BMC Neuroscience

Research article

Activation of muscarinic acetylcholine receptors elicits pigment granule dispersion in retinal pigment epithelium isolated from bluegill

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Published: 13 July 2004

BMC Neuroscience 2004, **5**:23 doi:10.1186/1471-2202-5-23

Received: 04 March 2004
Accepted: 13 July 2004

This article is available from: http://www.biomedcentral.com/1471-2202/5/23

Abstract

Background: In fish, melanin pigment granules in the retinal pigment epithelium disperse into apical projections as part of the suite of responses the eye makes to bright light conditions. This pigment granule dispersion serves to reduce photobleaching and occurs in response to neurochemicals secreted by the retina. Previous work has shown that acetylcholine may be involved in inducing light-adaptive pigment dispersion. Acetylcholine receptors are of two main types, nicotinic and muscarinic. Muscarinic receptors are in the G-protein coupled receptor superfamily, and five different muscarinic receptors have been molecularly cloned in human. These receptors are coupled to adenylyl cyclase, calcium mobilization and ion channel activation. To determine the receptor pathway involved in eliciting pigment granule migration, we isolated retinal pigment epithelium from bluegill and subjected it to a battery of cholinergic agents.

Results: The general cholinergic agonist carbachol induces pigment granule dispersion in isolated retinal pigment epithelium. Carbachol-induced pigment granule dispersion is blocked by the muscarinic antagonist atropine, by the M_1 antagonist pirenzepine, and by the M_3 antagonist 4-DAMP. Pigment granule dispersion was also induced by the M_1 agonist 4-[N-(4-chlorophenyl) carbamoyloxy]-4-pent-2-ammonium iodide. In contrast the M_2 antagonist AF-DX 116 and the M_4 antagonist tropicamide failed to block carbachol-induced dispersion, and the M_2 agonist arecaidine but-2-ynyl ester tosylate failed to elicit dispersion.

Conclusions: Our results suggest that carbachol-mediated pigment granule dispersion occurs through the activation of M_{odd} muscarinic receptors, which in other systems couple to phosphoinositide hydrolysis and elevation of intracellular calcium. This conclusion must be corroborated by molecular studies, but suggests Ca^{2+}-dependent pathways may be involved in light-adaptive pigment dispersion.

Background

The retinal pigment epithelium (RPE) of teleost fishes undergoes diurnal changes in the position of its pigment granules, dispersing them into long apical projections in the light and aggregating them into the cell body in the dark (see [1]). These movements are coordinated with changes in the position of rod and cone photoreceptors and are thought to optimize light capture by the relevant photoreceptors (rods in the dark; cones in the light). The RPE is not itself sensitive to light, and several lines of evidence suggest that it relies on paracrine signals from the retina to accomplish appropriate movements (see [2]). Although light-adaptive pigment granule movements occur only in "lower" vertebrate classes, the question of how the retina communicates with the RPE is relevant to normal retinal and RPE function in many vertebrate species and may contribute to greater understanding of the function of the pineal organ as well. RPE is crucial for normal visual function, and defects in the RPE are associated with a number of diseases that lead to retinal degeneration and blindness (for an example, see [3]).

It was established 15 years ago that dopamine was an important light signal in the retina of green sunfish [2,4] and bullfrog [5]. In green sunfish, pharmacological studies indicated that dopamine works through D_2 receptors [2], which are negatively coupled to adenylyl cyclase and cause cAMP levels in cells to decrease [6]. However, work by others [7,8] raised the possibility that other neurochemicals could be involved in regulating light adaptation in fishes. The finding that the cholinergic agonist carbachol induces pigment granule dispersion in green sunfish was the first evidence that retinomotor movements can be elicited by activating acetylcholine receptors in addition to dopamine receptors [9].

Acetylcholine has been shown to act through two major types of receptors in other systems, nicotinic and muscarinic receptors (see [10]). Nicotinic receptors are ligand-gated ion channels, while muscarinic receptors belong to the G-protein coupled receptor superfamily of seven transmembrane domain proteins. Five types of muscarinic receptor (M_1–M_5) have been defined in mammals (see [10]), and recent studies have demonstrated that zebrafish have at least two muscarinic receptor genes [11]. Heterologous systems in which a single, cloned receptor-type is expressed in cell types not normally expressing muscarinic receptors have demonstrated the receptors to be coupled to multiple intracellular signaling pathways. In most native systems, M_1, M_3 and M_5 receptors are coupled to phosphoinositide hydrolysis and calcium mobilization while M_2 and M_4 receptors are coupled to adenylyl cyclase through $G_{inhibitory}$ proteins. Additionally, in some cases, M_2 receptors are also coupled to potassium channels (see [10,12,13]).

We report here that carbachol-induced pigment granule dispersion occurs in RPE isolated from bluegill (*Lepomis macrochirus*). Furthermore, our results using a pharmacological approach suggest that carbachol acts on one or more of the "odd" subtypes (M_1, M_3 or M_5) of muscarinic receptors to elicit light adaptive pigment granule dispersion. We suggest, therefore, that acetylcholine may act in concert with dopamine or other mechanisms which reduce cellular cyclic adenosine monophosphate levels to assure appropriate, light adaptive pigment granule movement in the retinas of fishes.

Results

Forskolin induces pigment granule aggregation

The adenylyl cyclase activator forskolin induced pigment granule aggregation in isolated RPE in a dose-dependent manner as determined by evaluating the pigment position using the pigment index (PI; see [14]) (Table 1). Cells incubated in 10 μM forskolin were significantly aggregated compared to control samples incubated in isolation buffer alone ($p < 0.05$). Therefore, in subsequent experiments tissue was induced to aggregate pigment by incubating tissue in 10 μM forskolin prior to treatment with cholinergic agents.

Table 1: Forskolin induces pigment aggregation in a dose-dependent manner.

