Ca^{2+} REQUIREMENTS AND BEYOND: SIGNAL TRANSDUCTION IN PIGMENT GRANULE MOTILITY OF RETINAL PIGMENT EPITHELIUM

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ABSTRACT

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Through the study of melanosome movement many general intracellular motility mechanisms have been discovered. Melanosomes are found in both melanophores and retinal pigment epithelial (RPE) cells. Both cell types possess the ability to respond to chemical agents by either trafficking melanosomes toward the nucleus (aggregation) or down the lengths of distal dendrites or processes (dispersion). Despite many similarities in regulation of motility, key differences exist between melanophores and RPE. For example, high levels of cAMP are necessary for melanophore dispersion, whereas increased $[cAMP]_i$ in RPE cells promotes aggregation. Why similar mechanisms drive different functions remains unclear.

In the RPE of bluegill (*Lepomis macrochirus*), dispersion can be induced by acetylcholine (or its analog carbachol) binding to M_{odd} muscarinic receptors on the RPE leading to a cascade of messengers ultimately causing pigment granules to move inside long processes among the outer segments of photoreceptors in the retina. In other systems, M_{odd} receptor activation has been shown to be linked to Ca^{2+} mobilization. Therefore, I hypothesized that an elevation in intracellular Ca^{2+} is required for carbachol-induced pigment granule dispersion. In this study I investigated the requirement for Ca^{2+}. I found that intracellular, but not extracellular, Ca^{2+} is necessary for carbachol-induced dispersion. This finding supports my hypothesis that an elevation in intracellular Ca^{2+} is required for carbachol-induced pigment granule dispersion. Furthermore, I studied possible calcium-dependent effectors. I found calcineurin and phosphodiesterase-4 to be necessary components of the carbachol-mediated dispersion pathway. Protein kinase C plays a minor role if any.

CHAPTER I

INTRODUCTION

At any given time, a cell conducts many intracellular operations that result in the movement of organelles and vesicles, e.g., neurotransmitter processing and release in neurons. Cells require organelle motility to function. Pigment cells are an excellent model in which to study cell motility because pigment granules are readily visible, move rapidly, and undergo reversible intracellular movement which can be easily manipulated experimentally (Barral and Seabra, 2004). Pigment granules, or melanosomes, are found in pigment cells such as melanophores and retinal pigment epithelial cells (RPE). Melanin, the pigment found inside melanosomes, absorbs light over a wide range of wavelengths (Liebman *et al.*, 1969). In the epidermis of fish, melanin protects underlying cells by absorbing DNA-damaging ultraviolet rays. In the RPE of many fish and amphibians, melanin is thought to protect photoreceptors from photobleaching (Back *et al.*, 1965; see Burnside and Laties, 1979; Douglas, 1982; Schraermeyer *et al.*, 1999).

Melanosomes are typically observed in cells in two states - aggregated or dispersed. In the aggregated state, the melanosomes are withdrawn from the peripheral processes and clustered near the cell body (Figure 1A-B), while in the dispersed state,

melanosomes are positioned away from the body (in RPE, down the lengths of apical processes as shown in Figure 1C-D). In both melanophores and RPE, protection from light energy is heightened in the dispersed state. Figure 2 illustrates how dispersion leads to an increase in photon absorption.

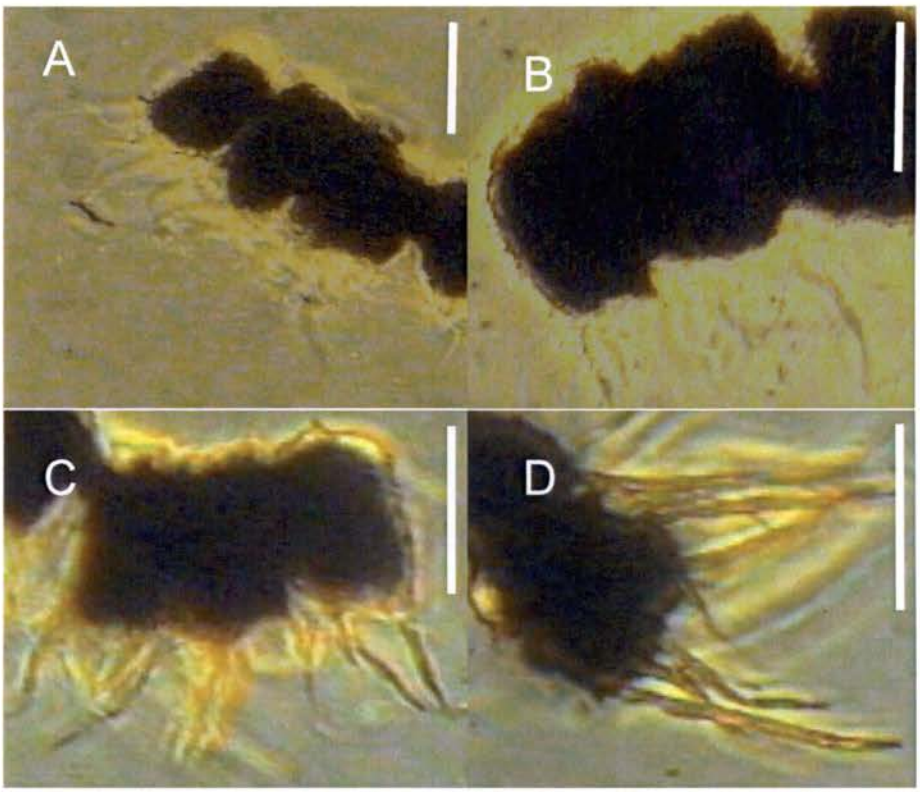

Figure 1. Phase contrast micrographs of RPE with aggregated and dispersed pigment granules. As seen in pictures A and B, aggregated RPE cells possess phase-dark apical processes. Compared to aggregated cells, the dispersed RPE in pictures C and D have phase-bright, thick processes, filled with pigment granules (although individual granules can not be resolved). The scale bar represents 25 micrometers.

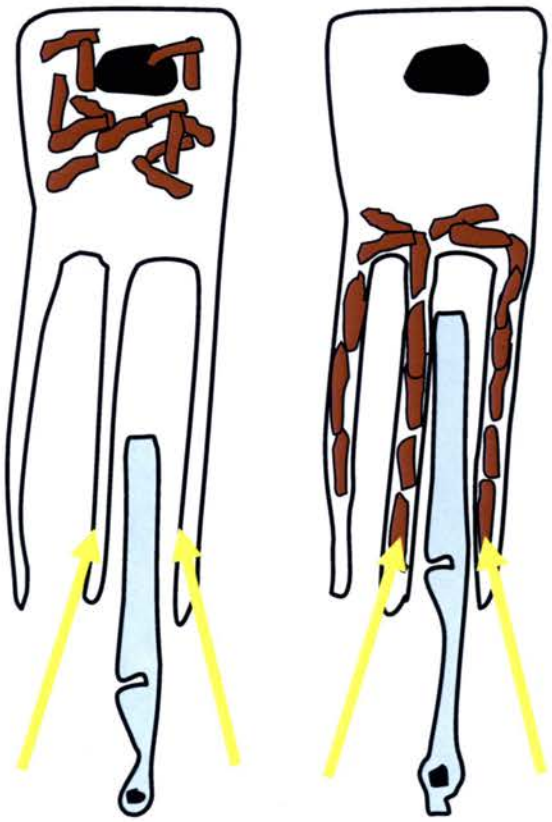

Figure 2. Light and dark adaptive retinomotor movements of RPE cells. In the dark, rod photoreceptors move down to the distal region of the RPE projections. In this state, they are able to absorb light (illustrated as yellow arrows). In bright light conditions, rods move into the RPE projections while the projections themselves fill with pigment granules. After light-adaptive retinomotor movements are completed, the rods are protected from photobleaching as photons are now absorbed by the pigment granules instead of rod outer segments. This mechanism is particularly important to organisms without the ability to adjust pupil diameter. In mammals, although some retinomotor movements occur, they are not as robust as seen in some amphibians and fish (Futter *et al.*, 2004; Burnside and Laties, 1986).

