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Calcium-independent regulation of pigment granule aggregation and dispersion in teleost retinal pigment epithelial cells

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SUMMARY

In the eyes of teleosts and amphibians, melanin pigment granules of the retinal pigment epithelium (RPE) migrate in response to changes in light conditions. In the light, pigment granules disperse into the cells' long apical projections, thereby shielding the rod photoreceptor outer segments and reducing their extent of bleach. In darkness, pigment granules aggregate towards the base of the RPE cells. In vitro, RPE pigment granule aggregation can be induced by application of nonderivatized cAMP, and pigment granule dispersion can be induced by cAMP washout. In previous studies based on RPE-retina co-cultures, extracellular calcium was found to influence pigment granule migration. To examine the role of calcium in regulation of RPE pigment granule migration in the absence of retinal influences, we have used isolated RPE sheets and dissociated, cultured RPE cells. Under these conditions depletion of extracellular or intracellular calcium ($[Ca^{2+}]_o$, $[Ca^{2+}]_i$) had no effect on RPE pigment granule aggregation or dispersion. Using the intracellular calcium dye fura-2 and a new dye, fura-pe3, to monitor calcium dynamics in isolated RPE cells, we found that $[Ca^{2+}]_i$ did not change from basal levels when pigment granule aggregation was triggered by cAMP, or dispersion was triggered by cAMP washout. Also, no change in $[Ca^{2+}]_i$ was detected when dispersion was triggered by cAMP washout in the presence of 10 μM dopamine, a treatment previously shown to enhance dispersion. In addition, elevation of $[Ca^{2+}]_i$ by addition of ionomycin neither triggered pigment movements, nor interfered with pigment granule motility elicited by cAMP addition or washout. Since other studies have indicated that actin plays a role in both pigment granule dispersion and aggregation in RPE, our findings suggest that RPE pigment granule migration depends on an actin-based motility system that is not directly regulated by calcium.

Key words: RPE, Pigment granule migration, Calcium, Fura-pe3

INTRODUCTION

Intracellular transport occurs in all eukaryotic cells. Force production for transport may be provided by motor proteins moving along microtubules or actin filament tracks, or by assembly and disassembly of cytoskeletal components (see Wadsworth, 1993; Cramer et al., 1994, for reviews). Intracellular transport is often regulated in time and space by messenger molecules that coordinate motile events with physiological demands. The regulatory mechanisms for intracellular transport are only beginning to be understood.

Our laboratory has been using pigment granule migration in the teleost retinal pigment epithelium (RPE) as a model with which to study the regulation and mechanisms of force production for intracellular particle transport (Burnside et al., 1982; Bruenner and Burnside, 1986; Garcia and Burnside, 1994; King-Smith et al., 1995). In the RPE of teleosts and amphibians, melanin pigment granules migrate into and out of the RPE cells' long apical projections in response to changes in light condition (see Burnside and Nagle, 1983, for review). Pigment granules disperse into apical projections in the light and aggregate to the base of the RPE cells in the dark. Since the apical projections interdigitate with rod photoreceptor outer segments, pigment dispersion reduces the extent of rod photopigment bleach in bright light (Back et al., 1965).

Although we do not yet understand the mechanism by which light regulates pigment granule position in RPE cells, several observations suggest that some extracellular signal from the retina is required, and that cAMP plays an important role in the intracellular signaling pathway. In the absence of retina, light has no effect on RPE pigment position in isolated RPE sheets or dissociated RPE cells (Bruenner and Burnside, 1986). However, treatments that elevate cAMP induce RPE pigment granule aggregation both in vivo and in isolated RPE preparations (Burnside et al., 1982; Burnside and Basinger, 1983; Bruenner and Burnside, 1986). Intracellular cAMP may be elevated either by stimulating endogenous adenylate cyclase with forskolin or by applying exogenous nonderivatized cAMP (Garcia and Burnside, 1994); both trigger pigment granule aggregation in isolated teleost RPE sheets and cells (Bruenner and Burnside, 1986; Garcia and Burnside, 1994).