Concentration	*Pigment Index ± SEM*	*n*
0 μM	0.98 ± 0.00	6
0.1 μM	0.93 ± 0.00	3
1 μM	0.89 ± 0.01	3
10 μM	0.75 ± 0.02	2
100 μM	0.71	1

RPE was isolated from dark-adapted bluegill and incubated 45 minutes in increasing concentrations of forskolin. Tissue fixed prior to incubation in forskolin had a pigment index of 0.97 ± 0.00 (n = 6 fish). Maximal aggregation was attained by concentrations as low as 10 μM.

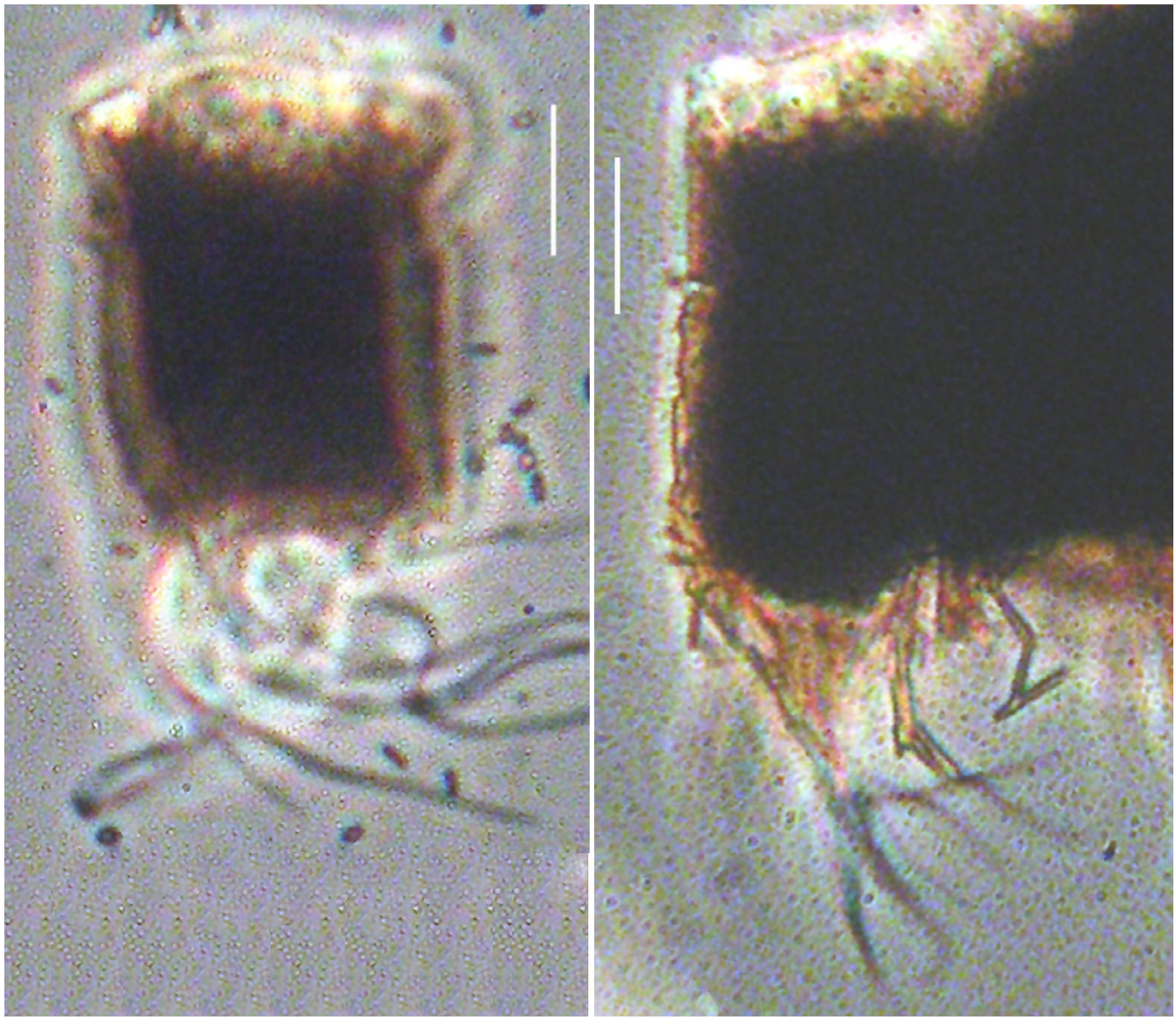

Figure 1

Phase contrast images illustrate RPE with pigment granules aggregated (left) and RPE with pigment granules dispersed (right). RPE were isolated from bluegill and treated with 10 μM forskolin for 45 minutes to induce aggregation. A subset was fixed overnight and prepared for microscopy. In a second subset forskolin was washed out, and RPE were treated with 100 nM carbachol to induce dispersion. After 45 minutes, the second subset was fixed overnight and prepared for microscopy. Images were obtained using a digital camera focused through the oculars of a Zeiss microscope. Scale bars = 10 μm.

Carbachol induces pigment granule dispersion by activating muscarinic receptors

The application of the general cholinergic agonist carbachol to isolated RPE that had been pretreated with forskolin caused pigment granule dispersion in a dose-dependent manner (Figures 1 and 2). The pigment index is the ratio of the length of the cell occupied by pigment to the total length of the cell, and approaches unity as the pigment granules become increasingly dispersed. Cells treated with 10 nM carbachol had significantly higher pigment indices (PI = 0.84 ± 0.04; n = 6) than control cells incubated in the absence of carbachol (PI = 0.73 ± 0.03; n = 6) ($p < 0.05$); although, the latter underwent slight, statistically significant dispersion, as well, relative to the forskolin-treated cells. Cells treated with concentrations greater than 10 nM did not disperse significantly further.