Pigment Granule Motility in Melanophores

Although much remains to be discovered, many components of the signaling pathways involved in melanosome motility have been identified. In general, aggregation in melanophores can be induced by melatonin, norepinephrine, and acetylcholine (ACh) (see Aspengren *et al.*, 2003; Hayashi and Fujii, 1994; Kumazawa and Fujii, 1984; Reed and Finnin, 1972). Norepinephrine bound to α-adrenergic receptors causes increases in $[Ca^{2+}]_i$ and decreases in $[cAMP]_i$ (Fujii, 1961; Novales and Fujii, 1970). Elevations in $[Ca^{2+}]_i$ may also be involved in cholinergic responses in catfish (*Parasilurus asotus*) melanophores since acetylcholine binds M_3 muscarinic receptors in this system, presumably activating the IP_3-Ca^{2+} pathway (Hayashi and Fujii, 1994). In many studies, aggregation in melanophore correlates with decreases in $[cAMP]_i$ and rises with $[Ca^{2+}]_i$ (Sammak *et al.*, 1992; Magun, 1973; Rozdzial and Haimo, 1986).

Sammak *et al.* (1992) suggest a possible function of lowering cAMP is deactivation of the cAMP-dependent protein kinase A (PKA). In 2005, Deacon *et al.* found that in *Xenopus* melanophores PKA deactivation results in the transient activation of ERK (extracellular signal-regulated kinase). ERK appears to be a key component when melatonin is used to induce aggregation.

A rise in intracellular Ca^{2+} often accompanies the aggregation of pigment granules in melanophores; however, a requirement for Ca^{2+} has not been found in all studies (Sammak *et al.*, 1992; Thaler and Haimo, 1992; Patil and Jain, 1993). For example, Sammak *et al.* (1992) found that blocking the rise in the $[Ca^{2+}]_i$ with BAPTA did not block epinephrine-induced aggregation in the melanophores of angelfish (*Pterophyllum scalare*) even though epinephrine stimulates a rise in $[Ca^{2+}]_i$. In contrast,

Thaler and Haimo (1992) found that epinephrine-induced aggregation did require Ca^{2+} in the melanophores of the African cichlid *Tilapia mossambica* since chelating Ca^{2+} with BAPTA prevented pigment movement. Therefore, the function of Ca^{2+} as a signaling intermediary may vary by species.

A number of Ca^{2+}-dependent mediators of pigment granule movement have been identified in melanophores. In the African cichlid *Tilapia mossambica*, Thaler and Haimo (1990) have shown that calcineurin, a Ca^{2+}-calmodulin-dependent protein phosphatase, mediates pigment granule aggregation. Thaler and Haimo's results also suggest that calcineurin dephosphorylates proteins targeted for phosphorylation by cAMP-dependent protein kinase. However, no specific substrate was identified (see also Haimo and Thaler, 1994).

As described earlier, elevation of cAMP is required for pigment granule dispersion in melanophores. Dispersion can be induced by the application of melanocyte-stimulating hormone (MSH) (Shizume *et al.*, 1954; Lerner, 1959; Daniolos *et al.*, 1990) which results in an increase of intracellular cAMP (Sammak *et al.*, 1992; Daniolos *et al.*, 1990; Rozdzial and Haimo, 1986). Following the rise in $cAMP_i$, activated PKA relays the signal for dispersion (Lynch *et al.*, 1986; Reilein *et al.*, 1998). Although the relevant targets for phosphorylation by PKA in melanophores are unknown, one candidate is the microtubule-dependent motor protein kinesin. Kinesin's ATPase activity has been observed to be turned on by PKA-mediated phosphorylation in neurons (Matthies *et al.*, 1993).

There is no rise in $[Ca^{2+}]_i$ associated with dispersion (Haimo and Thaler, 1994). Nevertheless, activation of protein kinase C (PKC) can result in dispersion in some

systems (Reilein et al., 1998). Ca^{2+} promotes PKC activity however Ca^{2+}-independent PKC isoforms exist (Newton, 1995). In *Xenopus* melanophores, Reilein *et al.* (1998) demonstrated partial dispersion can be induced by treatment with phorbol 12-myristate 13-acetate (PMA), which activates PKC. Using a recombinant peptides inhibitory to PKC, Reilein *et al.* (1998) also found PMA-mediated dispersion inhibited in transfected cells. Sugden and Rowe (1992), also studying *Xenopus* melanophores, had similar results, finding pigment granule dispersion in response to PMA. Inhibiting PKC with Ro 31-8220, also known as bisindolylmaleimide IX, did not inhibit MSH-mediated dispersion suggesting that PKC-mediated dispersion is part of a signaling pathway separate from the MSH-mediated path (Sugden and Rowe, 1992).

The Role of the Cytoskeleton in Aggregation and Dispersion

In melanophores, microtubules provide the path for bidirectional pigment granule motility. Microtubule-based aggregation involves dyneins whereas dispersion is powered first by kinesin (see Barral and Seabra, 2004). Activity of dynein and kinesin motor proteins are coordinated in that their activities do not occur simultaneously (Gross *et al.*, 2002). Upon reaching the periphery during dispersion, pigment granules dissociate from microtubules and attach to microfilaments (Barral and Seabra, 2004; Burnside, 1976; Murray and Dubin, 1975). The attachment of granules to actin requires a tripartite complex of Rab27a, melanophilin, and a myosin motor (Barral and Seabra, 2004). In *Xenopus* melanophores, myosin V terminates dynein-driven motility causing pigment granules to remain at the cell periphery (Gross *et al.*, 2002). In the RPE of *Rana pipiens pipiens*, actin is found in the apical processes; whereas, microtubules are not apparent (Murray and Dubin, 1975). In the RPE of green sunfish (*Lepomis cyanellus*)

microtubules are found in projections and have their plus-ends proximal (Troutt and Burnside, 1988) but are not thought to function in pigment granule motility (King-Smith *et al.*, 1997). Instead of kinesin/dynein activity, pigment granule migration in the RPE is thought to depend solely on myosin activity and actin polymerization/ depolymerization (King-Smith *et al.*, 1997).

Beyond melanosome motility in the RPE, Rab27a and MyRIP (the form of melanophilin found in RPE and other cells) have been shown to be important in adrenal chromaffin cell secretory pathways, thus illustrating that some observations made in RPE and melanosomes can be generalized to other systems (Desnos *et al.*, 2003).

Pigment Granule Motility in the RPE

With the exception of the cytoskeletal and motor protein components of RPE and melanophore pigment granule motility mechanisms, most other mediators appear inverted with respect to function (Table 1). For example, high $[cAMP]_i$ leads to dispersion in melanophores, but in RPE high $[cAMP]_i$ leads to aggregation (Keith *et al.*, 2006; García, 1993; Burnside and Ackland, 1984; Burnside and Basinger, 1983; Burnside *et al.*, 1982). Experimentally applying forskolin, an adenylyl cyclase activator, raises $[cAMP]_i$ and induces aggregation in isolated RPE cells (García and Burnside, 1994; Dearry and Burnside, 1988; Bruenner and Burnside, 1986; Dearry and Burnside, 1985).

Pigment granule migration in the RPE is not influenced by light directly. For example, when isolated RPE cells are exposed to light, dispersion does not occur (Snyder and Zadunaisky, 1976). Therefore, extracellular molecular mediators must signal the initiation of pigment granule motility (Liebman *et al.*, 1969). As in melanophores, several different agents have been identified that induce pigment granule movement. For

Table 1. A brief[1] comparison of melanophore/RPE mediators. The examples below illustrate key differences in the regulation of melanophore and RPE pigment granule motility. Each mediator is listed with species and reference. One salient difference between melanophores and RPE is the function of cAMP. In melanophores, a rise in $[cAMP]_i$ is required for dispersion. In RPE $[cAMP]_i$ must be lowered in order for dispersion to occur. Another inverted relationship is found in adrenergic and muscarinic receptor activation. Why such differences exist remains to be determined. It is important to note that exceptions to these generalizations exist when examining different species.

