissue was incubated, gyrated (60 rpm) and gassed for 45 minutes (Figure 4) (García, 1998; González *et al.*, 2004).

Pharmacological Agents

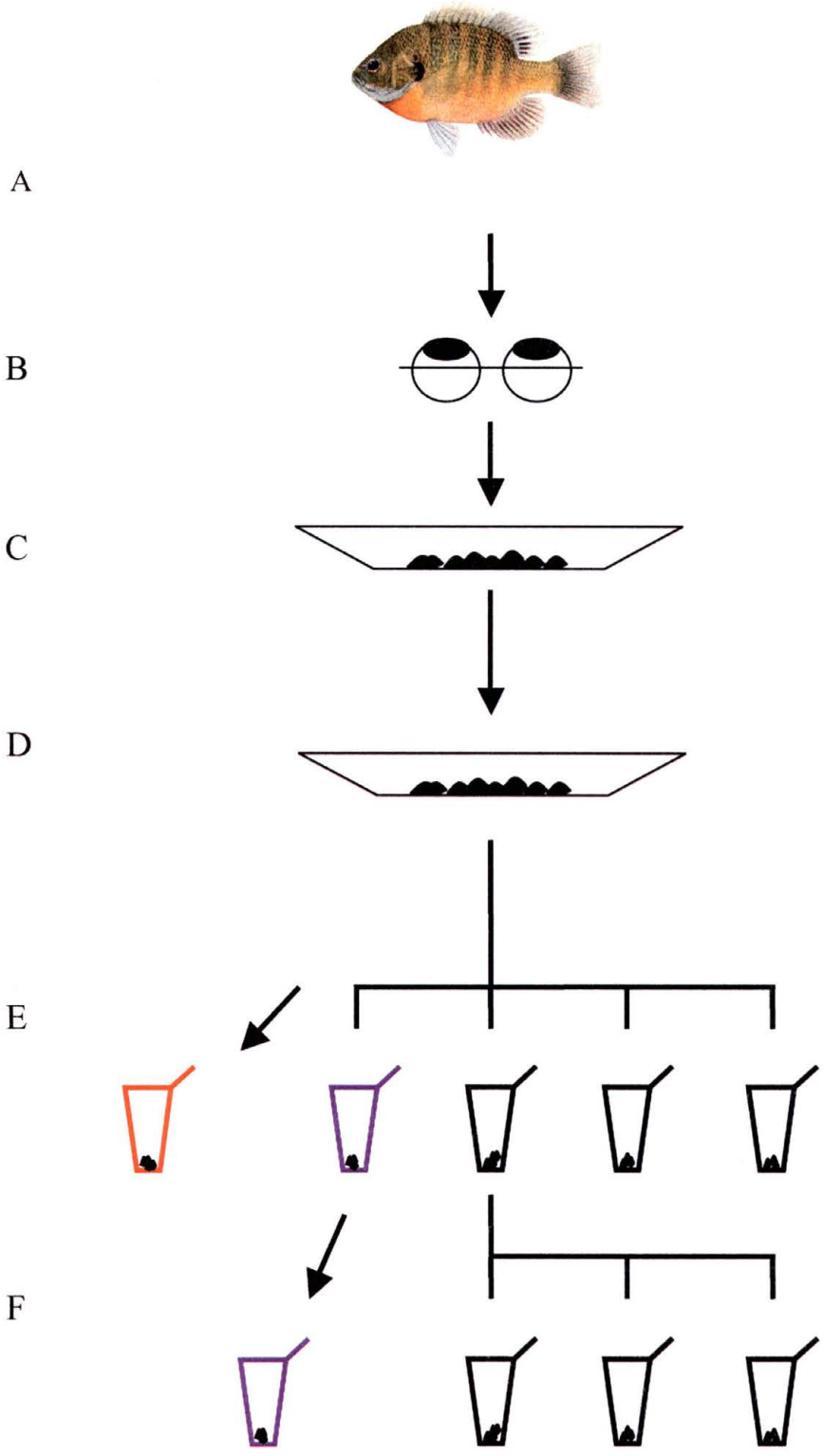
The first drugs tested were telenzepine, methoctramine, and p-FHHSiD; M_1, M_2, and M_3 antagonists, respectively. I then used U73122, a PLC inhibitor, and 2-APB, an IP_3 receptor antagonist. The drugs used in this study are listed in Table 2 along with their suppliers. Drugs were added to incubation tubes from 10X stock solutions just prior to addition of carbachol.

Table 2. Pharmacological agents used to elucidate the muscarinic receptor subtype and second messenger pathway involved in carbachol-induced pigment granule dispersion.

Drug	Target/Effect	Concentration Range Tested	Supplier
Telenzepine dihydrochloride	M_1 antagonist[1]	10 μM – 1 pM	Tocris, Ellisville, MO
Methoctramine tetrahydrochloride	M_2 antagonist[2]	10 μM – 100 pM	Sigma-Aldrich, St. Louis, MO
p-Fluorohexahydro-sila-difenidol (p-FHHSiD)	M_3 antagonist[3]	10 μM – 1 pM	Sigma-Aldrich, St. Louis, MO
U-73122	Phospholipase C inhibitor[4]	10 μM – 10 nM	Sigma-Aldrich, St. Louis, MO
2-Aminoethoxydiphenyl borate (2-APB)	IP_3-receptor antagonist[5]	10 μM – 100 pM	Tocris, Ellisville, MO

[1] Araujo dos Santos *et al.*, 2003.
[2] Mansfield *et al.*, 2003.
[3] Jankovic *et al.*, 2004.
[4] Mogami *et al.*, 1997.
[5] Ma *et al.*, 2003.

Fig. 4. Diagram of the experimental layout. The following illustration is a diagram of the experimental layout used throughout the experimentation. (A) Bluegill fish were dark adapted for 30-60 minutes, 6 hours into their light cycle. (B) The eyes were removed and hemisected under dim light (< 2 lux). (C) RPE was removed into a weigh boat using Ringer's buffer. (D) RPE was treated with 10 μM forskolin for 45 minutes. (E) RPE was then washed with Ringer's buffer and divided equally into five 1.7 ml microcentrifuge tubes. The first tube of each treatment was then fixed (which served as an aggregated control) (RED). The four remaining tubes were treated with 100 nM carbachol, and in the last three tubes, an antagonist or inhibitor, for 45 minutes, and were then fixed. (F) The first tube of this grouping, or the second tube overall, served as a dispersed control (PURPLE).