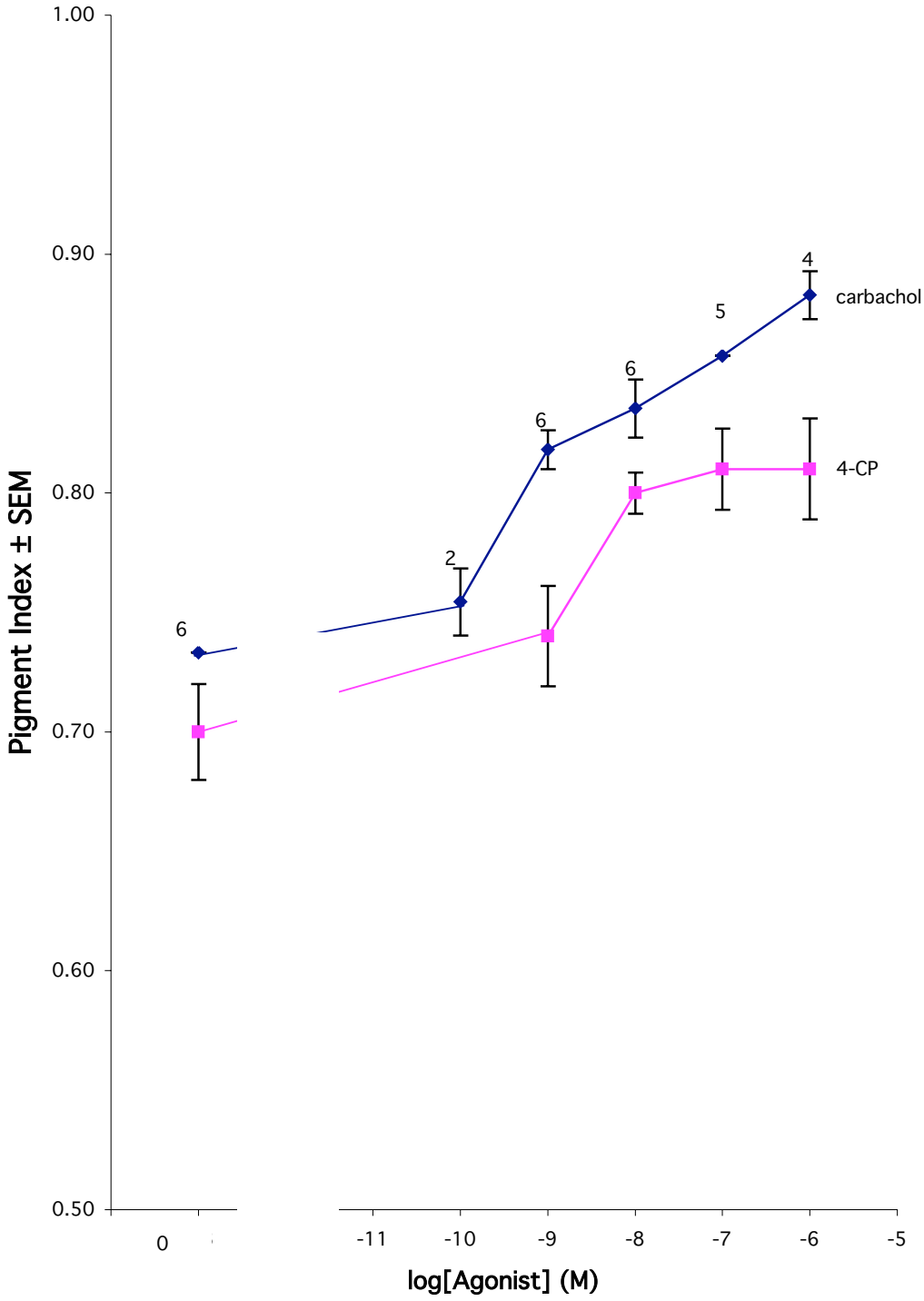

Figure 2
Carbachol induces pigment granule dispersion in isolated RPE. RPE were isolated from bluegill and, following treatment with forskolin to induce aggregation, were subjected to increasing concentrations of either carbachol or the M1 receptor agonist 4-[N-(4-chlorophenyl) carbamoyloxy]-4-pent-2-ammonium iodide (n = 4 at each concentration, except as noted for carbachol). Both agonists induced dose-dependent, saturable pigment granule dispersion with the former eliciting maximal dispersion at concentrations as low as 1 nM and the latter at concentrations as low as 10 nM. In contast, the M2 agonist arecaidine but-2-ynyl ester tosylate did not effectively stimulate pigment granule dispersion (see Table 2).

Even though the maximal effect of carbachol was seen with the 10 nM treatment, 100 nM carbachol was used in subsequent experiments testing antagonist effects, to ensure maximal response.

In order to examine if muscarinic receptors were involved in mediating carbachol-induced dispersion, we tested the ability of atropine, a muscarinic antagonist that blocks M_1–M_5 receptors, to inhibit carbachol-induced dispersion. We found atropine to be a highly effective blocker of carbachol-induced dispersion (Figure 3). Indeed, at concentrations as low as 10 pM, atropine completely inhibited carbachol-induced dispersion. This inhibition is illustrated by the observation that the mean pigment index of cells treated in carbachol alone (PI = 0.83 ± 0.02) is significantly higher compared to the mean pigment index of cells treated concurrently with 10 pM atropine and 100 nM carbachol (PI = 0.69 ± 0.03) ($p < 0.05$).

Carbachol exerts its effects through an M-odd receptor

A battery of pharmacological agents was tested to better characterize the receptor subtype involved in carbachol-induced pigment dispersion. Firstly pirenzepine, an M_1 blocker, was examined and found to be effective at blocking carbachol-induced dispersion (Figure 3) with 10 nM pirenzepine treatment (PI = 0.67 ± 0.02) being significantly more aggregated than the control incubated in carbachol alone (PI = 0.81 ± 0.01). However, at concentrations greater than 10 nM, pirenzepine-based inhibition was not significantly increased, suggesting receptor saturation at 10 nM pirenzepine.

Secondly, after discovering the M_1 blocker pirenzepine was effective in inhibiting carbachol-induced dispersion, the efficacy of the M_1 agonist, 4-[N-(4-chlorophenyl) carbamoyloxy]-4-pent-2-ammonium iodide, hereafter referred to as 4-CP, in inducing pigment granule dispersion was examined. 4-CP is a potent stimulator of pigment granule dispersion (Figure 2). Indeed, the pigment granule position (PI = 0.80 ± 0.01) in the 10 nM treatment was more dispersed than cells treated in buffer alone (PI = 0.70 ± 0.01) ($p < 0.05$). In fact, 10 nM 4-CP induced the maximal response ($p < 0.05$), as greater concentrations did not cause pigment granules to disperse significantly further.