Melanophores	
Aggregation	Dispersion
↑ **$[Ca^{2+}]_i$**, *Tilapia mossambica* (see Haimo and Thaler, 1994)	↓ **$[Ca^{2+}]_i$**, *Tilapia mossambica* (see Haimo and Thaler, 1994)
↓ **$[cAMP]_i$**, *Tilapia mossambica* (see Haimo and Thaler, 1994)	↑ **$[cAMP]_i$**, *Tilapia mossambica* (see Haimo and Thaler, 1994); *Pterophyllum scalare* (Sammak *et al* , 1992); *Xenopus* (Daniolos *et al* , 1990)
↓ **PKA**, *Xenopus* (Reilein *et al.*, 1998)	↑ **PKA**, *Tilapia mossambica* (Rozdzial and Haimo, 1986)
↑ **PP2B**, *Tilapia mossambica* (see Haimo and Thaler, 1994)	↓ **PP2B**, *Tilapia mossambica* (see Haimo and Thaler, 1994)
M_{odd} muscarinic receptor activation, *Kryptopterus bicirrhis* (Hayashi and Fujii, 1994)	↑ **PKC** *Xenopus* (Reilein *et al.*, 1998; Sugden and Rowe, 1992)
α_2-adrenergic activation, *Labrus ossifagus* (Andersson *et al.*, 1984)	
RPE	
Aggregation	Dispersion
↑ **$[cAMP]_i$**, *Lepomis cyanellus* (Burnside *et al.*, 1982); *Lepomis macrochirus* (Keith *et al* , 2006)	↓ **$[cAMP]_i$**, *Lepomis cyanellus* (Dearry and Burnside, 1988)
↑ **PKA**, *Lepomis cyanellus* (García, 1993)	↓ **PKA**, *Lepomis cyanellus* (García, 1993)
↓ **PP2B**, (García, 1993)	**M_{odd} muscarinic receptor activation**, *Lepomis macrochirus* (González, *et al.*, 2004)
	α_2-adrenergic activation, (Dearry and Burnside, 1988)

[1]- This table is not meant to summarize the entire literature but merely provide a few key examples of where melanophores and RPE cells differ. Exceptions do occur among different species.

example, the application of catecholamines and their agonists (epinephrine, phenylephrine, clonidine, apomorphine, and dopamine) onto isolated RPE cells induces dispersion (Dearry and Burnside, 1985; Bruenner and Burnside, 1986). Of the catecholamines, dopamine is thought to act through D2 receptors that inhibit adenylyl cyclase (Dearry and Burnside, 1988). With adenylyl cyclase inhibited, $[cAMP]_i$ decreases and dispersion ensues. These results show that the role for cAMP is reversed when comparing RPE cells and melanophores.

Whereas in melanophores acetylcholine causes aggregation (Hiyashi and Fujii., 1994), García (1998) reported that the acetylcholine analog carbachol induces dispersion in green sunfish (*Lepomis cyanellus*) RPE. González *et al.* (2004) extended this finding to bluegill (*Lepomis macrochirus*) RPE and further reported that muscarinic M_{odd} receptor activation leads to pigment granule motility. Later it was found that the native ligand acetylcholine can induce pigment granule dispersion (Phatarpekar *et al.*, 2005). Following M_{odd} receptor activation, phospholipase C is activated (Phatarpekar *et al.*, 2005), cleaving PIP_2 to generate diacylglycerol and inositol trisphosphate (IP_3). When carbachol and antagonists to the IP_3 receptor were applied simultaneously, dispersion was inhibited (Phatarpekar *et al.*, 2005).

In other systems, the IP_3 receptor has been found within the membrane of the endoplasmic reticulum. With ligand bound to the IP_3 receptor, Ca^{2+} stored within the ER lumen is released into the cytosol (see Irvine, 2002). Extrapolating these observations to regulation of pigment granule movement in RPE, one might infer a role for Ca^{2+} in regulating pigment granule dispersion in RPE. However, King-Smith *et al.* (1996) were unable to demonstrate a role for Ca^{2+} in either pigment granule dispersion or aggregation.

Rather, they found showed that when dispersion is experimentally induced by cAMP washout in RPE isolated from green sunfish (*Lepomis cyanellus*), there was no significant rise in intracellular Ca^{2+} levels. This finding may not, however, rule out a role for Ca^{2+} in a physiological setting involving receptor activation receptor activation, if the physiological role for Ca^{2+} precedes a decrease in intracellular cAMP. Therefore, carbachol-induced dispersion may still require Ca^{2+}. **Therefore, the hypothesis driving my study was that Ca^{2+} is required for carbachol-induced dispersion in RPE cells isolated from bluegill,** which I examined using calcium chelators, calcium channel blockers and calcium-free media. Furthermore, if calcium were required, calcium-dependent effectors would also be expected to play a role in pigment granule dispersion; therefore, intracellular mediators that lead to pigment granule dispersion were also explored.

CHAPTER II

MATERIALS AND METHODS

Experimental animals

The experimental animals were maintained as described previously (González *et al.,* 2004). All experiments were performed following protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Texas State University- San Marcos (Protocol # 069744F82). Bluegill (*Lepomis macrochirus*) were purchased from Johnson Lake Management, San Marcos, TX. All fish were acclimated at least two weeks prior to experimentation in indoor, aerated aquaria on a 12 hour: 12 hour (light/dark) cycle.

Extraction of RPE

To extract RPE, fish were captured 6 hours into the light cycle and dark adapted in a room void of light. In the dark room, the fish were allotted thirty minutes to adjust to the darkness in aerated aquaria. After thirty minutes, the spinal cord was severed, and the fish were double-pithed under dim, incandescent light (≤ 2 lux). Light was measured using a Lutron LX-101 lux meter (Lutron, Coopersburg, PA). The eyes were then removed and hemisected along the equatorial axis. The anterior portion of the eye was discarded. The retina was removed from the eyecup, and the RPE was flushed out using

bicarbonate-buffered Ringer prepared the day of the experiment (see González *et al.*, 2004). All chemicals were purchased from Sigma-Aldrich, St. Louis, MO. The Ringer's solution contained 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, 1.12 mM $MgSO_4$, 1 mM EGTA, and 1.8 mM $CaCl_2$, titrated to pH of 7.4 using 1 M NaOH. The buffer was gassed with 95% air/ 5% CO_2 for at least fifteen minutes prior to the dissection and throughout the experiment to maintain a pH of 7.2. For one experiment, Ca^{2+}-free Ringer was prepared as above with the omission of $CaCl_2$.

Drug Treatments

Once the RPE had been isolated, excess Ringer was removed, and forskolin (10 μM) was applied to induce aggregation. The cells were then incubated for 45 minutes in a humidified chamber gassed with a mixture of 95% air and 5% CO_2 on a gyratory shaker (50 rpm) as in González *et al.* (2004).

After incubation in forskolin, approximately one third of the collected RPE was fixed using a 2X stock solution of fixative prepared in phosphate buffered saline (PBS). PBS was prepared as 137 mM NaCl, 2.7 mM KCl, 4.3 mM $NaH_2PO_4 H_2O$, and 1.4 mM KH_2PO_4 in purified water (resistivity 18 Mohms). Purified water was obtained from a NANOpure Infinity Laboratory Water System. After dilution with an equal volume of Ringer's solution containing tissue, the final concentration of fixative was 0.5% glutaraldehyde, 0.5% paraformaldehyde, and 0.8% potassium ferricyanide.

The remaining cells were divided between two weigh boats and washed clean of forskolin using Ringer. This procedure entailed pipetting Ringer's solution into and out of the weigh boats; about 3 volumes were exchanged. Tissue pieces for both treatment

groups were then placed in microcentrifuge tubes containing either 50 μL of 1 μM carbachol and 450 μL of Ringer's solution or 50 μL of 1 μM carbachol, 400 μL of Ringer's solution, and 50 μL of 10X experimental drug (see **Pharmacological Agents** below). Following an additional 45' incubation, the remaining samples were fixed as described above (Figure 3).

Pharmacological Agents

To study the requirement for extracellular and intracellular Ca^{2+}, Verapamil and BAPTA-AM (both of which were prepared freshly the day of experimentation) were used, respectively. Cypermethrin (from 1 nM stock solution) was used to inhibit calcineurin (PP2B). Staurosporine, bisindolylmaleimide II, and phorbol esters were used to investigate a role for PKC activity. Staurosporine (Sigma) was divided into 10 μM aliquots in DMSO. Bisindolylmaleimide II (Sigma) was dissolved in DMSO to make a 1 mM stock solution, which was then divided into aliquots and frozen. Phorbol 12-myristate 13-acetate (Sigma) was prepared as a 100 μM stock and divided into aliquots. Rolipram was used to inhibit PDE4. Rolipram was prepared the day of experimentation. All inhibitors prepared in DMSO were stored at -20°C, then diluted in Ringer's solution just before use. A complete list of can be found in Table 2.