A
B
C
D
E
F

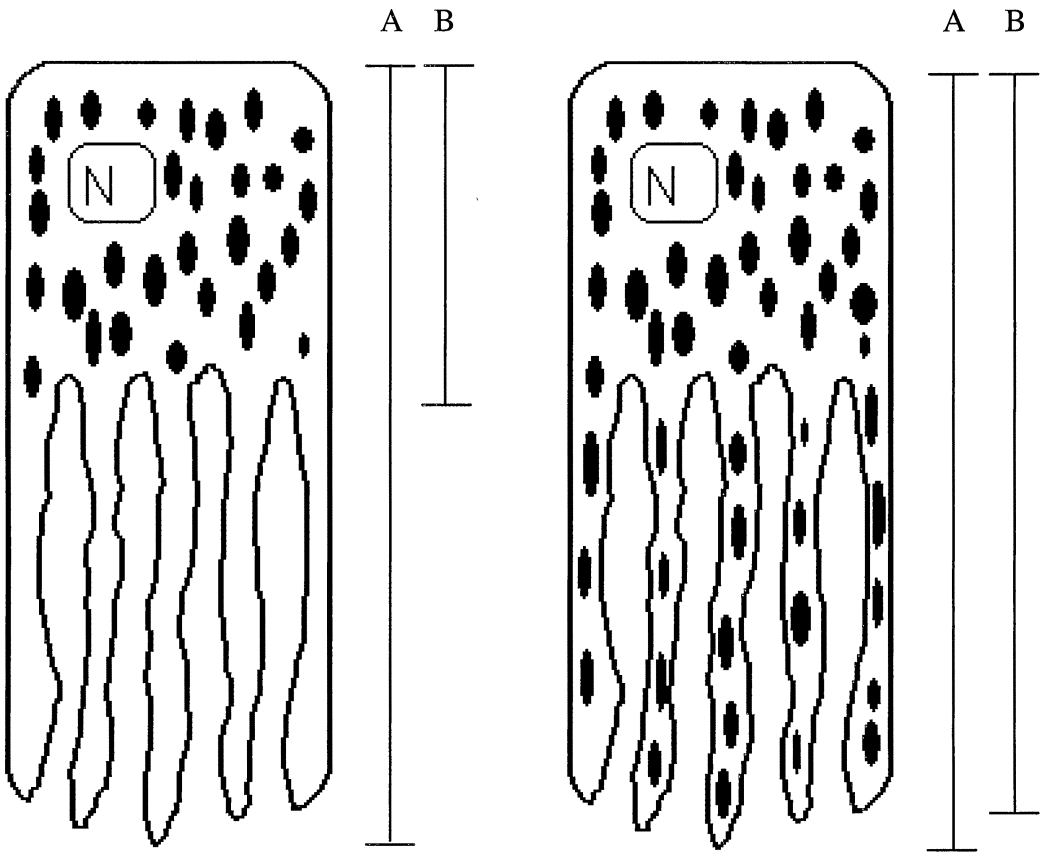
Tissue Fixation

Tissue was fixed by ımmersion ın 0.5% glutaraldehyde, 0.5% paraformaldehyde, and 0.8% potassium ferricyanıde in PBS buffer for a minimum of 12 hours prior to measuring. The tissue, once in the fixative, was stored at 4°C.

Measuring Pigment Indices

Sheets or pieces of RPE were placed on a microscope slide and chopped with a 22 mm^2, No. 1 coverslip. RPE cells were then mounted on the microscope slide and viewed using a Zeiss microscope equipped with phase contrast optics. Images were obtained using an Olympus BH2-RFCA phase contrast microscope. Pigment index (PI) was recorded for cells that met the following criteria: (1) the cell was at least 30 micrometers in length (as measured using an ocular micrometer), (2) the cell had a phase-bright base, and (3) the cell had at least 3 intact apical processes. The PI is a ratio of the length from the phase-bright basal membrane to the most dispersed pigment to the total length of the cell (phase-bright basal membrane to the tip of the apical process) (Figure 5). Thirty cells were measured from each fish for each treatment. N = number of fish.

Fig. 5. Diagram illustrating how PI was measured. The two cells shown are the same cells shown in Figure 2. After the criteria described in the Methods are met, the initial measure (A) is taken. This measurement is the total length of the cell, from the phase-bright basal membrane to the tip of the longest process. The second measurement (B) is from the phase-bright membrane to the most dispersed pigment granule. The first measurement is divided by the second measurement to yield the pigment index (PI = B/A).


A
B
N
A
B
N

Statistics and Data Analysis

Dose response curves were created in order to determine the effects of the pharmacological agents on pigment granule position, using the mean pigment index. Error bars represent ± two standard errors of mean (2*SEM). To evaluate differences among means for individual pharmacological treatments and carbachol and forskolin, single factor analysis of variance (ANOVA) was used. For comparison between two individual means, a Student's T-test was performed. Following an ANOVA, Tukey's HSD post hoc analyses were performed in order to determine if significant differences occurred among experimental treatments. A P value of < 0.05 was assumed to indicate significant difference. All of the statistical analysis was performed using S-PLUS 6.1 for Windows (2002).

To determine the efficacy of the M_1 and M_3 antagonists, values for pIC_{50} were estimated using Microsoft Excel along with XLfit4 (www.idbs.com/xlfit4/). A Boltzmann sigmoidal curve was fitted to each data set. 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}.

RESULTS

Treatment of RPE with forskolin resulted in pigment indices ranging from 0.64-0.74 (0.69 ± 0.01) while carbachol treatments resulted in pigment indices between 0.85-0.92 (0.88 ± 0.01). Phase contrast images were obtained illustrating aggregation and dispersion of pigment (Figure 6).

Telenzepine, an M_1 muscarinic receptor antagonist, blocked carbachol-induced pigment granule dispersion (Figure 7). The mean PI of forskolin treated cells (PI = 0.69 ± 0.01; n = 4) was significantly different ($p < 0.001$) from the PI of carbachol-treated cells (PI = 0.89 ± 0.01; n = 4). RPE treated with telenzepine at concentrations as low as 1 nM were significantly ($p < 0.001$) less dispersed than carbachol-treated controls, the former having a mean PI = 0.82 ± 0.02; n = 3. At lower concentrations, there were no significant differences between telenzepine-treated cells and carbachol-treated controls ($p = 0.1034$). The maximal effect of telenzepine was seen at concentrations of 100 nM and higher. An estimated pIC_{50} value for telenzepine was approximately 8.5 (Figure 8).

Methoctramine, a muscarinic receptor antagonist which is selective for M_2 was unable to block carbachol-induced pigment granule dispersion at all the concentrations examined, 100 pM to 10 μM (Figure 9). Cells treated with carbachol alone (PI = 0.90 ± 0.02; n = 3) were significantly more dispersed ($p < 0.001$) than forskolin treated cells (PI = 0.67 ± 0.03).

RPE treated with p-FHHSiD, an antagonist selective for M_3 muscarinic receptors, at concentrations as low as 10 nM were significantly ($p < 0.001$) less dispersed than carbachol-treated controls, the former having a mean PI = 0.79 ± 0.01; n = 3 (Figure 10).

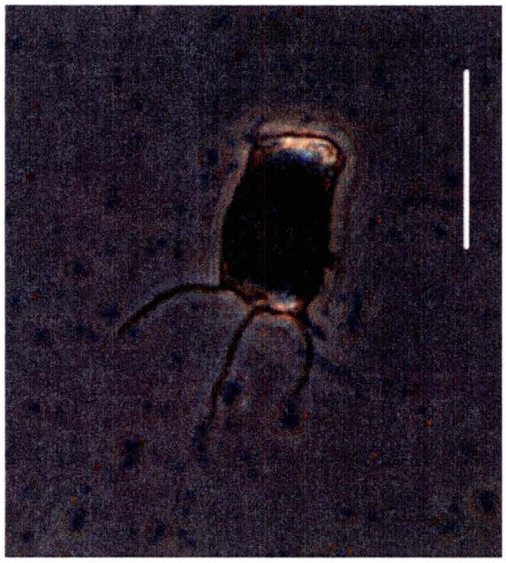
At lower concentrations, there were no significant differences between p-FHHSiD-treated cells and carbachol-treated controls ($p = 0.2076$). Since p-FHHSiD was dissolved in 10% ethanol, a vehicle control was run with 10% EtOH and carbachol (PI = 0.86 ± 0.02; n = 3). PI results were not significantly different from the carbachol-treated controls ($p = 1.0$). Maximal effect of p-FHHSiD was seen at concentrations of 1 μM and higher. The pIC_{50} was estimated to be 7.2 (Figure 11).

Since M_{odd} receptors appeared to be involved in pigment granule dispersion, U73122, a PLC inhibitor, was used to test whether carbachol activated PLC. The mean PI for forskolin treated cells (PI = 0.70 ± 0.04; n = 3) was significantly different ($p < 0.001$) from the PI for carbachol-treated cells (PI = 0.89 ± 0.03; n = 3). RPE 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 = 0.80 ± 0.05; n = 3 (Figure 12). At a concentration as low as 10 nM, there were no significant differences between U73122-treated cells and carbachol-treated controls ($p = 0.05$).

The observation that the PLC inhibitor blocked carbachol-induced dispersion suggested that PLC activity is involved in mediating carbachol-induced dispersion. Since PLC may result in the release of intracellular Ca^{2+} via IP_3-receptor activation, I tested whether the IP_3-receptor antagonist, 2-APB, could block carbachol-induce dispersion (Figure 13). Forskolin treated cells (PI = 0.72 ± 0.01; n = 4) were significantly different from carbachol treated cells (PI = 0.87 ± 0.02; n = 3). 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. When the effects of 100 pM 2-APB was examined (PI = 0.88 ± 0.02; n=3), no significant difference with carbachol was seen (p = 0.80).

Fig. 6. Phase contrast micrographs of aggregated (top) and dispersed (bottom) pigment granules. Images were taken using an Olympus BH2-RFCA at 400X. The scale bar represents approximately 30 micrometers. The images were taken at 400x.