Next the M_3 antagonist 4-DAMP was tested and was found effective in blocking carbachol-induced dispersion (Figure 3). Maximal inhibition occurred in the 10 nM treatment group (PI = 0.65 ± 0.02), with cells incubated in this treatment having significantly more aggregated pigment than the control cells incubated in carbachol alone (PI = 0.75 ± 0.00) ($p < 0.05$).

Following examination of agents selective for M_{odd} receptors, the effects of M_{even} selective agents were examined. The ability of the M_2 blocker, AF-DX 116, and the M_4 inhibitor tropicamide to inhibit carbachol-induced pigment dispersion was examined. AF-DX 116 was not effective in blocking carbachol-induced dispersion (Figure 3). The pigment index of the control cells treated with carbachol, but with no inhibitor (PI = 0.87 ± 0.01) was greater than that from cells treated in forskolin alone (PI = 0.68 ± 0.01) ($p < 0.05$). Yet, the pigment indices of RPE treated with both AF-DX 116 and carbachol were not significantly different from pigment indices of the cells treated with carbachol alone. Similarly, there was no significant difference between the pigment index of control cells treated with carbachol and those treated with both carbachol and tropicamide.

Finally the M_2 agonist arecaidine but-2-ynyl ester tosylate (arecaidine) did not induce pigment granule dispersion. Neither the pigment indices of the control cells incubated in 0.01% DMSO, nor the pigment indices of cells treated in arecaidine were significantly different from cells treated in forskolin alone (Table 2).

Discussion

Here we show that carbachol induces pigment granule dispersion in RPE isolated from bluegill (*Lepomis macrochirus*). The maximal effect of carbachol was seen at concentrations as low as 10 nM. This result is consistent with García's [9] earlier study in which carbachol induced pigment granule dispersion in isolated RPE of green sunfish (*Lepomis cyanellus*). Using bluegill, we have shown that carbachol-induced pigment granule dispersion is not species-specific and that carbachol-induced pigment dispersion is dose-dependent and saturable, indicating a receptor-mediated process. Atropine, a general muscarinic antagonist, was shown to block pigment granule dispersion, suggesting muscarinic receptors mediate carbachol-induced dispersion. Furthermore, because M_1 and M_3 antagonists strongly inhibited pigment granule dispersion induced by carbachol and because the M_1 agonist 4-CP induced pigment granule dispersion, the involvement of M_1 or M_3 receptors is suggested. The involvement of an M_5 receptor cannot be addressed pharmacologically at this time since no M_5-selective agents are currently commercially available.

Although the results from the pharmacological studies with muscarinic receptor agonists and antagonists are internally consistent with the interpretation that M_{odd} receptors are involved in mediating carbachol-induced dispersion (M_{odd} reagents are effective; M_{even} are not), drug studies in embryonic chick have illustrated that pharmacological results taken by themselves can be misleading. Pirenzepine, although described as an M_1-selective