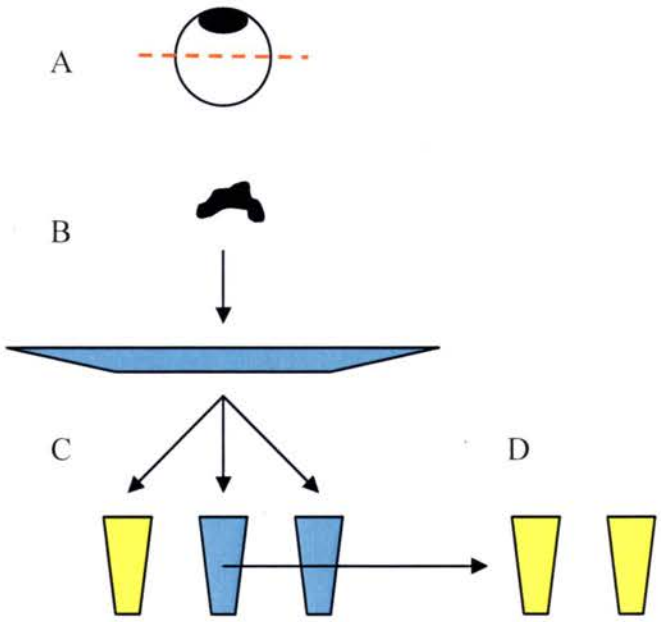

Figure 3. Layout of experimental procedure. Eyes were removed and hemisected (A). RPE was flushed from the posterior eyecup using Ringer. RPE was then incubated in FSK medium (10 μM) for 45' to induce aggregation (B). Cells were then divided into three microcentrifuge tubes (C). One sample was immediately fixed (yellow tube). The remaining cells were either placed in medium containing carbachol (100 nM) or medium containing both carbachol and inhibitor. The latter two samples underwent an additional 45' incubation. Following incubation, the remaining cells were fixed (D). All samples were refrigerated overnight prior to further processing. All treatments were replicated a minimum of three times using three fish (n=3).

Table 2. Pharmacological agents used to decipher ions and molecular mediators involved in carbachol-induced pigment granule dispersion. Drug concentrations used are justified by the articles referenced. All agents were purchased from Sigma-Aldrich (St. Louis, MO)

Drug	Target/effect	Concentration	Reference
Verapamil	L-type Ca^{2+} channel antagonist	10 μM, 100 μM[1]	Patil and Jain, 1993
BAPTA-AM	Intracellular Ca^{2+} chelator	1 μM[2], 30 μM	Thaler and Haimo, 1990.
Cypermethrin	PP2B inhibitor	100 pM[3]	Enan and Matsumara, 1992
Staurosporine	PKC inhibitor	100 nM, 0.5 μM[4], 1 μM	Reilein *et al.* 1998
Bisindolylmaleimide II	PKC inhibitor	20 nM, 200 nM, 2 μM[5]	Mahata *et al.*, 2002
Phorbol 12-myristate 13-acetate	PKC activator	1 μM[6], 10 μM, 100 μM	Reilein *et al.*, 1998
Rolipram	PDE4 inhibitor	10 μM[7]	Fleming *et al.*, 2004

Microscopy, calculation of pigment index, and statistical methods

Fixed RPE was placed onto a slide and chopped into smaller fragments for analysis. For each condition, thirty cells were measured from a minimum of three fish. Measurements were made using a Zeiss phase-contrast light microscope equipped with an ocular micrometer. Cells were measured if they appeared to be whole, that is, if they had the phase-bright base typical of an intact cell and long apical processes giving an overall length of at least 50 μm. Furthermore, cells were only measured if they had at least three apical processes.

A pigment index ratio (PI) was determined by comparison of total length of a cell to the distance from the base to the farthest dispersed pigment granule (Figure 4). Analysis of variance (ANOVA) was performed among the mean pigment indices of each treatment group. Differences were considered significant when $p<0.05$. Tukey *post hoc* analysis was performed to determine which treatments differed.

Statistical analysis was performed using the computer program S-Plus 7.0. Prior to analysis, the two control conditions (FSK and carbachol-alone) were combined from all experiments. When experimental conditions were found statistically significantly different from the carbachol control condition, a percent inhibition was calculated. To calculate percent inhibition, first the difference between experimental treatment and FSK treatment was found. This value was then divided by the difference between FSK and carbachol conditions and multiplied by 100. Finally, the resulting value was subtracted from 100 to yield percent inhibition. A complete list of PI values for each experiment is in the appendix.

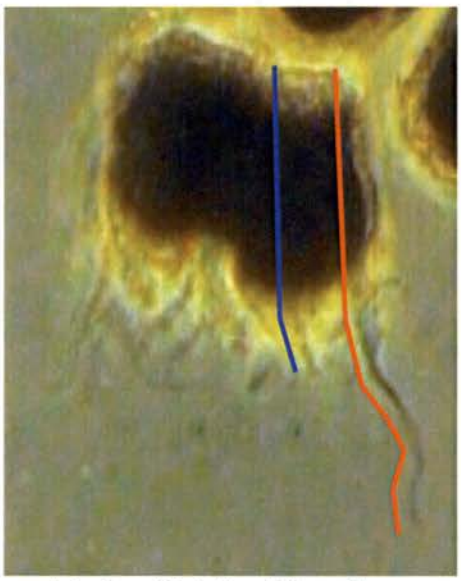

Figure 4. Measuring pigment index. Total cell length was measured (red line). The cell was then measured from base to most dispersed pigment granule (blue line). Pigment index was calculated by dividing the second measurement by the first (PI = blue / red).

CHAPTER III

RESULTS

To test whether an elevation in intracellular Ca^{2+} was required for carbachol-induced pigment granule dispersion, I treated cells with the membrane-permeant Ca^{2+} chelator BAPTA-AM. When cells were incubated with 100 nM carbachol in the presence of 1 μM BAPTA-AM or 30 μM BAPTA-AM, the resulting PI values were not statistically significantly different from the PI of cells treated with FSK but were different from PI of cells treated with carbachol alone (Figure 5). The maximum concentration of DMSO used during incubation (5% vol/vol) did not alter PI values (Figure 6).

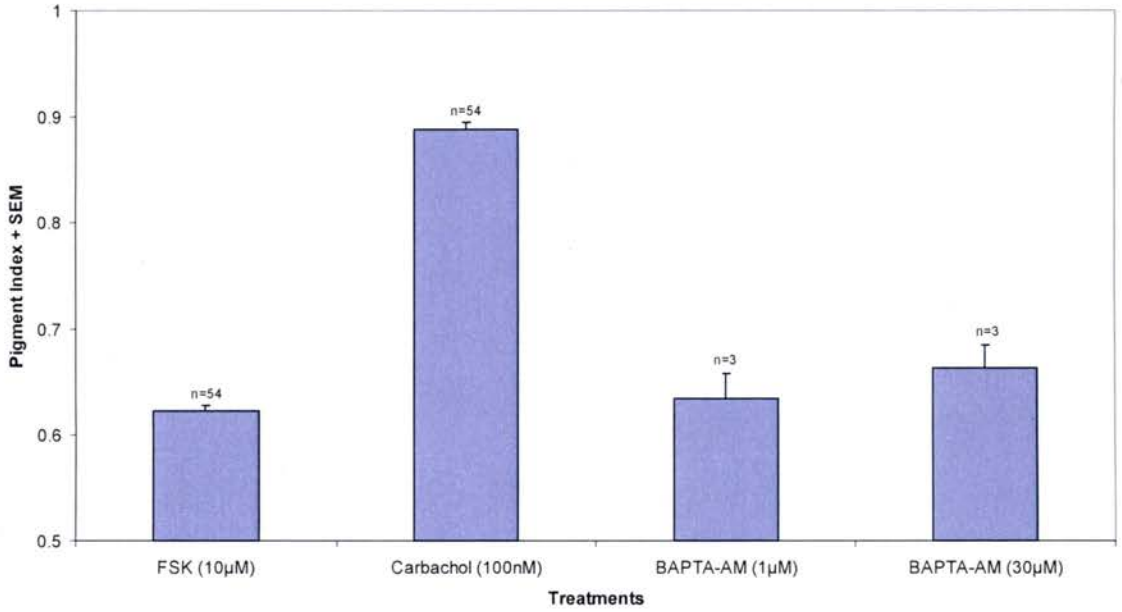

Figure 5. BAPTA blocks carbachol-mediated dispersion. Isolated RPE cells were first treated with 10 μM forskolin for 45' to induce aggregation. Following aggregation, carbachol (100 nM) was tested along with BAPTA-AM. The mean pigment index of cells treated with BAPTA-AM was significantly different than the mean pigment index of cells treated with carbachol alone. Dispersion was inhibited 95% in cells treated with 1 μM BAPTA and 82% in those treated with 30 μM BAPTA. No statistically significant differences were found between the FSK, BAPTA at 1 μM, or BAPTA at 30 μM treatments. The error bars represent the standard error of the mean (SEM).