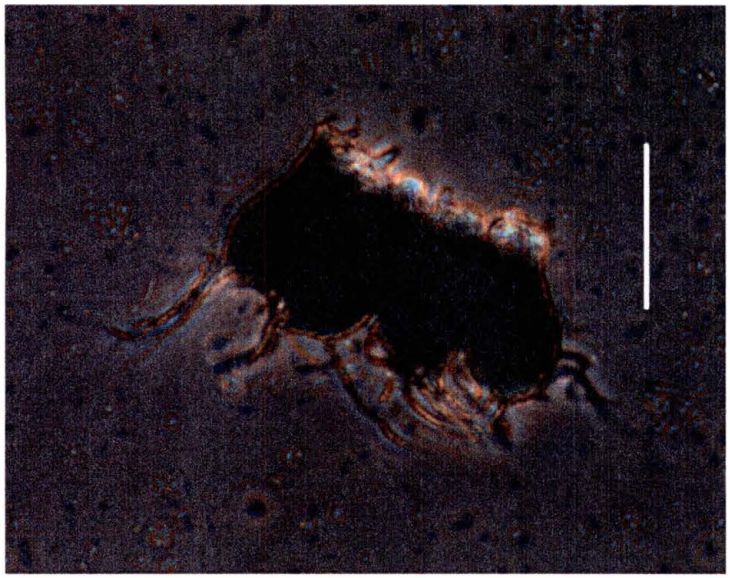
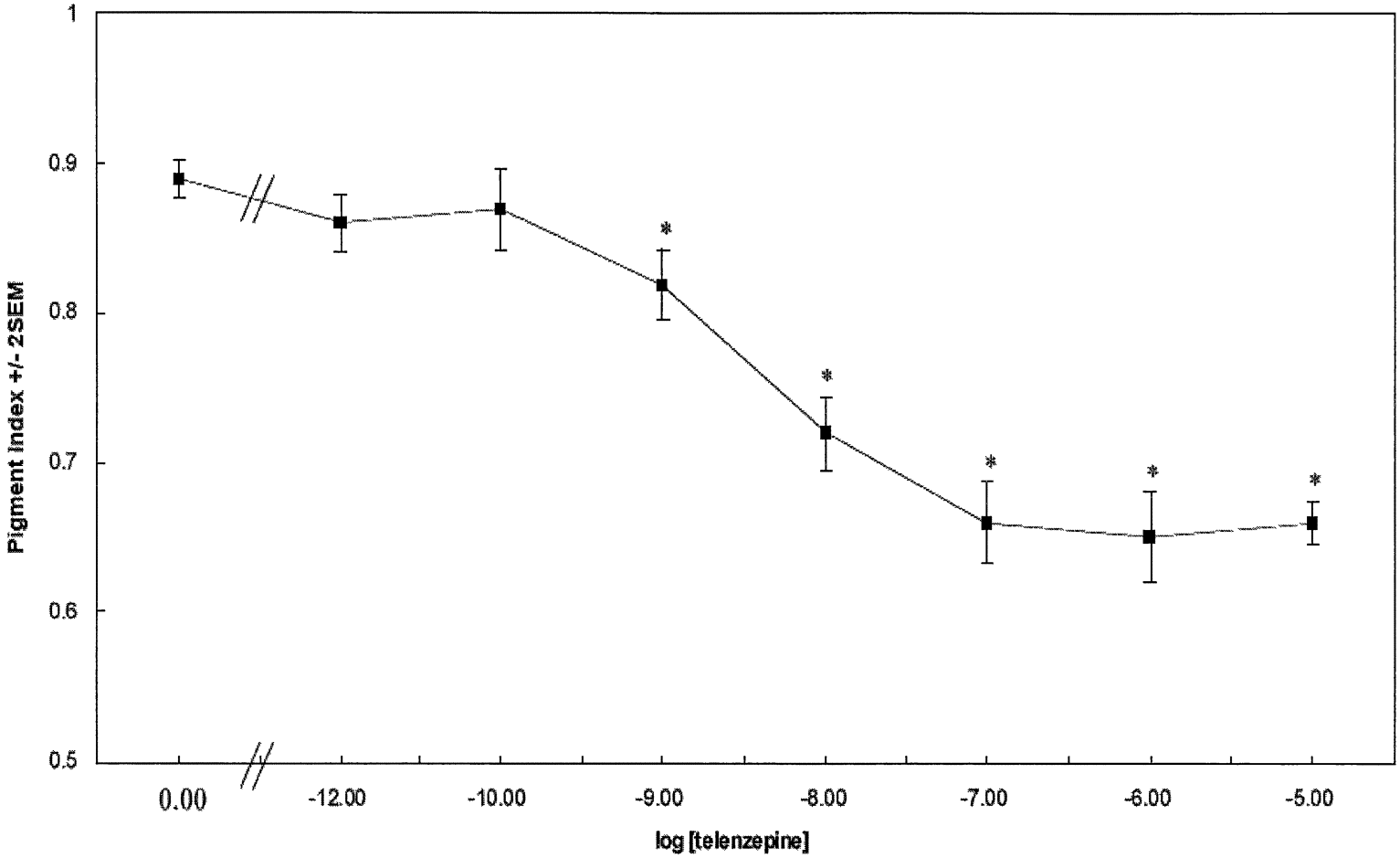


Fig. 7. Telenzepine blocked carbachol-induce pigment granule dispersion. RPE cells were isolated from bluegill and treated with 10 μM forskolin to induce pigment granule aggregation. Following pigment aggregation, 0.1 μM carbachol was tested along with telenzepine, an M_1 antagonist. All samples had an $n = 3$ with the exception of 0.1 μM telenzepine ($n = 4$). Telenzepine, at concentrations as low as 1 nM, showed significant difference from 0.1 μM carbachol. Hatch-marks on the graph and on the X-axis indicate discontinuity in the scale. Asterisks indicate treatments that yielded PIs significantly different from treatments with carbachol ($p < 0.001$).


1
0.9
0.8
0.7
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0.5
Pigment Index +/- 2SEM
0.00
-12.00
-10.00
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-8.00
-7.00
-6.00
-5.00
log [telenzepine]
*
*
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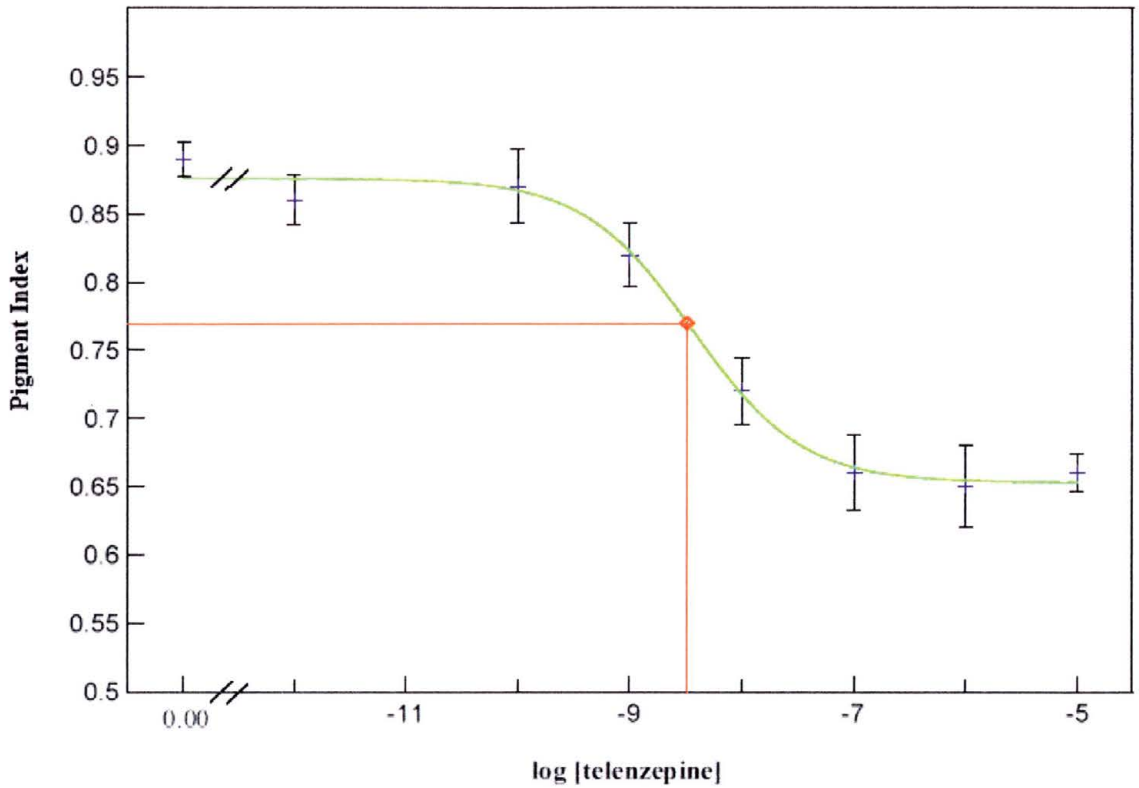
*
*