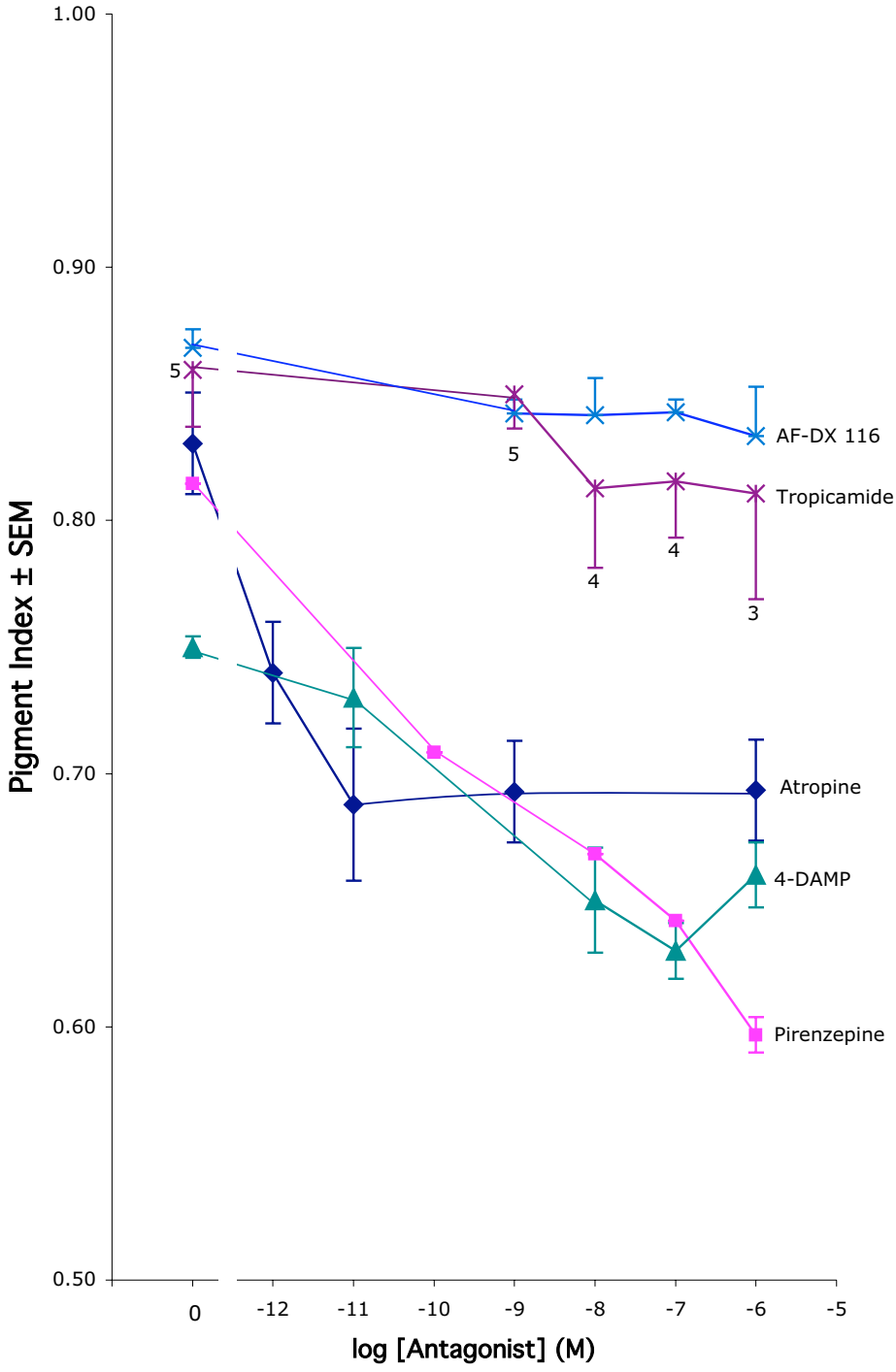

Figure 3

Atropine and M_{odd} muscarinic agonists block carbachol-induced pigment granule dispersion. RPE were isolated from bluegill, and following treatment with forskolin were subjected to 100 nM carbachol and varying concentrations of muscarinic receptor antagonists (n = 4, except as noted for tropicamide). The general muscarinic receptor antagonist atropine completely blocked carbachol-induced dispersion at concentrations as low as 10 pM. The M1 antagonist pirenzepine and the M3 antagonist 4-DAMP also blocked dispersion at concentrations as low as 10 nM. In contrast, the M2 antagonist AF-DX 116 and the M4 antagonist tropicamide were ineffectual at blocking carbachol-induced pigment dispersion, even at concentrations as high as 1 μM.

Table 2: Arecaidine but-2-ynyl ester tosylate does not induce pigment granule dispersion

Concentration	*Pigment Index ± SEM*	*n*
0 nM	0.74 ± 0.01	4
1 nM	0.77 ± 0.03	4
10 nM	0.80 ± 0.02	4
100 nM	0.78 ± 0.02	4
1 μM	0.78 ± 0.01	4

RPE was isolated from dark-adapted bluegill and, following incubation for 45 minutes in 10 μM forskolin, was incubated an additional 45 minutes in increasing concentrations of arecaidine but-2-ynyl ester tosylate or in a DMSO control. At the end of incubation in forskolin, RPE had a pigment index of 0.72 ± 0.02 (n = 4 fish); the 0.01% DMSO control had a pigment index of 0.69 ± 0.02 (n = 2 fish).

antagonist in both mammals and trout [15,16], avidly binds M_2 receptors in chick [16] and may bind M_4 receptors in other systems as well [17]. Furthermore, Hsieh and Liao [11] have reported that pirenzepine binds the M_2 receptor they have isolated from zebrafish with a submicromolar dissociation constant. Therefore, a molecular characterization of the receptor subtype(s) expressed by bluegill RPE must be done before a definitive assignment can be made. These studies are currently underway in our laboratory.

Given the ambiguity associated with the pharmacology of muscarinic receptors in non-mammalian vertebrates, the question of which muscarinic receptor is involved in carbachol-induced pigment granule dispersion remains open. Based on our current knowledge of the signaling pathways involved in regulating pigment granule movement, the simplest model for muscarinic receptor involvement would invoke an M_{even} receptor negatively coupled to adenylyl cyclase through $G_{inhibitory}$ proteins (Figure 4). According to this model, activation of the receptor would lead to inhibition of adenylyl cyclase, resulting in decreased $cAMP_i$ levels due to degradation by phosphodiesterases, efflux via organic anion transporters, or both. As a consequence of decreased $cAMP_i$, the activity of cAMP-dependent protein kinase (PKA) would diminish. The activity of protein phosphatases would then tip the balance between phosphoproteins and their dephosphorylated counterparts toward the latter, and this, in turn, would lead to pigment granule dispersion.

Consistent with this model are the observations that treatments expected to elevate $cAMP_i$ induce pigment granule aggregation in RPE isolated from bluegill (Table 1), green sunfish [2,14,18], and blue-striped grunt [19]. Furthermore, King-Smith et al. [20] demonstrated that simply washing away exogenously applied, extracellular cAMP was sufficient to induce pigment granule dispersion in both isolated RPE sheets and dissociated RPE cells. Importantly, elevation of cAMP caused aggregation and washout of cAMP caused dispersion irrespective of the external or internal concentrations of calcium. We have also observed exogenous cAMP induces aggregation in RPE isolated from bluegill, and its washout is sufficient to induce dispersion (García, unpublished observations). Presumably, in both bluegill and green sunfish RPE removing extracellular cAMP not only eliminated the supply of cAMP available for import via organic anion transporters [18], but also reversed the gradient, favoring its export. In addition, microinjection of the PKA inhibitor PKI_{5-24} amide into dissociated cells isolated from green sunfish resulted in pigment granule dispersion (García, unpublished observations).

The central importance of cAMP in regulating pigment granule position is also suggested by studies conducted on dermal melanophores of tilapia (*Tilapia mossambica*) [21-25], black tetra (*Gymnocorymbus ternetzii*) [26], and angelfish (*Pterophyllum scalare*) [27] among others (reviewed in [28]). In each of these cases, increased $cAMP_i$ is associated with pigment granule dispersion and decreased $cAMP_i$ with pigment granule aggregation. Studies done on permeabilized melanophores from tilapia demonstrated that the direction of pigment granule movement was dictated by the addition and removal of cAMP [22] and that pigment granule dispersion could be induced by addition of the catalytic subunit of PKA [25]. Similarly, in melanophores isolated from black tetra cAMP levels measured immunohistochemically tracked closely with extent of pigment granule movement toward the plus-end of microtubules during pigment granule dispersion [26]. Sammak et al. [27] observed fluxes in $cAMP_i$ as well as Ca^{2+} levels in melanophores isolated from angelfish, but found that only the former were necessary and sufficient to influence pigment granule position, whereas the latter were neither necessary nor sufficient to influence pigment granule movement. Similarly, Kotz and McNiven [29] presented pharmacological evidence suggesting that reducing