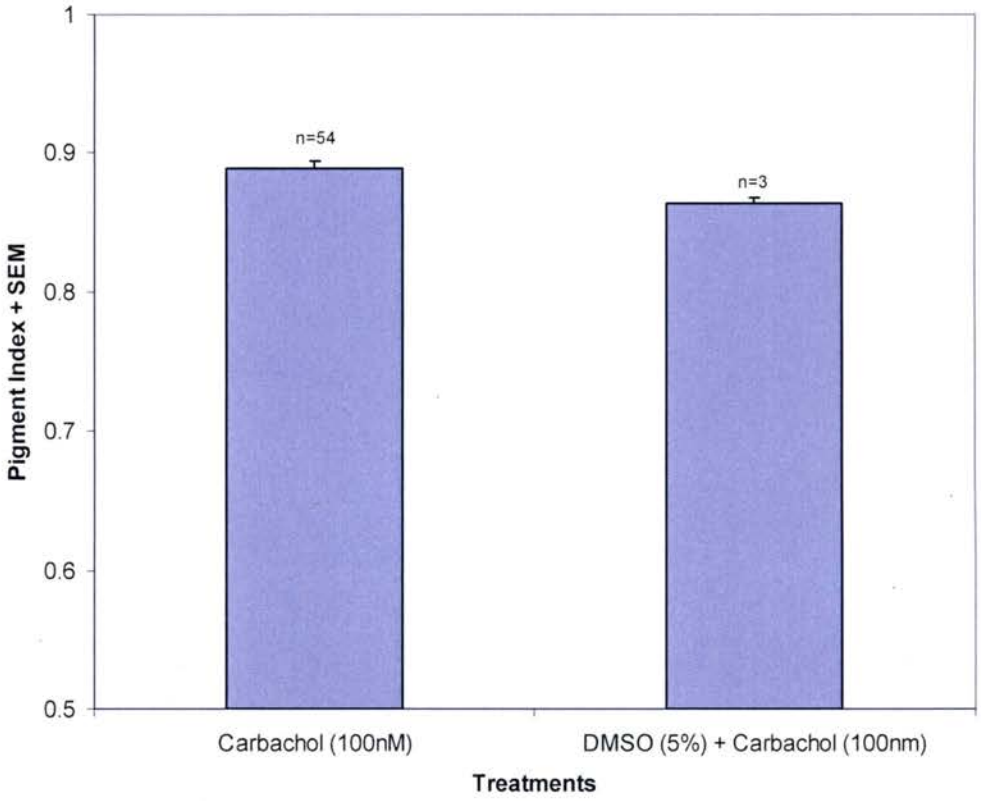

Figure 6. DMSO did not affect pigment granule dispersion. Carbachol-mediated dispersion is not affected by DMSO. No statistically significant differences exist between pigment indices of cells treated with carbachol in the absence or presence of DMSO (5% vol/vol).

To test the importance of extracellular Ca^{2+} in carbachol-induced pigment granule dispersion, verapamil, an L-type Ca^{2+} channel antagonist, was tested for its ability to block carbachol-induced dispersion. Verapamil failed to block carbachol-induced dispersion at both 10 μM and 100 μM. The difference in pigment index (PI) between cells with and without the Ca^{2+} channel blocker was not statistically significant (Figure 7). Also, the differences between the pigment indices for either condition (carbachol or carbachol plus verapamil) and the FSK condition were statistically significant.

To confirm that an influx of extracellular Ca^{2+} was not required, RPE cells were isolated and challenged to dispersion in the presence or absence of extracellular Ca^{2+} following treatment with forskolin (FSK). RPE were treated with 10 μM FSK to induce pigment granule aggregation. Carbachol (100 nM) was used to induce pigment granules to disperse. The difference in pigment index (PI) between cells with and without extracellular Ca^{2+} available was not statistically significant (Figure 7). However, the difference between either condition (with and without Ca^{2+}) and the 10 μM FSK condition was statistically significant.

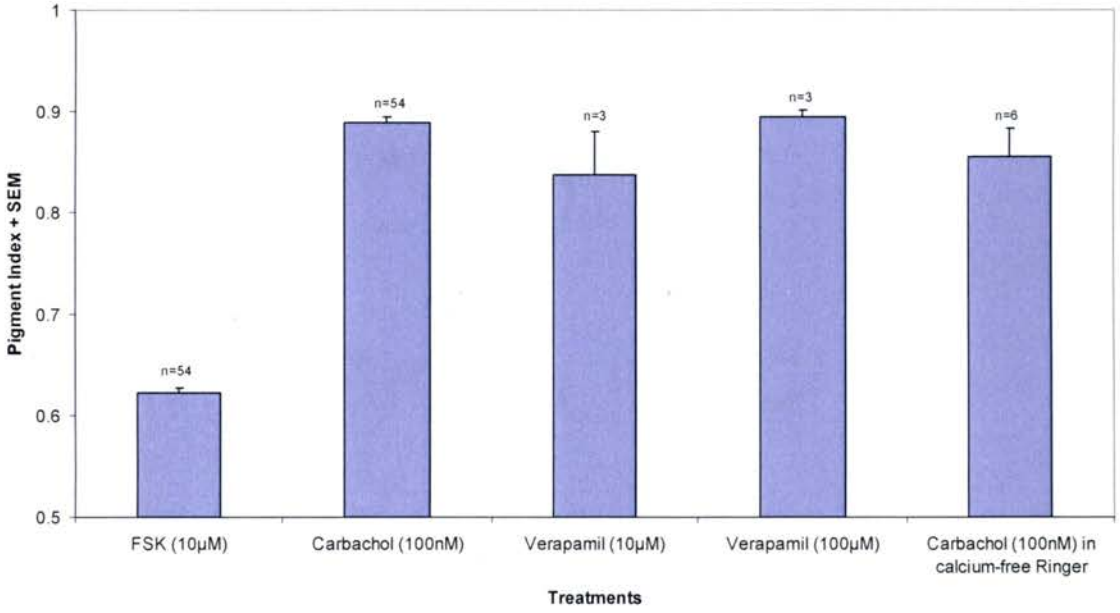

Figure 7. Extracellular Ca^{2+} is not required for carbachol-induced dispersion. Isolated RPE cells were first treated with 10 μM forskolin for 45' to induce aggregation. Following aggregation, carbachol (100 nM) was either applied to cells in normal Ringer or in Ca^{2+}-free Ringer. A separate sample of cells was treated with carbachol plus Verapamil (10 μM, 100 μM). Aside from the forskolin treated samples, none of the treatments yielded statistically significantly different pigment indices.

Since my results using BAPTA-AM suggested a role for intracellular Ca^{2+}, I wanted to test for possible downstream, Ca^{2+}-dependent mediators. I first tested whether protein kinase C (PKC) is required for carbachol-induced dispersion by using two inhibitors (staurosporine and bisindolylmaleimide II). Cells treated with carbachol in the absence or presence of 100 nM staurosporine were found to differ from the cells treated with FSK, but were not different from each other (Figure 8). When bisindolylmaleimide II (20 nM, 200 nM, 2 μM) was applied with carbachol, dispersion was partially inhibited only in the 200 nM condition (Figure 9). Exhibiting a 20% inhibition in dispersion, the cells treated with 200 nM bisindolylmaleimide II were statistically significantly different from both FSK and carbachol treatments. To test whether PKC was sufficient to induce dispersion, PMA was applied to RPE. Following FSK treatment, PMA (without carbachol) did not elicit PI values that were significantly different from FSK-aggregated cells (Figure 10).

Another Ca^{2+}-dependent effector molecule tested was the protein phosphatase calcineurin. The calcineurin inhibitor cypermethrin (100 pM) completely inhibited carbachol-induced pigment granule dispersion (Figure 11). The cypermethrin condition was not significantly different from the FSK condition but was the different from the carbachol condition.

I then tested for PDE4 activity during carbachol-induced dispersion. At 10 μM, rolipram completely blocked carbachol-induced dispersion (Figure 11). The rolipram condition was not significantly different from the FSK condition but was different from the carbachol condition.