Fig. 8. The pIC_{50} value was obtained for telenzepine using the data from the previous figure. In order to calculate the pIC_{50}, a Boltzmann sigmoidal curve was fitted to each data set. The highest and lowest Y-value obtained from the fitted curve was used to calculate the midpoint. Hatch-marks on the graph and on the X-axis indicate discontinuity in the scale. The pIC_{50} value estimated was 8.5.


Pigment Index
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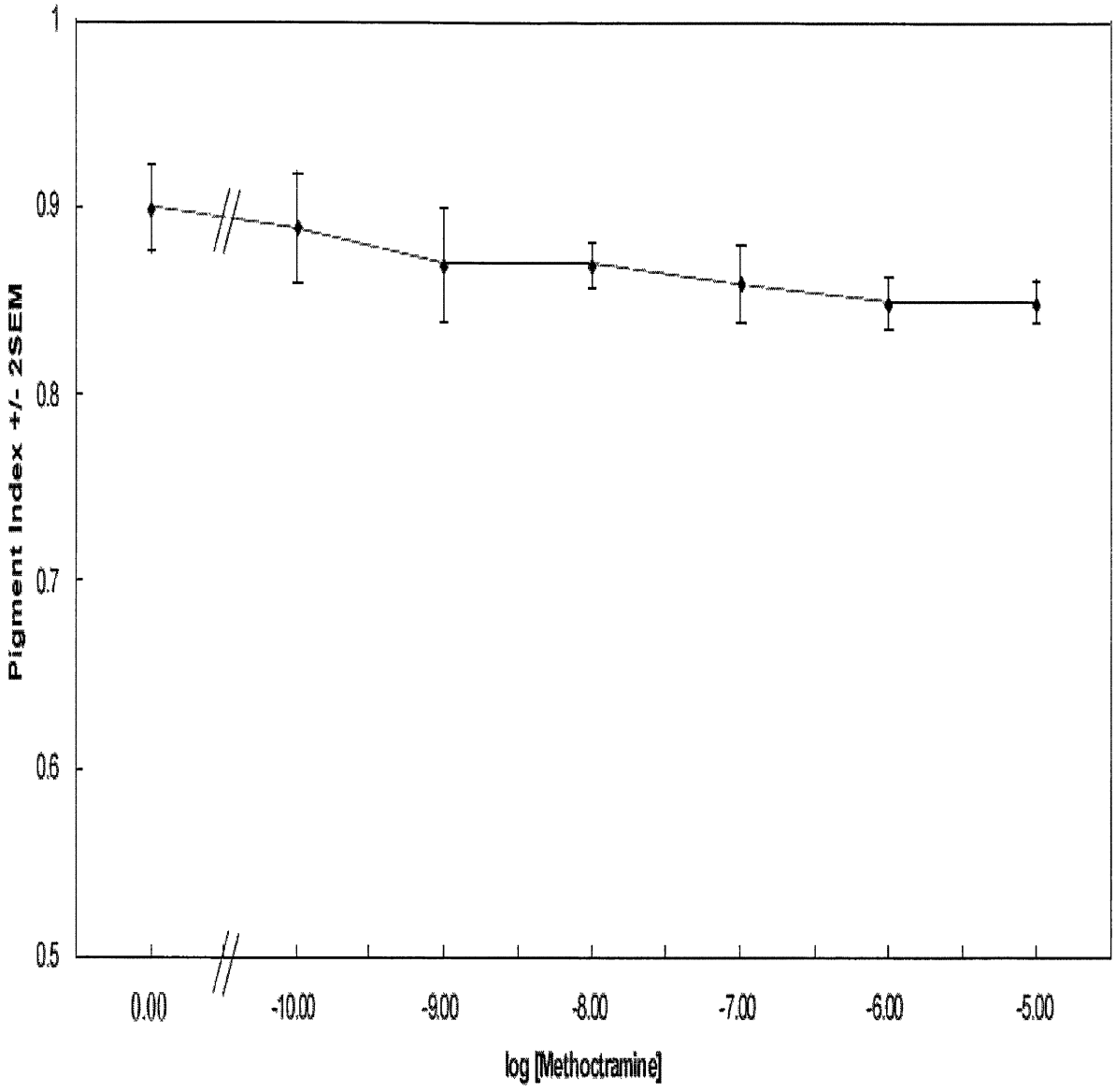
-9
-7
-5
log [telenzepine]

Fig. 9. Methoctramine unable to block carbachol-induced pigment granule dispersion. RPE cells were isolated from bluegill and treated with 10 μM forskolin to induce pigment granule aggregation. Following pigment aggregation, 0.1 μM carbachol was tested along with methoctramine, an M_2 antagonist. All samples had an n of 3. Methoctramine-treated cells did not show a significant difference from 0.1 μM carbachol-treated cells at any of the concentrations tested. Hatch-marks on the graph and on the X-axis indicate discontinuity in the scale.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Pigment Index +/- 2SEM
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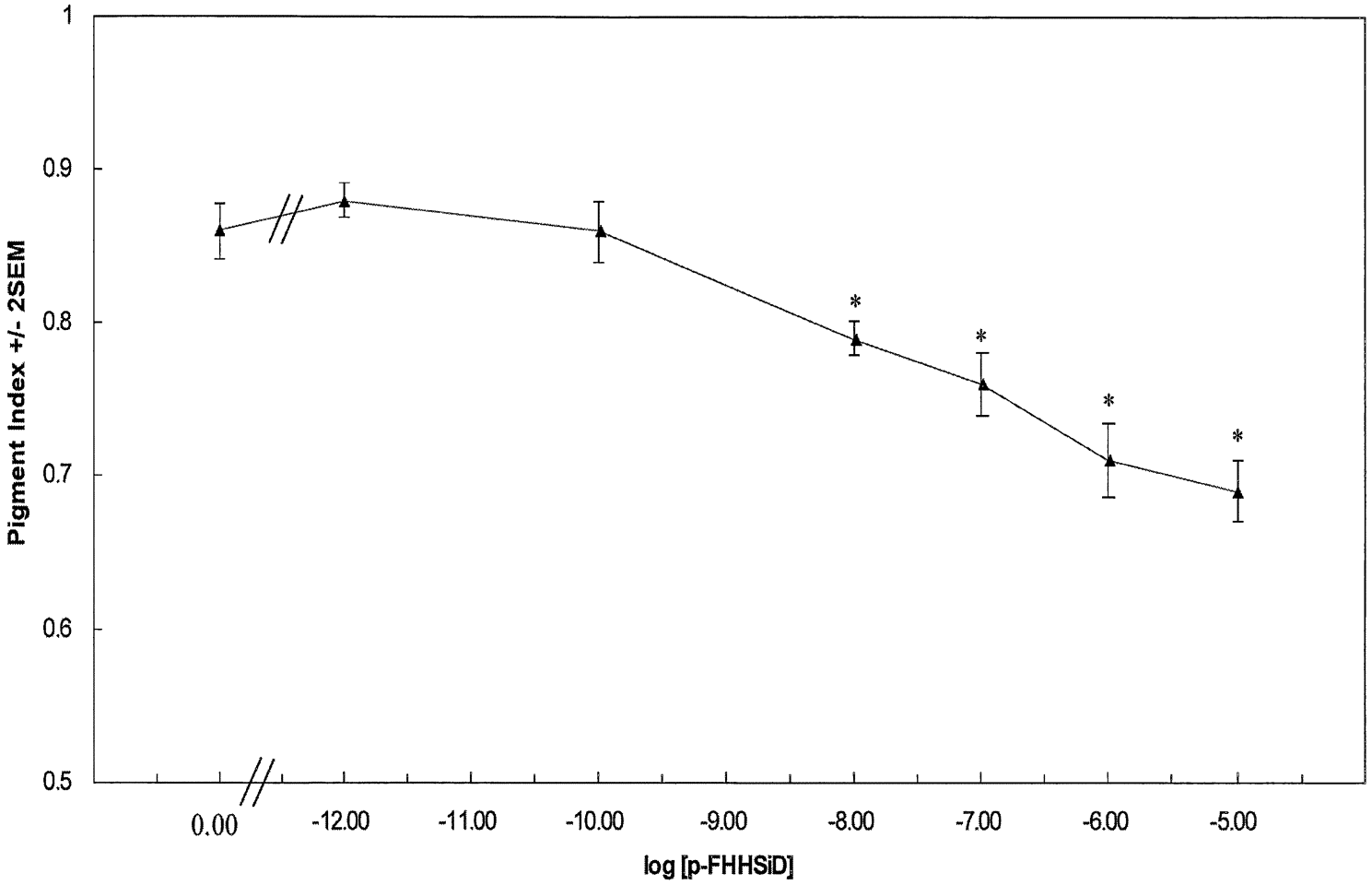
-7.00
-6.00
-5.00
log [Methoctramine]