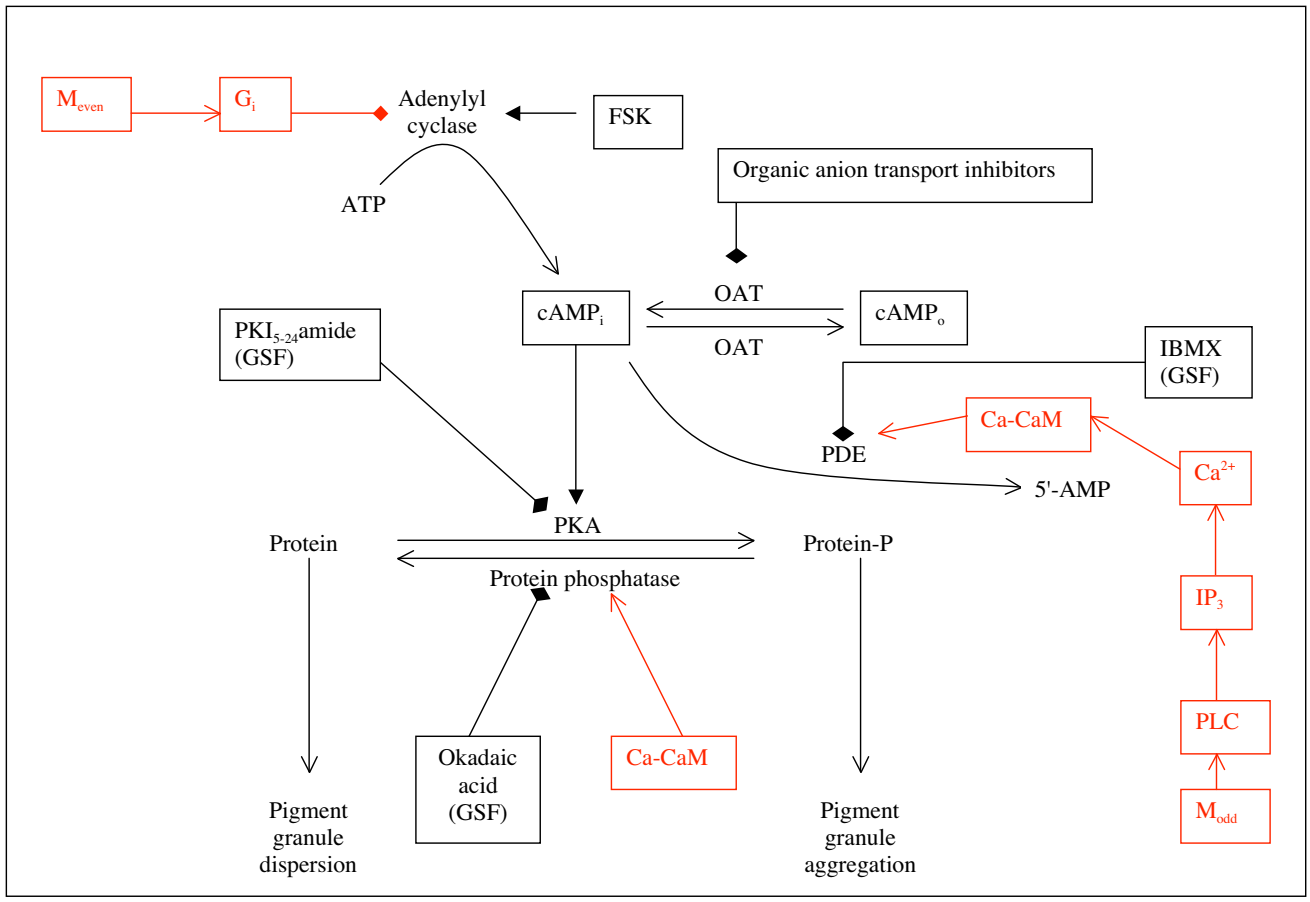

Figure 4

Schematic diagram illustrating second messenger pathways possibly involved in regulating pigment granule movement in RPE and entry points for muscarinic regulation. Most evidence suggests a central role for cAMP-dependent phosphorylation events in the induction of pigment granule aggregation in retinal pigment epithelium. Shown in black boxes in this schematic diagram are players in this pathway that have been manipulated experimentally in RPE isolated from bluegill, green sunfish or both. Pathways shown in red indicate possible regulatory inputs from muscarinic receptor activation (see text for a more extensive explanation as well as references). Abbreviations used are as follows (listed alphabetically): **5'-AMP**, 5'-adenosine monophosphate; **Ca^{2+}**, calcium; **Ca-CaM**, calcium-calmodulin complex; **$cAMP_i$**, intracellular cyclic adenosine monophosphate; **$cAMP_o$**, extracellular cyclic adenosine monosphosphate; **FSK**, forskolin; **G_i**, inhibitory GTP-binding protein; **GSF**, green sunfish (indicating that this experimental manipulation was only performed on RPE isolated from green sunfish); **IBMX**, isobutylmethylxanthine (a phosphodiesterase inhibitor); **IP_3**, inositol trisphosphate; **M_{even}**, muscarinic acetylcholine receptor subtype 2 or 4; **M_{odd}**, muscarinic acetylcholine receptor subtype 1, 3, or 5; **OAT**, organic anion transporter; **PDE**, phosphodiesterase; **PKI_{5-24}amide**, cAMP-dependent protein kinase inhibitory peptide (amino acids 5–24) with an amide adduct; **PLC**, phospholipase C; **Protein-P**, a phosphoprotein; **arrows terminating in diamonds** signify inhibitory effects; **arrows terminating in triangles or barbs** signify stimulatory effects.

$cAMP_i$ was sufficient to stimulate aggregation in squirrelfish melanophores, while Ca^{2+} dynamics appeared variable and unimportant.