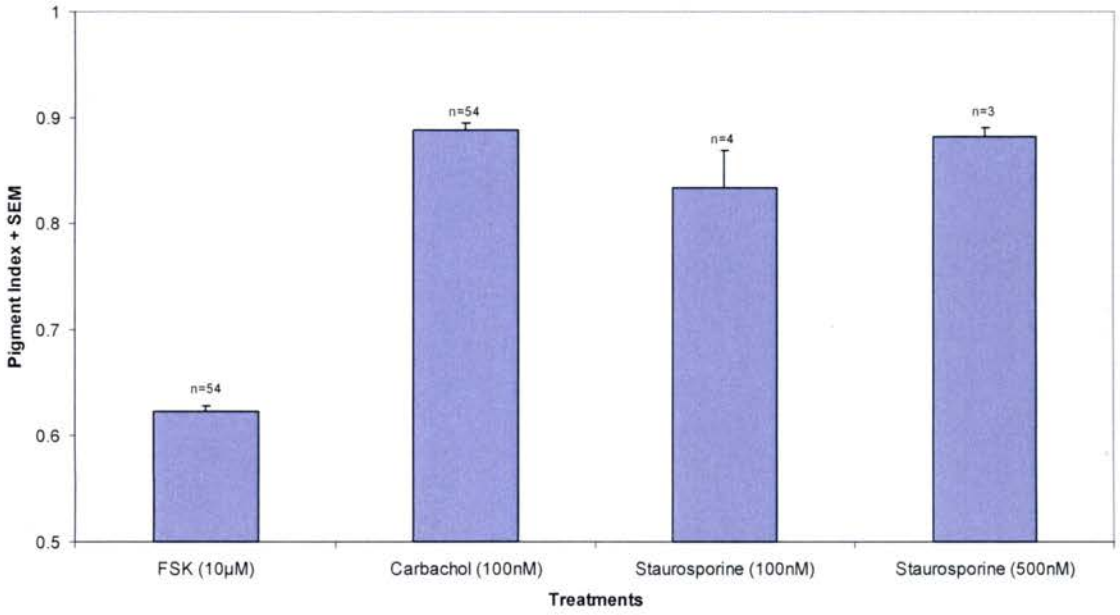

Figure 8. Staurosporine does not interfere with dispersion. Isolated RPE cells were first treated with 10 μM forskolin for 45' to induce aggregation. Following aggregation, carbachol (100 nM) was tested along with staurosporine (100 nM and 500 nM), a PKC-inhibitor. Cells treated with staurosporine were not significantly different than cells treated with carbachol alone.

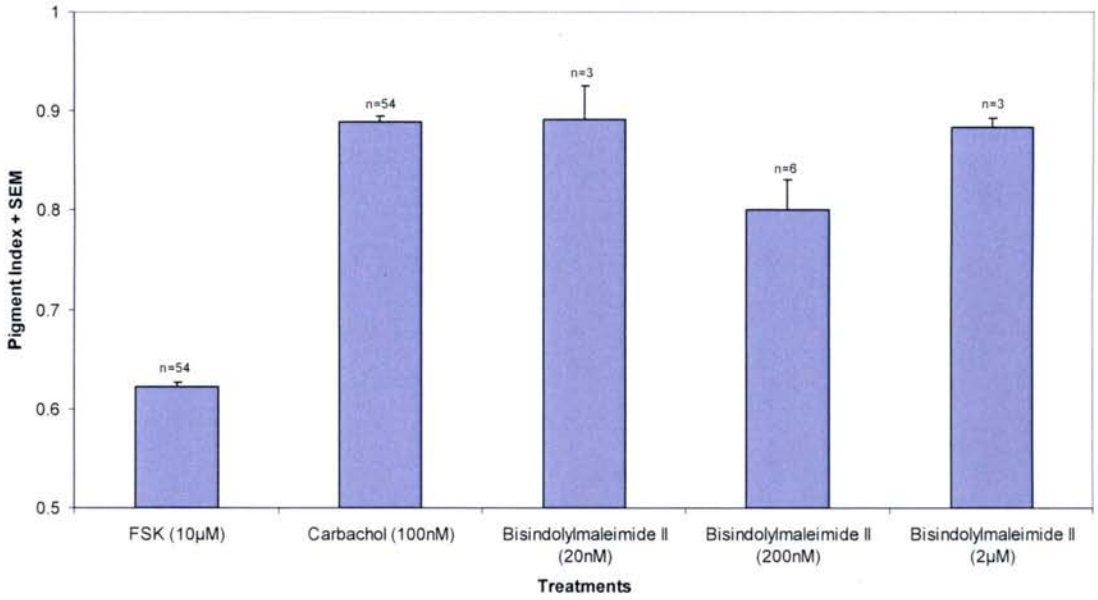

Figure 9. Bisindolylmaleimide II does not block dispersion. Isolated RPE cells were first treated with 10 μM forskolin for 45' to induce aggregation. Following aggregation, carbachol (100 nM) was tested along with bisindolylmaleimide II, a PKC inhibitor. The pigment index of cells treated with bisindolylmaleimide II (at 20 nM and 2μM) was not significantly different at any concentration tested from the pigment index of cells treated with carbachol alone. At 200 nM, bisindolylmaleimide treated cells exhibited a 20% inhibition with respects to dispersion.

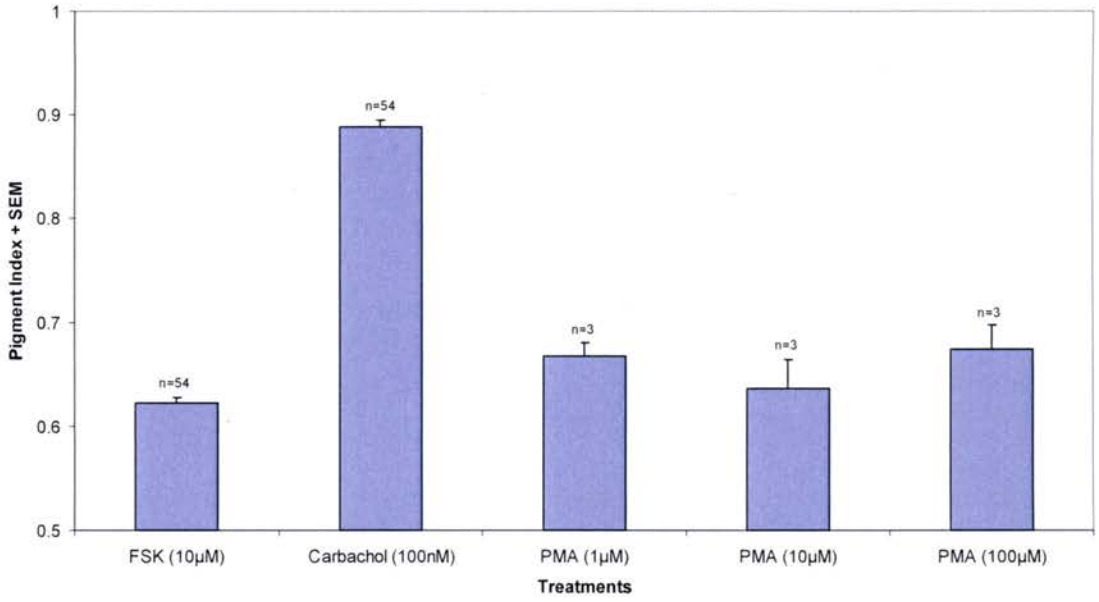

Figure 10. PKC-activation does not induce dispersion. Isolated RPE cells were first treated with 10 μM forskolin for 45' to induce aggregation. Following aggregation, cells were either treated with carbachol (100 nM) or treated with PMA, a PKC activator. The pigment indices of cells treated with PMA were significantly different from the pigment index of cells treated with carbachol and were not significantly different from the pigment index of cells treated with forskolin.