Fig. 10. p-FHHSiD blocked carbachol-induce pigment granule dispersion. RPE cells were isolated from bluegill and treated with 10 μM forskolin to induce pigment granule aggregation. Following pigment aggregation, 0.1 μM carbachol was tested along with p-FHHSiD, an M_3 antagonist. All samples had an n of 3. p-FHHSiD treated cells showed significant difference from 0.1 μM carbachol-treated cells at concentrations as low as 10 nM. Hatch-marks on the graph and on the X-axis indicate discontinuity in the scale. Asterisks indicate treatments that yielded PIs significantly different from treatments with carbachol ($p < 0.001$).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0.9
0.8
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Pigment Index +/- 2SEM
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-11.00
-10.00
-9.00
-8.00
-7.00
-6.00
-5.00
log [p-FHHSiD]
*
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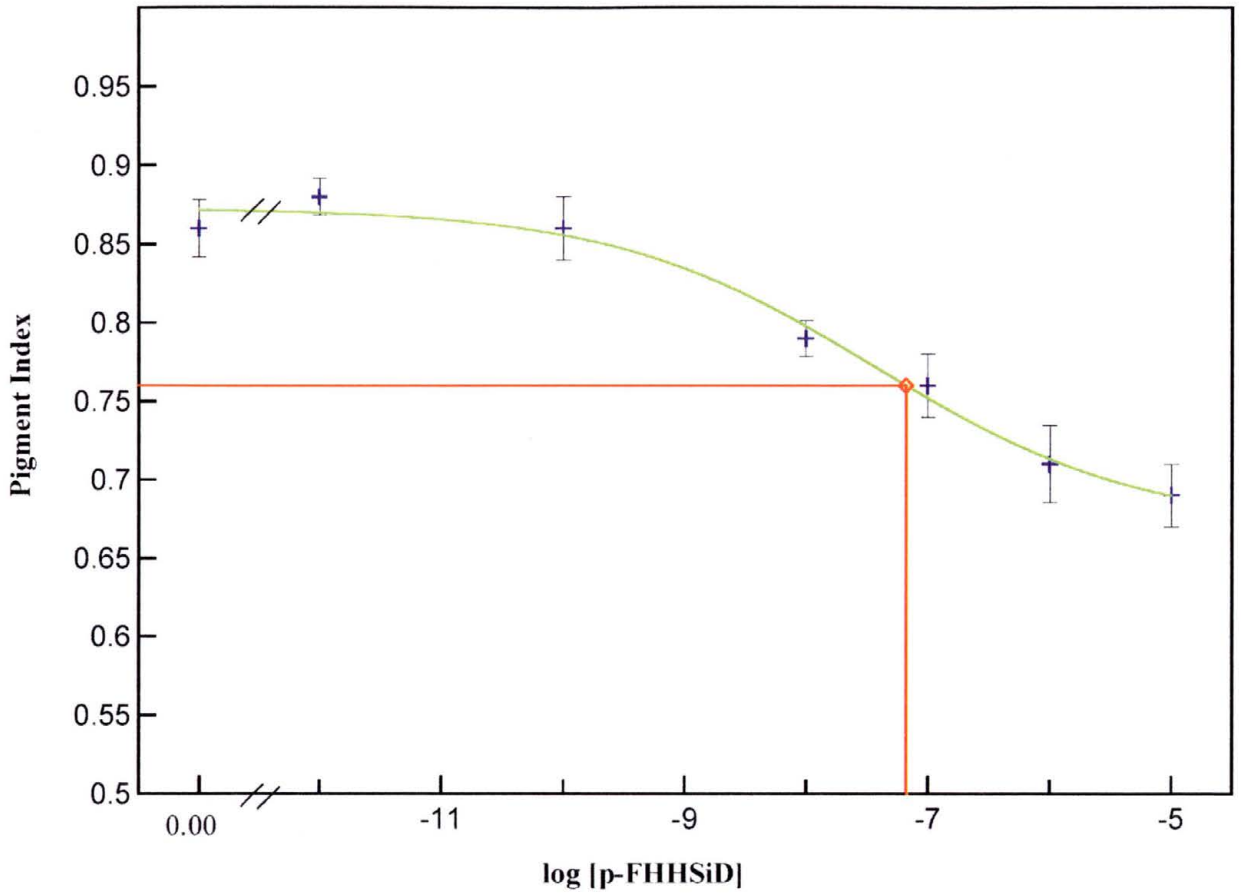
Fig. 11. The pIC_{50} value was obtained for p-FHHSiD using the data from the previous figure. In order to calculate the pIC_{50}, a Boltzmann sigmoidal curve was fitted to the data set. The highest and lowest Y-value obtained from the fitted curve were used to calculate the midpoint. Hatch-marks on the graph and on the X-axis indicate discontinuity in the scale. The pIC_{50} value obtained was 7.2.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0.9
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0.8
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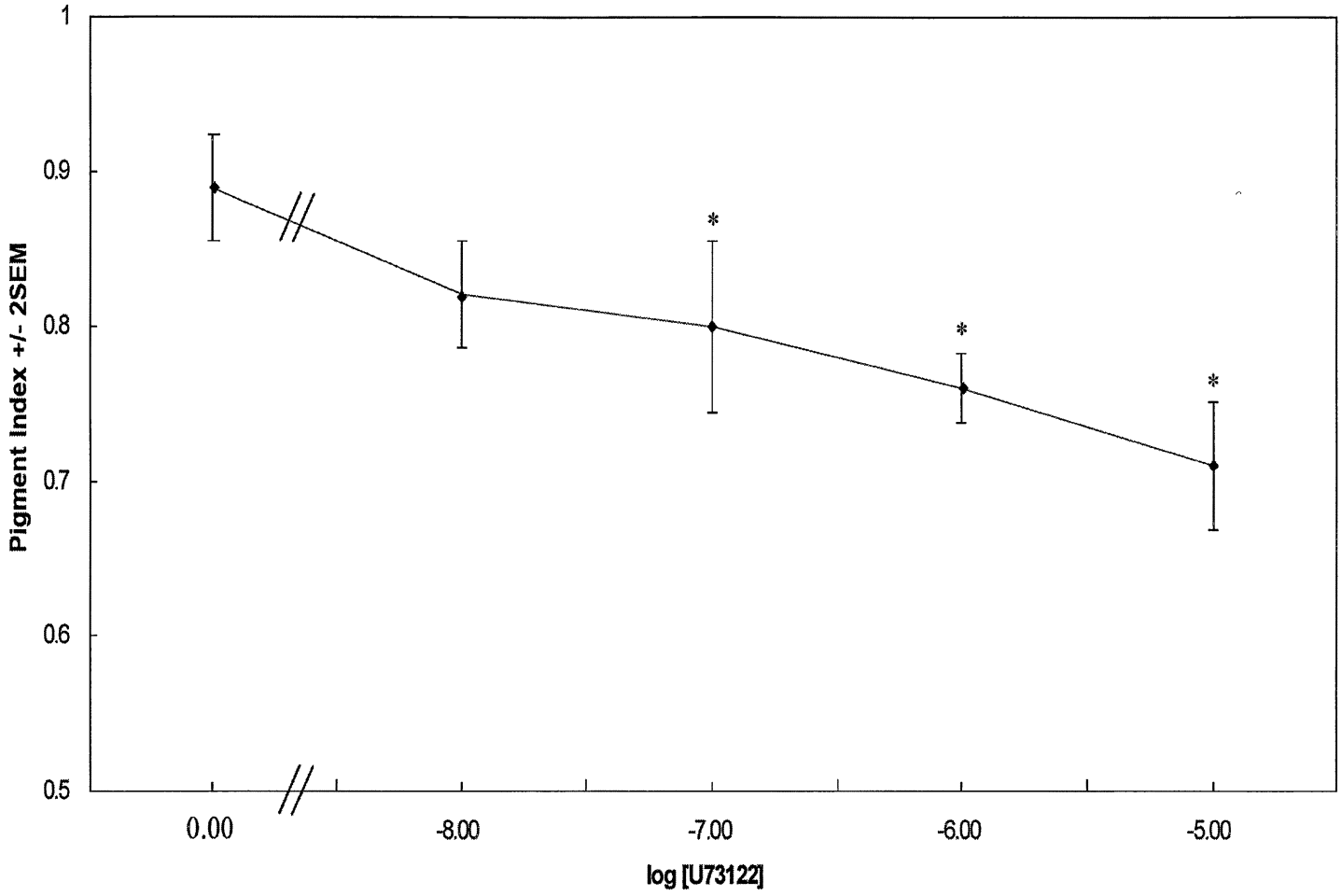
-11
-9
-7
-5
log [p-FHHSiD]