Since the effect of exogenous nonderivatized cAMP (but not that of forskolin) is blocked by anion transport inhibitors, it seems likely that extracellular cAMP can enter RPE cells through anion transporters and, once inside the cell, trigger pigment granule aggregation. Thus, cAMP may participate as both an extra- and an intracellular messenger in regulation of RPE pigment granule migration (Garcia and Burnside, 1994). A possible source of exogenous cAMP is the retina, since retinal cAMP levels have been shown to increase in darkness in many vertebrate species (see Lolley, 1980, for review). In green sunfish, cAMP levels are three times higher in dark- than in light-adapted retinas (Burnside et al., 1982; Dearry and Burnside, 1985). These findings are consistent with previous reports that increasing intracellular cAMP levels can produce increases in extracellular cAMP (cf. Barber and Butcher, 1981).

In RPE-retina co-cultures or isolated RPE sheets, pigment granule dispersion can be induced by dopamine, acting through receptors of the D2 family (Dearry and Burnside, 1985, 1988, 1989). Since receptors of the D2 family usually inhibit adenylate cyclase, the effect of dopamine on pigment granule migration is also consistent with a role for cAMP. In many cell types that use cAMP as an intracellular messenger, calcium also plays a role in the signaling pathway, either opposing the action of cAMP or acting synergistically with cAMP (see Rasmussen, 1981, for review). It is not clear whether calcium is involved in regulating pigment granule migration in RPE cells. Although several investigators have reported calcium effects on RPE pigment granule migration, the site of calcium action was ambiguous in these studies, since they were performed using in vitro preparations that included not only the RPE but also the neural retina (Dearry and Burnside, 1984; Snyder and Zadunaisky, 1976; Mondragon and Frixione, 1989). In a preliminary study of isolated RPE cells from the green sunfish, *Lepomis cyanellus*, it was found that varying extracellular calcium had no affect on RPE pigment granule aggregation or dispersion (Bruenner and Burnside, 1986). To investigate the role of calcium in regulating RPE pigment granule migration more thoroughly, we have extended these initial studies to examine the effects of experimentally altering intracellular and extracellular calcium on pigment granule aggregation and dispersion in isolated RPE sheets and dissociated RPE cells from *Lepomis cyanellus*.

We report here that alteration of intra- or extracellular calcium had no effect on pigment granule aggregation or dispersion in RPE sheets or dissociated cells. For these studies aggregation and dispersion were triggered by cAMP application or washout, respectively, or cAMP washout in the presence of dopamine, which enhances the extent of pigment granule dispersion (Dearry and Burnside, 1989; Garcia and Burnside, 1994). In addition, we used the intracellular calcium indicator dye, fura-2, and a newly introduced dye, fura-pe3, to monitor intracellular calcium levels in isolated RPE cells when aggregation was induced by cAMP, or when dispersion was induced by cAMP washout with or without dopamine. Under these conditions we could detect no change in intracellular calcium during pigment granule aggregation or dispersion. Our results suggest that calcium changes are not required for triggering pigment granule dispersion or aggregation in teleost RPE cells.

MATERIALS AND METHODS

Experimental animals

Green sunfish, *Lepomis cyanellus*, were purchased from commercial suppliers (Fenders Fish Hatchery; Baltic, Ohio) and maintained in dechlorinated tapwater in indoor aquaria on a 14 hour:10 hour (light:dark) light cycle and fed fish chow. Animals were held in the laboratory for a minimum of two weeks prior to experimentation.

Isolation of RPE sheets and dissociated cells

RPE sheets were isolated as described previously (Garcia and Burnside, 1994). Saline used for sheet isolation was a modification of Earle's Ringer: 116.3 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.12 mM $MgSO_4$, 1.0 mM Na_2HPO_4, 25.5 mM glucose, 24.0 mM $NaHCO_3$, 3.0 mM HEPES, 1.0 mM ascorbic acid, 1.0 mM EGTA. EGTA buffered free calcium levels to a final concentration of 2.4 μM at pH 7.2 (Steinhardt et al., 1977). pH was initially adjusted to 7.4 and dropped to 7.2 after 30 minutes of gassing with 95% CO_2/5%O_2. This medium had a lower free calcium concentration than standard Earle's Ringer (2.4 μM compared to 1.8 mM); it was used in these experiments, since it had been found to be more conducive to pigment granule aggregation in RPE sheets compared to regular calcium Ringer (Dearry and Burnside, 1984).