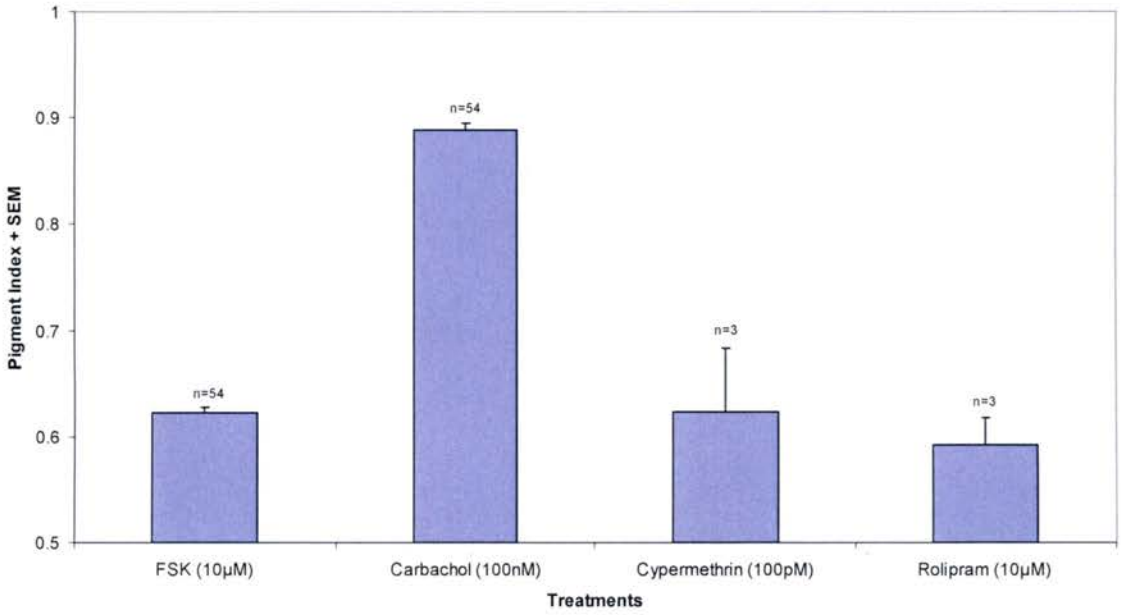

Figure 11. PP2B and PDE4 are active during carbachol-induced dispersion. Isolated RPE cells were first treated with 10 μM forskolin for 45' to induce aggregation. Following aggregation, carbachol (100 nM) was tested along with either the calcineurin inhibitor cypermethrin (100 pM) or the PDE4 inhibitor rolipram (10 μM). Cypermethrin-treated cells experienced 100% inhibition in dispersion; whereas, rolipram treated cells experienced 113% dispersion inhibition. Cells treated with either cypermethrin or rolipram were not significantly different from cells treated with forskolin alone.

CHAPTER IV

DISCUSSION

The results support the hypothesis, indicating that elevation of intracellular Ca^{2+} required for carbachol-induced pigment granule dispersion. Furthermore, the necessary Ca^{2+} may be derived entirely from intracellular stores and that one effector activated by Ca^{2+} that may be relevant is the protein phosphatase calcineurin. In addition, PDE activity is required or permissive in order for pigment granule dispersion to occur. In contrast, our results using inhibitors and activators of PKC suggest that the activity of this enzyme is neither necessary for carbachol-induced pigment granule dispersion nor sufficient to drive pigment granule dispersion.

Ca^{2+}-dependent carbachol-induced dispersion

Carbachol-mediated dispersion requires downstream activation of the IP_3 receptor (Phatarpekar *et al.*, 2005), suggesting that intracellular Ca^{2+} stores are accessed following muscarinic receptor activation (González *et al.*, 2004). Chelating intracellular Ca^{2+} with BAPTA completely blocks carbachol-induced dispersion. This Ca^{2+} is most likely coming from intracellular stores (possibly regulated by the IP_3 receptor) and not from the extracellular environment since carbachol-induced dispersion is blocked by the IP_3 receptor antagonist 2-aminoethoxydiphenyl borate (Phatarpekar *et al.*, 2005) and

eliminating extracellular Ca^{2+} or blocking its entry across the plasma membrane failed to inhibit dispersion. There are many Ca^{2+} requiring mediators that may be involved in carbachol-induced dispersion, for example the Ca^{2+}-calmodulin-dependent protein phosphatase calcineurin. Since cypermethrin inhibits calcineurin activity, finding that cypermethrin blocks carbachol-induced pigment granule dispersion suggests that calcineurin may be involved. When FSK is applied to cells, cAMP levels increase (Dearry and Burnside, 1985), leading to PKA activation (García, 1993) and eventual aggregation of pigment granules. In the RPE pigment motility system, the substrate phosphorylated by PKA is yet unknown. With numerous substrates possible, knowing the exact timing and localization of PKA activation will be necessary to determine which substrate is relevant. Prior to dispersion, cAMP levels drop and likewise PKA activity. Increased Ca^{2+} concentrations inside the cell may lead to calcineurin activation that may remove phosphate groups from substrates of PKA, enabling pigment granule dispersion to occur. My finding that cypermethrin inhibits carbachol-induced dispersion is consistent with this model.

As stated before, inhibition of PLC blocks carbachol-induced dispersion (Phatarpekar et al., 2005). Since PLC cleaves PIP_2 into IP_3 (leading to Ca^{2+} mobilization) and DAG, and DAG and Ca^{2+} together activate protein kinase C, protein kinase C seemed likely to be a mediator of carbachol-induced dispersion. This hypothesis seemed the more likely since in some species, pigment dispersion in melanophores appears to be regulated by PKC (Sugden and Rowe, 1992; Reilein *et al.*, 1998). However, my results from three separate experiments do not strongly support a role for PKC in carbachol-induced dispersion. Treatment with the PKC activator PMA failed to induce pigment

granule dispersion. Neither of the PKC inhibitors tested (bisindolylmaleimide and staurosporine) inhibited carbachol-induced pigment granule dispersion at most concentrations tested. Cells treated with bisindolylmaleimide II at 200 nM experienced a 20% inhibition in dispersion relative to the carbachol-treated cells when data from the latter were compiled from all experiments. However, when comparison was made only to the carbachol-treated cells from the experiment in which the 200 nM concentration was tested, the difference in mean pigment indices was not found to be statistically significant. This case was the only one in which the compilation of the data yielded a different statistical outcome. Nevertheless, it remains possible that PKC has a role in carbachol-mediated dispersion which parallels the main pathway linking receptor activation to pigment granule movement. It should be mentioned that definitive evidence that PKC is expressed in bluegill RPE has yet to be found. Unpublished work by an undergraduate in the laboratory suggested that PKC-α and PKC-β were expressed in bluegill brain, but not in bluegill RPE (García, personal communication). These findings are consistent with work reported by others. In the retina of zebrafish, PKC labeling is observed in the neural retina, but not in the retinal pigment epithelium (Yazulla and Studholme, 2001).

Possible role of PDE4 in pigment granule dispersion

Dearry and Burnside (1986) found that pigment granule aggregation can be induced with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX). In the present study, carbachol did not induce dispersion when rolipram, a selective PDE4 inhibitor, was also applied. Although PDE4 activity is required for dispersion, it is most likely not regulated by Ca^{2+} or Ca^{2+}-dependent mediators. Instead, PDE4 is regulated by

PKA (Sette *et al.*, 1994). When FSK is applied to cells, cAMP levels increase which likely leads to PKA activation. A possibly relevant target PKA can phosphorylate is PDE4 and its phosphorylation increases its activity. PDE4 then degrades cAMP thus reducing PKA activity (Bender and Beavo, 2006). In rat embryo fibroblasts, this mechanism is thought to be a timing loop that regulates the duration of PKA activity (Fleming *et al.*, 2004).

Summary

Although much is still unknown, my results have increased our understanding of the pathway from carbachol to pigment granule dispersion. First, carbachol binds an M_{odd} muscarinic receptor leading to PLC activation (González *et al.*, 2004; Phatarpekar *et al.*, 2005). PLC then cleaves PIP_2 into DAG and IP_3. A role for DAG in pigment movement in RPE has not been revealed. In contrast, IP_3 binds its receptor, thus releasing Ca^{2+} from intracellular stores (Phatarpekar *et al.*, 2005). Ca^{2+} released from such stores is sufficient for downstream mediator activation; in other words, extracellular Ca^{2+} is not required. Increasing cytosolic Ca^{2+} may lead to calcineurin activation. As a phosphatase, calcineurin may remove phosphate groups added by PKA. This dephosphorylation alters the structure of some unknown protein. PDE4 works to degrade cAMP and inactivate PKA. How PDE4 activity is regulated in the RPE is not understood. Refer to Figure 11 for the proposed carbachol-induced dispersion pathway.