Fig. 12. PLC inhibition blocked carbachol-induced pigment granule dispersion. RPE cells were isolated from bluegill and treated with 10 μM forskolin to induce pigment granule aggregation. Following pigment aggregation, 0.1 μM carbachol was tested along with U73122, a PLC inhibitor. All samples had an n of 3. U73122 showed a significant difference from 0.1 μM carbachol at concentrations as low as 100 nM. Hatch-marks on the graph and on the X-axis indicate discontinuity in the scale. Asterisks indicate treatments that yielded PIs significantly different from treatments with carbachol ($p < 0.001$).


1
0.9
0.8
0.7
0.6
0.5
Pigment Index +/- 2SEM
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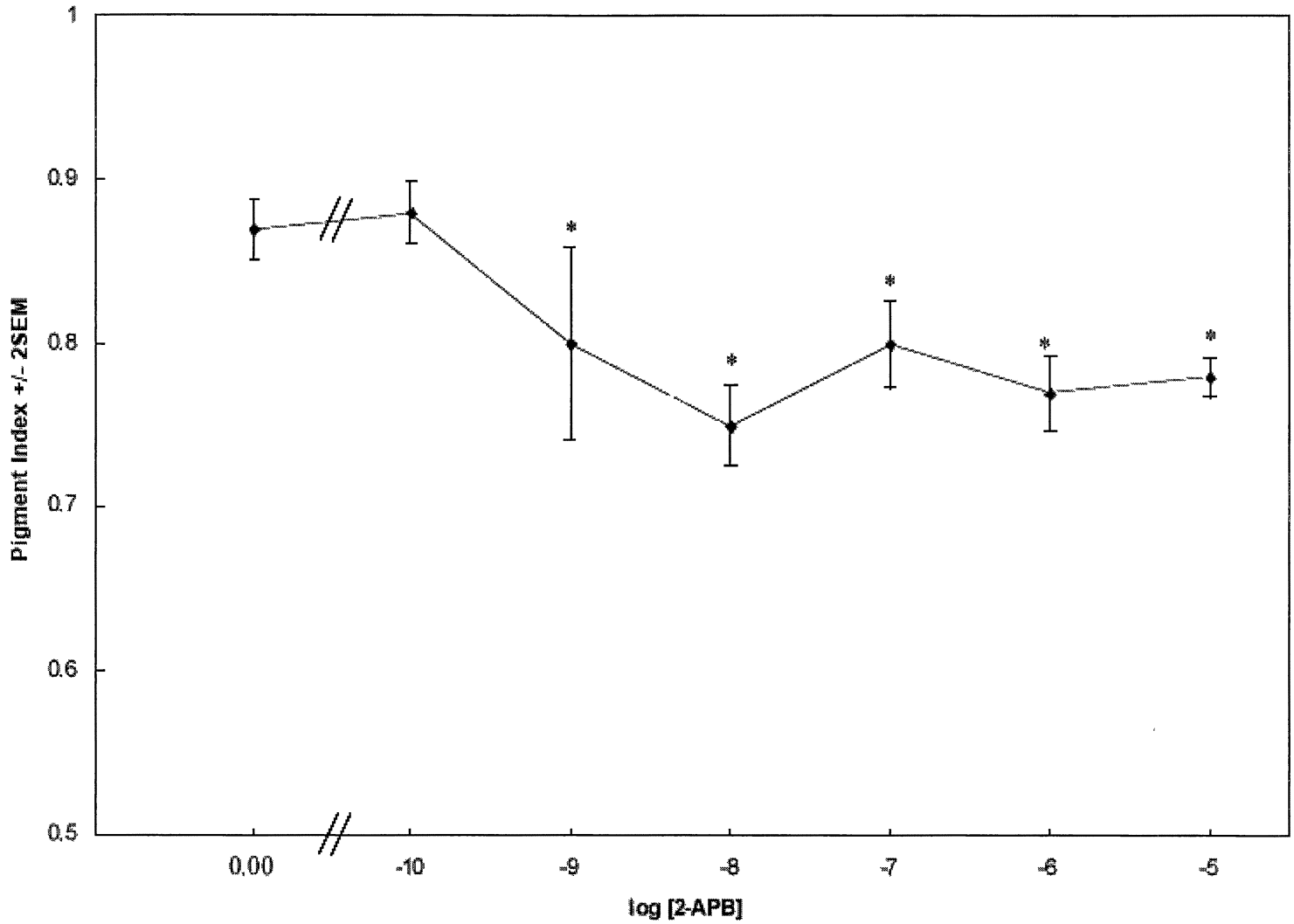
*
*
0.00
-8.00
-7.00
-6.00
-5.00
log [U73122]

Fig. 13. 2-APB blocked carbachol-induce pigment granule dispersion. RPE cells were isolated from bluegill and treated with 10 μM forskolin to induce pigment granule aggregation. Following pigment aggregation, 0.1 μM carbachol was tested along with 2-APB, an IP_3-receptor antagonist. All samples had an n of 3 with the exception of forskolin and 100 nM 2-APB in which n = 4. 2-APB showed a significant difference from 0.1 μM carbachol at concentrations as low as 1 nM. Hatch-marks on the graph and on the X-axis indicate discontinuity in the scale. Asterisks indicate treatments that yielded PIs significantly different from treatments with carbachol ($p < 0.001$).


1
0.9
0.8
0.7
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0.5
Pigment Index +/- 2SEM
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log [2-APB]
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DISCUSSION

My objective was to use pharmacological agents to uncover the signaling pathway associated with carbachol-induced pigment granule dispersion. My results are consistent with those of González *et al.* (2004) and support the conclusion that M_{odd} receptors seem to be involved in pigment granule dispersion. Furthermore, I have shown that when a phospholipase C inhibitor was applied, it blocked pigment granule dispersion at concentrations as low as 100 nM. Since the hydrolysis of PIP_2 by PLC leads to the release of Ca^{2+} stored in the endoplasmic reticulum via IP_3, an IP_3-receptor antagonist was tested; it too blocked pigment granule dispersion at concentrations as low as 1 nM.