Although the simplest model for muscarinic regulation would be through G-protein-mediated inhibition of adenylyl cyclase, a model involving activation of phospholipase C must also be considered. While evidence exists in heterologous expression systems for M_1 receptor-based inhibition of adenylyl cyclase [17], M_{odd} receptor activation is more typically associated with activation of phospholipase C. In general, phospholipase C catalyzes the conversion of phosphatidylinositol bisphosphate to diacylglycerol and inositol trisphosphate (IP_3). IP_3, in turn,

activates ligand-gated Ca^{2+} channels located on intracellular storage organelles, leading to release of Ca^{2+} from these stores, and an elevation of cytoplasmic free Ca^{2+}. Cytoplasmic Ca^{2+} typically binds to and activates calmodulin, which modulates the activities of a number of enzymes, including certain phosphodiesterases (e.g. PDE1) as well as some protein phosphatases (e.g. calcineurin). In the former case, increasing phosphodiesterase activity would decrease cAMP levels, leading to decreased PKA activity and decrease in the rate of phosphorylation of PKA-target proteins. In addition, if protein phosphatases were activated, the tendency for target proteins to be dephosphorylated would be further enhanced. It should be noted that calcineurin has been shown to be involved in regulating pigment granule movement in melanophores isolated from tilapia [25]. In either case (or both cases), the balance would be shifted to dephosphorylated proteins which, though not yet identified, seem to favor pigment granule dispersion.

The results we present herein are consistent with this model in that agents characterized as M_{odd}-selective in other systems affected the pigment granule movement under study, while M_{even}-selective agents did not [10]. However, one wonders how to reconcile these results with those reported for green sunfish RPE [20] or melanophores [27,29] showing that Ca^{2+} transients are neither necessary nor sufficient for pigment granule movement in either direction, i.e. aggregation or dispersion. One possibility is that the Ca^{2+} "requirement" for pigment granule dispersion in RPE can be bypassed or overruled by other mechanisms. For example, King-Smith et al. [20] observed that in RPE isolated from green sunfish pigment granule dispersion initiated by cAMP-washout could be accomplished in the absence of extracellular Ca^{2+}, following depletion of intracellular stores by incubating RPE sheets in Ca^{2+}-free medium, or damping changes in intracellular Ca^{2+} by infiltrating the cells with the Ca^{2+}-chelator BAPTA. In any of these cases, $cAMP_i$ may have been attenuated in the cells by efflux through the activity of organic anion transporters in addition to basal activity of phosphodiesterases. Thus, a requirement for Ca^{2+}-based reduction in either cAMP or phosphoproteins could be bypassed by a mechanism involving cAMP-efflux. RPE isolated from green sunfish seem to have a high basal phosphatase activity since treatment with okadaic acid is sufficient to induce pigment granule aggregation (García, unpublished results).

King-Smith et al. [20] also showed that elevating Ca^{2+} by treating cells with ionomycin was not sufficient to prevent aggregation when 1 mM cAMP was added to the medium. Earlier work done by García and Burnside [18] suggested that cAMP was imported into RPE cells via organic anion transporters. Thus, given a sufficient gradient for import, cAMP levels in the cell could remain high along with PKA activity, resulting in a continual phosphorylation of target proteins associated with pigment granule aggregation, even in the face of increased phosphodiesterase activity or phosphatase activity.

Also consistent with the results we report here suggesting that bluegill RPE express muscarinic receptors are the observations that human [30,31] and rat [32] RPE express muscarinic receptors. The receptors on human RPE have been linked to phosphoinositide hydrolysis [30,31], suggesting that they belong to the M_1, M_3 or M_5 subclass or some combination of these subclasses. Based on pharmacological studies, Feldman *et al*. [30] concluded that M_3 receptors are the subtype present on human RPE.

Conclusions

The pharmacological studies reported herein indicate the involvement of muscarinic acetylcholine receptors in carbachol-induced pigment granule dispersion in RPE isolated from bluegill, and further suggest that they belong to the odd-subtypes. This conclusion requires corroboration from on-going molecular studies and suggests further experiments to look at downstream signaling pathways.

Methods

Fish maintenance

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

Isolation of RPE and drug treatments

All experiments were carried out in dim, incandescent light (≤2 lux). In order to facilitate isolation of RPE, fish were dark-adapted for thirty minutes in a light-tight box, prior to dissection during subjective midday (about 6 hours after light onset). Fish were killed by severing the spine followed by double pithing. Eyeballs were removed and hemisected, and the cornea, lens, vitreous humor, and the neural retina were discarded. RPE sheets were flushed out of the eyecup by applying a steady stream of modified Ringer's solution (isolation buffer). The isolation buffer contained 24 mM $NaHCO^-_3$, 3 mM HEPES (free acid), 116 mM NaCl, 5 mM KCl, 1 mM $NaH_2PO_4.H_2O$, 26 mM dextrose, 1 mM ascorbic acid, 0.8 mM $MgSO_4$, 1 mM EGTA, and 0.9 mM $CaCl_2$. Free Ca^{2+} concentrations were estimated to be 10^{-5} M (see [9,18]). The isolation buffer was gassed with a mixture of 95% air and 5% CO_2 for at least 15 minutes prior to and throughout the dissection to maintain the pH at 7.2. The RPE sheets were divided into 6 samples and incubated in 24

Table 3: Cholinergic agonists and antagonists used in this study

Agonist	*Site of Action*	*Induced Pigment Granule Dispersion*
Carbachol	M1-M5	Yes
4-Chlorophenyl	M1	Yes
Arecaidine but-2-ynyl ester tosylate	M2	No
Antagonist	***Site of Action***	***Blocked Pigment Granule Dispersion***
Atropine	M1-M5	Yes
Pirenzepine	M1	Yes
AF-DX 116	M2	No
4-DAMP	M3	Yes
Tropicamide	M4	No

RPE was isolated from dark-adapted bluegill and, following incubation for 45 minutes in 10 μM forskolin, was incubated an additional 45 minutes in increasing concentrations of agonist or in the presence of 100 nM carbachol and increasing concentrations of antagonists. The agonists and antagonists tested and their efficacy are presented.

well plates in a humidified chamber according to the regimens described below.