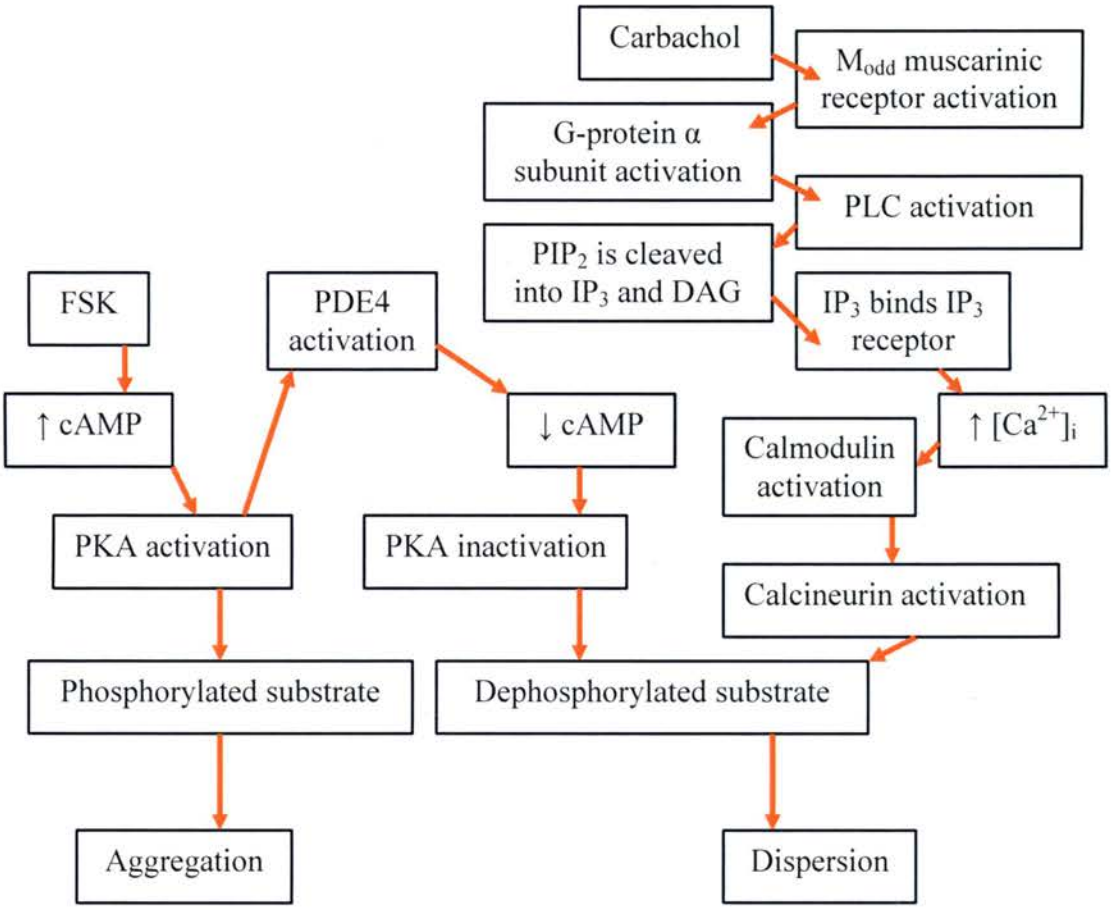

Figure 12. RPE carbachol-induced dispersion pathway. In conjunction with previous evidence, shown above is the proposed pathway that follows carbachol and forskolin (FSK) treatment. All of the above mediators have been tested with the exception of calmodulin. Also, although PDE4 is required for carbachol-induced dispersion, I do not imply that it is specific for this pathway. The specific phosphorylated/dephosphorylated substrate(s) is(are) unknown.

Future studies

My overall research goal was to reveal possible mediators involved in carbachol-induced pigment granule dispersion. I have found evidence supporting the Ca^{2+}-dependent calcineurin pathway which also requires PDE4 activity. To further validate the necessity of intracellular Ca^{2+}, Ca^{2+}-imaging studies will be essential in order to compare data with King-Smith *et al.* (1996). Testing whether treatment of cells with Ca^{2+} ionophores or releasing caged Ca^{2+} is sufficient to elicit dispersion following forskolin-induced pigment granule dispersion would help to determine whether elevating intracellular Ca^{2+} is sufficient to induce pigment granule dispersion.

Since calcineurin activity is dependent on activated calmodulin, inhibiting calmodulin with drugs like N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7) is another important future study to conduct (Klee *et al.*, 1998).

Aside from pharmacology, many molecular techniques may also prove useful in deciphering the carbachol-induced dispersion pathway. Via RNA-interference (siRNA), translation of calcineurin, PKC, PLC, and other mediators could be prevented thus revealing further evidence of their importance in the process of either dispersion or aggregation.

APPENDIX

Collective results. Shown below are the pigment indices and sample sizes of each experiment performed in this study. In every case, the values in black are not statistically different from one another but are different from the values in red. For example, BAPTA-AM conditions are similar statistically to FSK conditions but are different from carbachol conditions. As described in the Methods, statistical analysis was performed by comparing each of the values listed in the third column with the combined FSK and carbachol control values.

FSK (10μM)	**Carbachol (100nM)**	**BAPTA-AM (1μM)**
0.63 ± 0.02; n = 3	0.88 ± 0.01; n = 3	0.63 ± 0.02; n = 3
FSK (10μM)	**Carbachol (100nM)**	**BAPTA-AM (30μM)**
0.64 ± 0.05; n = 6	0.97 ± 0.01; n = 6	0.66 ± 0.02; n = 3
FSK (10μM)	**Carbachol (100nM)**	**Carbachol (100nM) (Ca^{2+}-free Ringer)**
0.64 ± 0.01; n = 6	0.90 ± 0.01; n = 6	0.86 ± 0.03; n = 6
FSK (10μM)	**Carbachol (100nM)**	**Verapamil (10μM)**
0.54 ± 0.02; n = 3	0.84 ± 0.04; n = 3	0.84 ± 0.04; n = 3
FSK (10μM)	**Carbachol (100nM)**	**Verapamil (100μM)**
0.64 ± 0.01; n = 3	0.86 ± 0.01; n = 3	0.89 ± 0.01; n = 3
FSK (10μM)	**Carbachol (100nM)**	**Staurosporine (100nM)**
0.58 ± 0.04; n = 4	0.90 ± 0.03; n = 4	0.83 ± 0.04; n = 4
FSK (10μM)	**Carbachol (100nM)**	**Staurosporine (500nM)**
0.61 ± 0.004; n = 3	0.86 ± 0.01; n = 3	0.88 ± 0.01; n = 3
FSK (10μM)	**Carbachol (100nM)**	**Bisindolylmaleimide II (20nM)**
0.64 ± 0.03; n = 3	0.88 ± 0.02; n = 3	0.89 ± 0.03; n = 3
FSK (10μM)	**Carbachol (100nM)**	**Bisindolylmaleimide II (200nM)**
0.63 ± 0.01; n = 6	0.87 ± 0.02; n = 6	0.8 ± 0.03; n = 6
FSK (10μM)	**Carbachol (100nM)**	**Bisindolylmaleimide II (2μM)**
0.62 ± 0.02; n = 3	0.88 ± 0.01; n = 3	0.88 ± 0.01; n = 3
FSK (10μM)	**Carbachol (100nM)**	**PMA (1μM)**
0.63 ± 0.02; n = 3	0.89 ± 0.01; n = 3	0.67 ± 0.01; n = 3
FSK (10μM)	**Carbachol (100nM)**	**PMA (10μM)**
0.64 ± 0.03; n = 3	0.88 ± 0.02; n = 3	0.64 ± 0.03; n = 3
FSK (10μM)	**Carbachol (100nM)**	**PMA (100μM)**
0.63 ± 0.02; n = 3	0.88 ± 0.01; n = 3	0.67 ± 0.02; n = 3
FSK (10μM)	**Carbachol (100nM)**	**Cypermethrin (100pM)**
0.59 ± 0.02; n = 3	0.88 ± 0.02; n = 3	0.62 ± 0.06; n = 3
FSK (10μM)	**Carbachol (100nM)**	**Rolipram (10μM)**
0.62 ± 0.04; n = 3	0.94 ± 0.03; n = 3	0.59 ± 0.03; n = 3
FSK (10μM)	**Carbachol (100nM)**	**DMSO (5%) + Carbachol**
0.61 ± 0.04; n = 3	0.86 ± 0.01; n = 3	0.86 ± 0.00; n = 3

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