Sheets from 3-5 fish were divided into groups and placed in 35 mm dishes or 24-well plates (Falcon) containing 0.8 ml culture medium in a humidified, gassed (95%O_2/5% CO_2) chamber and rotated at 50-60 rpm; treatments were initiated immediately after dissection and collection of sheets. Drugs used in experiments were added from 10× stock solutions. BAPTA, acetyl-methoxy ester (BAPTA-AM; Molecular Probes, Eugene, OR) was prepared fresh each day from frozen 10× stock solutions. At the end of each experimental treatment, sheets were fixed overnight after dilution with 5× concentrated fixative (final concentration 0.5% glutaraldehyde, 0.5% formaldehyde, 0.8% $K_3Fe(CN)_6$ in 0.1 M phosphate buffer, pH 7.0; Bruenner and Burnside, 1986). Fixative was made fresh on the day of the experiment.

Dissociated RPE cells were obtained from sheets of RPE as described previously (King-Smith et al., 1995). For scanning electron microscopy, isolated cells were cultured on 12 mm round coverslips, fixed as described above, and treated with osmium tetroxide (1% in 0.1 M phosphate buffer) for one hour. Cells were rinsed in distilled water, dehydrated in ethanol, processed in a critical-point dryer (Samdri PVT-3B, Tousimis, Rockville, MD). Samples were mounted on stubs, sputter-coated with gold (Polaron, San Jose, CA), and viewed on a DS 130 scanning electron microscope (Topcon Technologies Inc., Paramus, NJ) at 14 kV. For microinjection, cells were plated onto coverslips glued to 35 mm plastic Petri dishes that had a 18 mm hole cut out of the bottom. Prior to injection, 3-5 ml culture medium (without BSA) was added to each dish.

Calculation of pigment index

Pigment granule position was determined from RPE sheets as described by Garcia and Burnside (1994). Using this technique, cells that were totally dispersed had PI values approaching 1.00; the PI of aggregated cells ranged from 0.60 to 0.75. Thirty cells were measured per sample and the mean PI was calculated. These means were then averaged, and standard errors were calculated. Data are presented as the mean of several fish ± standard error of the mean (s.e.m.), where *N* represents the number of fish in each treatment. Cells that were lysed or had a total cell length less than 30 μm were not measured, and samples were discarded if fewer than 30 cells were measurable.

Dissociated RPE cells assumed a stellate configuration after culture, having apical projections extended radially from the cell body. The pigment index for dissociated cells was determined by taking the ratio of the area occupied by pigment granules to the total area of the cell with its projections. Total area was measured by connecting the tips of projections to form a polygon; the area occupied

by pigment granules was measured by connecting the distal-most pigment granule in each projection to form a polygon. Measurements were made using an image analysis program (Optomax, Inc, Burlington, MA). The mean PI from 20 cells was determined for each sample, and means and standard errors for each treatment were calculated as described above for sheets. The criterion for measurement was that cells had to have apical projections extending into each quadrant of a Cartesian plane.

Experiments using fura-2

Needles for microinjection of fura-2 were prepared from 1 mm glass capillary tubes containing a glass filament (WPI, Sarasota FL), washed in hot nitric acid and rinsed in distilled water. Needles were pulled on a flaming Brown-type horizontal micropipette puller (model P 80/PC; Sutter Instrument Co., Novato, CA) and backfilled with 5.4 mM fura-2 (pentapotassium salt; Molecular Probes) in either phosphate buffered saline (114 mM KCl, 20 mM NaCl, 3 mM $MgCl_2$, 3 mM NaH_2PO_4, pH 7) or a HEPES-buffered salt solution (50 mM HEPES, 0.1 mM $MgCl_2$, pH 7). No difference in results was observed in experiments using either of the two buffers. Typically 3-4 cells were microinjected and assayed in each experiment, and cells were used for up to 6-7 hours after isolation.

Photometric measurement of fura-2 fluorescence was carried out as described by Steinhardt et al. (1994). Epi-illumination at 340, 350 or 357 nm and 385 nm was provided by a xenon lamp with alternating interference filters. Use of the 357 filter tended to decrease the range between ratios for zero-calcium and saturated-calcium standards. Prior to each experiment, background emission at 340-357 and 385 nm was measured from uninjected cells and subtracted from subsequent measurements from injected cells.