Pharmacological Support of Previous Findings

González *et al.* (2004) showed that carbachol-induced pigment granule dispersion was blocked by an M_1 antagonist, pirenzepine, and by an M_3 antagonist, 4-DAMP. Carbachol-induced pigment granule dispersion was not blocked with AF-DX 116 and tropicamide, M_2 and M_4 antagonists, respectively. When I tested the effects of telenzepine and p-FHHSiD, M_1 and M_3 antagonists, respectively, my results demonstrated the ability of two more M_{odd} antagonists to block pigment granule dispersion. Methoctramine, M_2 antagonist, did not block pigment granule dispersion. Receptor antagonists show varying degrees of selectivity, and nonselective inhibition among subtypes can occur. I estimated pIC_{50} values from data obtained by Mr. González and from my research to try to elucidate the relative binding affinities of the drugs and

the pattern seen. The IC_{50} value is the concentration at which 50% inhibition is observed (Wyska *et al.*, 2003) and is presumed to reflect the binding affinity of the drug for the receptor. From Mr. González's data, I estimated the pIC_{50} of pirenzepine to be 9.6, while 4-DAMP had a pIC_{50} of approximately 10.3. From my results, I estimated a pIC_{50} for telenzepine of 8.5 (Figure 8) and p-FHHSiD of 7.2 (Figure 11). Based on these estimates, the rank order of antagonist efficacy was 4-DAMP > telenzepine > pirenzepine > p-FHHSiD. Eglen *et al.* (2001) describes the affinity constants derived from radioligand binding studies on human recombinant receptors. From their work, it is seen that the pattern of 4-DAMP > pirenzepine > p-FHHSiD is consistent with M_1 and M_5 binding (Eglen *et al.*, 2001). According to Eglen *et al.* (2001), the rank order for M_3 is 4-DAMP > p-FHHSiD > pirenzepine. Hsieh and Liao (2002) reported that the profile muscarinic antagonist binding in zebrafish corresponded with human, with the exception of pirenzepine. This could be due to the threonine present at the ligand-binding site. Chicken M_2 also has a much higher affinity for pirenzine than human M_2, the threonine again is present in the ligand-bind site for chicken (Hsieh and Liao, 2002). I cannot discriminate with these data whether M_1 or M_5 is responsible for pigment granule, but my results are consistent with M_{odd} activation, and favor M_1 and M_5 over M_3.

Phatarpekar *et al.* (2004) isolated and sequenced bluegill M_5 and showed that this particular subtype was expressed in RPE and retina. Dr. Simon Durdan (unpublished results) showed that M_2 was expressed in retina but not RPE. The presence of M_1 and M_3 in RPE has not been ruled out.

Ability of a PLC Inhibitor to Block Pigment Granule Dispersion

With evidence supporting M_{odd} participation in pigment granule dispersion, I tested U73122, a PLC inhibitor, since M_{odd} receptors are known to couple through G_{11} to PLC. My results further supported the idea of pigment granule dispersion being mediated by M_{odd} receptors since U73122 blocked carbachol-induced dispersion. Examples of some of the possible effects of the inhibition of DAG and IP_3 could be the inhibition of the release of Ca^{2+}, with consequent failure to activate PKC and CaM and CaM-kinases (Farago and Nishizuka, 1990; Blumberg, 1991; Nelsestuen and Bazzi, 1991; Chin and Means, 2000; Broadley and Kelly, 2001) (Figure 14).

Possible Role of Ca^{2+} in Pigment Granule Dispersion

To further examine the second messenger pathway, 2-APB was used to antagonize the IP_3-receptor located on the endoplasmic reticulum. King-Smith *et al.* (1996), as described earlier, conducted a study where the effects of the depletion of intra- and extracellular Ca^{2+} from RPE were examined, and they concluded that the depletion had no effect on pigment movement in either direction *in vitro*. In part, they showed that increases in $[cAMP]_i$ was sufficient to induce aggregation and that the external and internal concentrations of Ca^{2+} were irrelevant. To address the possibility of the involvement of $[Ca^{2+}]_o$ in pigment dispersion, RPE sheets were treated with cAMP to cause aggregation. Cyclic AMP was washed out to cause dispersion with either control Ringer (2.4 mM free Ca^{2+}), Ca^{2+}-free Ringer containing 1 mM EGTA (EGTA Ringer, < 1 nM free Ca^{2+}), or Ringer containing 1.8 mM Ca^{2+} and 1 mM EGTA for a free calcium concentration of about 10^{-5}M. In all three cases, dispersion was seen in RPE. To address the possible involvement of $[Ca^{2+}]_o$ in pigment aggregation, RPE sheets with dispersed

pigment granules were placed in one of three Ringer's solutions (see above). Again, when cAMP was applied, aggregation was achieved with the RPE sheets in all three solutions. These data demonstrated that $[Ca^{2+}]_o$ is not required pigment movement in either direction (King-Smith *et al.*, 1996). Since required Ca^{2+} fluxes could have been derived from intracellular stores, King-Smith *et al.* (1996) examined the effects of $[Ca^{2+}]_i$ on pigment dispersion. RPE was treated with cAMP to cause aggregation, and then the cells were loaded with BAPTA, a Ca^{2+} chelator. Intracellular calcium did not prove to be necessary for pigment movement. In addition, the application of cAMP or washout did not lead to an increase in $[Ca^{2+}]_i$. This observation was achieved by using fura-2 (Ca^{2+} indicators). Using fura-pe3, localized Ca^{2+} signaling was examined. RPE cells, previously aggregated, were subjected to pigment dispersion by washout of cAMP in medium containing dopamine or RPE cells were subjected to cAMP to induce aggregation. By loading the cells with fura-pe3, fluorescence studies revealed that localized changes in $[Ca^{2+}]_i$ is not associated with pigment granule dispersion or aggregation. Although King-Smith *et al.* (1996) demonstrated that Ca^{2+} changes were neither essential nor sufficient for pigment movement, they mention that their results based on *in vitro* studies may skip Ca^{2+}-requiring steps involved *in vivo* in the endogenous cAMP regulatory pathway.

Thaler and Haimo (1990) showed that the activation of calcineurin, a CaM-activated phosphatase, causes pigment aggregation in scales isolated from *Tilapia mossambica*. Furthermore, they showed that permeabilized cell models aggregated pigment upon addition of calcineurin, where otherwise the cell would remain dispersed. In this study, an antibody specific for calcineurin was applied to the lysed cells, and it

inhibited pigment aggregation. Sammak *et al.* (1992) showed that in scales isolated from *Pterophyllum scalar*, angelfish, Ca^{2+} rose during, but was not required for, pigment movement in melanophores induced by epinephrine. Overall, the requirements for Ca^{2+} to accomplish pigment movement seem to vary among systems.

Proposed Pathway by Which Pigment Granule Movement Occurs in RPE

The ability of M_{odd} antagonists to block carbachol-induced pigment granule dispersion led me to investigate a pathway through which pigment movement in bluegill RPE could occur. I shall first discuss pigment granule aggregation in fish RPE. Pigment granule aggregation is induced by forskolin (Bruenner and Burnside, 1986; García and Burnside, 1994). Forskolin activates adenylyl cyclase (Seamon *et al.*, 1981). There are nine known isoforms of adenylyl cyclase in mammals (AC1-AC9), all of which are activated by forskolin except for AC9 (Hanoune and Defer, 2001). According to Völkel *et al.* (1996), AC7 was cloned from bovine RPE cDNA. AC7 is characterized as having a widespread tissue distribution and being unaffected by Ca^{2+} (Cooper, 2003).

To recapitulate, the pathway discussed in the introduction pertaining to the stimulation of PLC-β, which hydrolyzes PIP_2 into IP_3 and DAG, is the pathway by which M_{odd} receptors normally function. The second messenger IP_3 binds to receptors located on the intracellular surface of the endoplasmic reticulum, causing the release of Ca^{2+} (Zeng and Wess, 2000; Broadley and Kelly, 2001). Ca^{2+} can alter PKC, which can then be activated by the combination of Ca^{2+} and DAG. PKC can phosphorylate many proteins including phosphodiesterases (PDE), leading to their activation, which decreases cAMP levels (Farago and Nishizuka, 1990; Blumberg, 1991; Nelsestuen and Bazzi, 1991;

Broadley and Kelly, 2001; see González et al., 2004). As mentioned earlier, Ca^{2+} can exert effects through a number of effectors including calcineurin, a protein phosphatase (Klee *et al.*, 1998). Calcineurin can inhibit AC9 (Cooper, 2003). AC9 is unresponsive to forskolin (Hanoune and Defer, 2001). It is unlikely that this specific pathway is relevant to our system, however, since fish RPE clearly express a forskolin-sensitive AC (my results; García and Burnside, 1994). Nevertheless, it would be worthwhile to investigate the type of AC expressed in bluegill RPE. Ca^{2+} also can activate CaM, which activates CaM-kinases (Chin and Means, 2000). CaM also regulates plasma membrane Ca^{2+} pumps and various ion channels (Chin and Means, 2000).