Isolated RPE undergo pigment dispersion [18]; therefore, pigment granule aggregation in all samples was induced by a 45-minute treatment with the adenylate cyclase stimulator, forskolin (Calbiochem, La Jolla, CA). Dose response analysis of forskolin was carried out using forskolin (10 mM) resuspended in DMSO and then diluted serially in low calcium Ringer's solution to concentrations ranging from 0.1 μM to 100 μM. Control tissues were incubated in low calcium Ringer's alone or in low calcium Ringer's with 0.01% DMSO.

For experiments to test the efficacy of cholinergic agonists and antagonists, following isolation, tissue was treated for 45 minutes with 10 μM forskolin, after which forskolin was washed out 3 times using isolation buffer prior to further treatment with agonists and antagonists.

The effectiveness of various cholinergic agonists was evaluated by dose response analysis. Agonists (see Table 3 for list of agonists and antagonists) were prepared in isolation buffer or DMSO (arecaidine but-2-ynyl ester tosylate) and were applied following wash out of forskolin (see above). In order to maintain the pH (7.2) constant throughout the experiment, the tissue was incubated with agonist for 45 minutes in a humidified chamber gassed with a mixture of 95% air and 5% CO_2 on a gyratory shaker (50 rpm). Control tissue was incubated in low calcium Ringer's solution or for the experiments testing arecaidine in 0.01% DMSO. The cells were fixed by adding a 2 × stock solution of fixative to the isolation buffer to achieve a final concentration of 0.5% glutaraldehyde, 0.5% paraformaldehyde, and 0.8% potassium ferricyanide.

Separate experiments were conducted using antagonists, also prepared in isolation buffer, as a 2 × stock solution. After washing out the forskolin with isolation buffer, antagonists (see Table 3) were applied, immediately followed by application of carbachol (100 nM) in equal parts. After a 45-minute incubation, the RPE was fixed (see above).

Preparation of tissue for measurement and statistical analysis

After fixing the tissue overnight, individual RPE cells were dissociated by chopping the RPE sheets on a glass slide using a No. 1 coverslip. RPE fragments were then mounted on the slide and were viewed under a phase contrast microscope. The determination of pigment granule position was done by calculating pigment indices (PI) [14]. The pigment index is a ratio of the length of the cell occupied by pigment to the total length of the cell. Using an ocular micrometer, in most cases thirty cells per treatment per fish were measured and the mean PI was calculated. In four cases as few as nine cells per treatment per fish were measured, and the mean pigment index obtained was included in the analysis.

Dose response curves were plotted to determine if the agonist or antagonist treatment affected the pigment index. The pigment indices plotted are the average of the mean PI calculated (see above). The error bars represent the standard error of the mean. The n values represent the number of fish used in obtaining the data and is 4 fish unless otherwise noted. Statistical comparisons were made among pigment indices yielded from different

concentrations of a single drug, but were not made among pigment indices yielded from treatment with different drugs. To determine if the treatment means were significantly different, one-way analysis of variance (one-way ANOVA) followed by Tukey's multiple comparison test was used with comparisons made using the summary data (the means from each fish from each treatment). To test if dispersion occurred in control samples (no agonist applied), treatment means between the forskolin sample and the control sample were analyzed using a Student's t-test. All statistical analyses were utilized assuming that the pigment indices were normally distributed and variances between means were equal. Statistical significance was reported when $p < 0.05$.

List of abbreviations used

4-CP 4-[N-(4-chlorophenyl) carbamoyloxy]-4-pent-2-ammonium iodide

4-DAMP 4-diphenylacetoxy-N-methylpiperidine

5'-AMP 5'-adenosine monophosphate

AF-DX 116 11-[[[2-Diethylamino-*O*-methyl]-1-piperidinyl]acetyl]-5,11-dihydrol-6 *H*-pyridol [2,3-*b*][1,4]benzodiazepine-6-one

ANOVA analysis of variance

BAPTA 1,2-*bis*-(*o*-Aminophenoxy)ethane-N,N,N',N'-tetraacetic acid

Ca-CaM calcium-calmodulin complex

cAMP 3',5'-cyclic adenosine monophosphate

DMSO dimethylsulfoxide

EGTA Ethyleneglycotetraacetic acid

FSK forskolin

G_i inhibitory GTP-binding protein

GSF green sunfish

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IBMX isobutlymethlyxanthine

IP_3 1,4,5-inositol trisphosphate

M_{even} muscarinic acetylcholine receptor type 2, 4 or both

M_{odd} muscarinic acetylcholine receptor type 1,3, 5 or some combination

OAT organic anion transport

PDE phosphodiesterase

PI pigment index

PKA cAMP-dependent protein kinase

PKI_{5-24} amide PKA-inhibitory peptide (amino acids 5–24) with amide adduct

PLC phospholipase C

Protein-P phosphoprotein

RPE retinal pigment epithelium

rpm rotations per minute

SEM standard error of the mean

TX Texas

Authors' contributions

AG conducted most of the pharmacological experiments, the statistical analysis and prepared a first draft of the manuscript. EC carried out the experiments testing the efficacy of tropicamide. DG conceived of and oversaw the project, secured funding, substantially revised the discussion and prepared the manuscript for publication.

Acknowledgements

The authors are grateful for help with the statistical analysis provided by Drs. James R. Ott and Butch Weckerly. The authors also wish to thank Dr. Joseph Koke for help in preparing figure 1, and Drs. Koke and Simon Durdan and Mr. Prasad Phatarpekar for critically reading the manuscript. Finally, the authors thank Mr. Chad Copeland for proof-reading the final version. This study was funded primarily by a National Science Foundation research grant and REU supplements (IBN 00-77666, 01-32212, and 0228857) and a teacher enhancement grant (ESIE 9731321). Support was also provided by the National Institutes of Health through a Bridges to the Baccalaureate grant (GM 58375-01A1).

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