I propose that pigment granule dispersion works through M_{odd} receptors. Forskolin has been shown to activate adenylyl cyclase, which increases cAMP, leading to pigment granule aggregation, presumably by phosphorylating some key protein. When M_{odd} antagonists, a PLC inhibitor, and an IP_3-receptor antagonist were used, all were able to block pigment granule dispersion. If calcineurin activation is blocked, the dephosphorylation of various proteins could be hindered; thus, key phosphoproteins would remain phosphorylated and pigment dispersion would not be observed. Also, if activation of phosphodiesterase by the Ca^{2+}- CaM complexes is blocked, cAMP levels may not decrease as a result. It is important to note that it is unclear which isoform of adenylyl cyclase is present in bluegill RPE. Therefore, whether there is a direct or indirect role of Ca^{2+} in the activation or inhibition of adenylyl cyclase, it appears Ca^{2+} may be involved in pigment granule dispersion.

Future Studies and Conclusion

There is still more that can and should be done to better elucidate whether M_{even} or M_{odd} receptors are involved in pigment granule dispersion induced by carbachol. First and foremost, I would recommend that snake venom, specific for M_5 be used. Miyoshi and Tu (1999) report *Naja naja sputatrix* (Malayan spitting cobra) venom to have selectivity for M_5. Green mamba venom can also be purified in order to isolate a muscarinic receptor inhibitor (see Miyoshi and Tu, 1999). The reason we did not test such a selective inhibitor was due to unavailability of purified venom, and handling a snake in order to obtain such venom was not feasible.

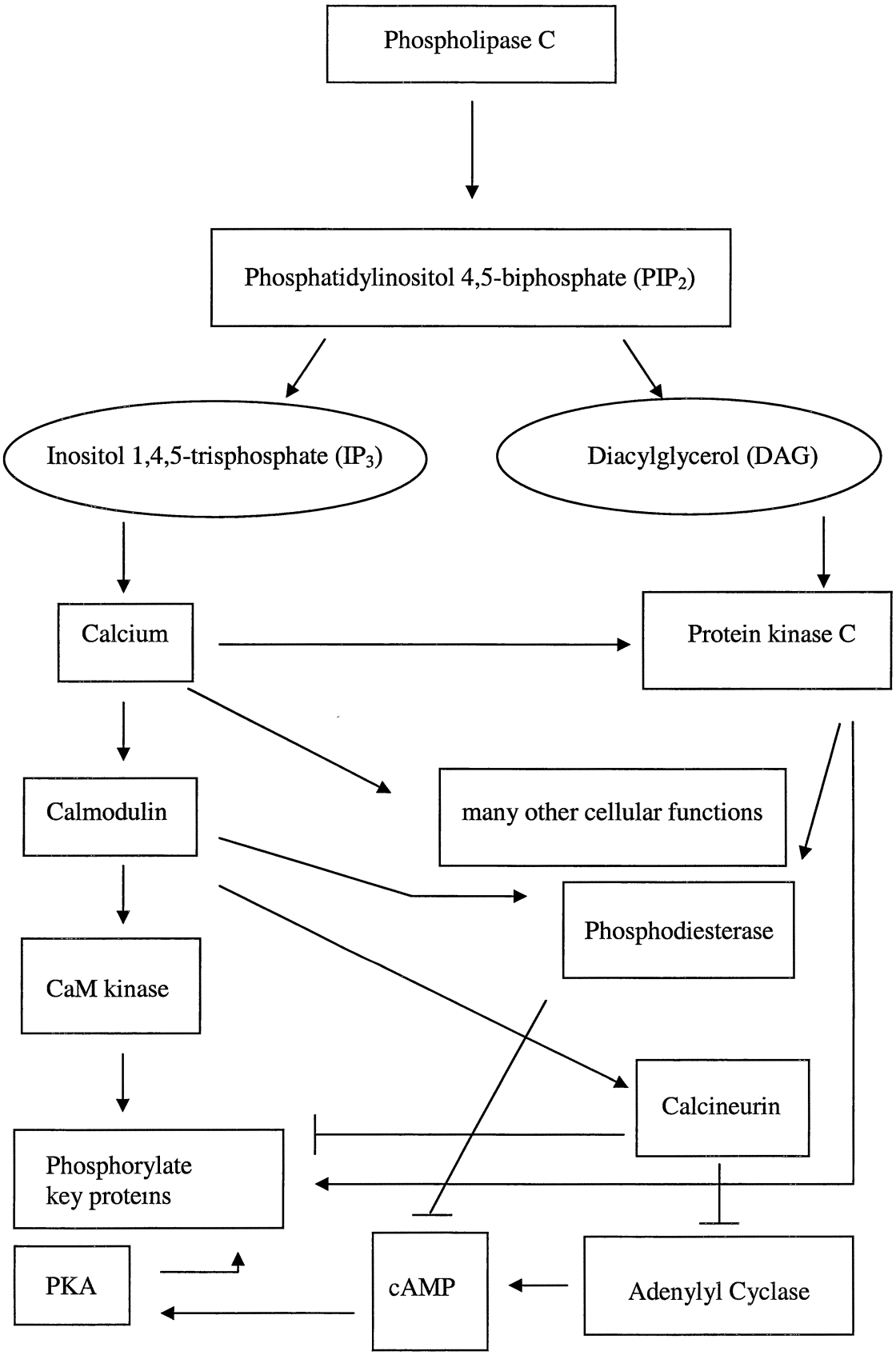
It is important to attempt to characterize the adenylyl cyclase isoform(s) present in bluegill RPE. The importance of characterizing adenylyl cyclase could clarify pigment movement induced by forskolin and whether adenylyl cyclase has any role in pigment dispersion. Along with this study I would use phorbol esters to activate PKC and see if there is any effect. Since PDE can be activated by PKC and CaM, it would be interesting to inhibit PDEs. Furthermore, it would be interesting to use an inhibitor of calcineurin such as cypermethrin (Enan and Matsumura, 1992) or cyclosporin.

It would be interesting to investigate the role of DAG. In order to conclude the involvement of calcium, RHC 80267 (DAG inhibitor) should be used. If inhibiting DAG does not effect pigment granule dispersion induced by carbachol, PKC could possibly be ruled out.

I think it would be interesting to conduct a study performed similar to that of King-Smith *et al.* (1996) using fura-2. RPE cells could be cultured and treated to disperse through the activation of muscarinic receptors, in which fura-2 could indicate a change in calcium.

In conclusion, I have added support to the work performed by González *et al.* (2004) showing that M_{odd} antagonists were able to block carbachol-induced pigment granule dispersion. Methoctramine (M_2 antagonist) was unable to block pigment granule dispersion. Not only did my results concur with these of González *et al.* (2004), but also I helped illuminate the second messenger pathway involved in pigment granule dispersion. Finally, I propose that Ca^{2+} is involved in pigment granule dispersion, furthermore, based on the results from studies with 2-APB, Ca^{2+} release from intracellular stores could be necessary for carbachol-induced pigment granule dispersion.

Fig. 14. Diagram of the M_{odd} pathway, which activates $G_{q/11}$. $G_{q/11}$ activates PLC. For the description of the pathway, see the Introduction.


Phospholipase C
Phosphatidylinositol 4,5-biphosphate (PIP_2)
Inositol 1,4,5-trisphosphate (IP_3)
Diacylglycerol (DAG)
Calcium
Protein kinase C
Calmodulin
many other cellular functions
Phosphodiesterase
CaM kinase
Calcineurin
Phosphorylate key proteins
PKA
cAMP
Adenylyl Cyclase

REFERENCE LIST

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