Calibration of fura-2 emission was performed prior to each experiment using zero-calcium and saturated-calcium standard solutions in 130 mM KCl, 10 mM NaCl, 10 mM MOPS, pH 7, containing 1 μm fura-2. Calcium concentration was calculated from emission ratios using calibration ratios (Tsien et al., 1985) and a K_d of 130 nM. Measurements were corrected for cell viscosity by multiplying by 0.85 (Poenie, 1990). The calculations of intracellular calcium concentrations make many assumptions, and the actual value of $[Ca^{2+}]_i$ should be considered an estimate. We observed in some experiments that calculated basal $[Ca^{2+}]_i$ based on in vitro calibration ratios were offscale in the negative direction. Emphasis should therefore be placed on qualitative changes in $[Ca^{2+}]_i$.

After microinjection of fura-2, the 340-357/385 emission ratio was monitored to ensure that the cell recovered from the injection. After the basal ratio stabilized, 1 mM cAMP was added to the medium as a 10× solution to trigger pigment granule aggregation. Emission ratios were measured every second for 15 to 30 minutes, or until pigment granule movement was complete. To monitor intracellular calcium concentration during dispersion, 1 mM cAMP was added to cells prior to injection, to aggregate pigment granules. After injection, cAMP was washed out with three changes of Ringer containing no cAMP. Some dispersion experiments were conducted by washing out cAMP in Ringer containing 10 μM dopamine (RBI, Natick, MA). Cells were photographed after injection and after pigment granule movements were completed using Plus X Pan film, ASA 120. In some experiments digitonin (10 μM) was added to the medium to permeabilize cells and elicit a rise in $[Ca^{2+}]_i$. To demonstrate that calcium transients were detectable in RPE cells, in some experiments 0.1 M KCl was added to cells after fura-2 injection to depolarize cells.

Experiments using fura-pe3

Isolated RPE cells were loaded for 1 to 2 hours with 5 μM fura-pe3-AM (Texas Fluorescent Laboratories, Austin, TX) in HEPES-buffered Ringer containing 1.8 mM calcium and 0.4% BSA. Background fluorescence was measured from areas on the coverslip without cells, and subtracted from intensity measurements. Ionomycin (Calbiochem, San Diego, CA) used in some experiments was added to cells from 10× stock solutions. Cells loaded with fura-pe3 were used both for photometric ratio measurements and ratio imaging experiments. In vitro calibrations were carried out using fura pe-3 standards as described above for fura-2 measurements. For ratio imaging, individual RPE cells were viewed using an epifluorescence Nikon microscope equipped with a 40× oil immersion lens. Images of cells were acquired using a silicon-intensified camera (SIT-68, Dage-MTI, Inc., Michigan City, IN), and processed using Image 1/FL software (Universal Imaging Corp., West Chester, PA). A filter wheel (Lambda-10, Sutter Instrument Co., Novato, CA) was used for fluorescence excitation at 340 and 380 nm. Images were collected every 6 to 15 seconds, and data were stored and played back on a optical memory disk recorder (Panasonic TQ 2028F, Secaucus, NJ). Attempts at in vitro calibration as were made for photometric fura measurements resulted in saturation of the SIT camera, so data are reported as the 340/380 fluorescence ratios.

We considered whether melanin pigment granules might have affected the emission spectrum of fura in RPE cells. Sammak et al. (1992), in their study of regulation of pigment granules motility in teleost melanophores, observed little effect of purified melanin preparations on the emission spectrum of fura-2; the predominant effect of melanin in pigment granules was a reduction in fluorescence intensity from the area of the melanophores where pigment granules were most concentrated. We also observed a reduction in fluorescence intensity in the central regions of RPE cell bodies containing fura-2 or fura-pe3, but observed no differences in the fluorescence ratios in cell bodies compared to those in less heavily pigmented regions such as the apical projections (see Results).

RESULTS

Pigment granule migration in cultured RPE sheets and isolated RPE cells

As previously reported, isolated RPE sheets from *Lepomis cyanellus* retained their apical/basal polarity after isolation (see Dearry and Burnside, 1988; Garcia and Burnside, 1994). In contrast, when dissociated cells were allowed to settle and attach to glass coverslips, they spread out their apical projections around the cell body in a radial orientation (Fig. 1).

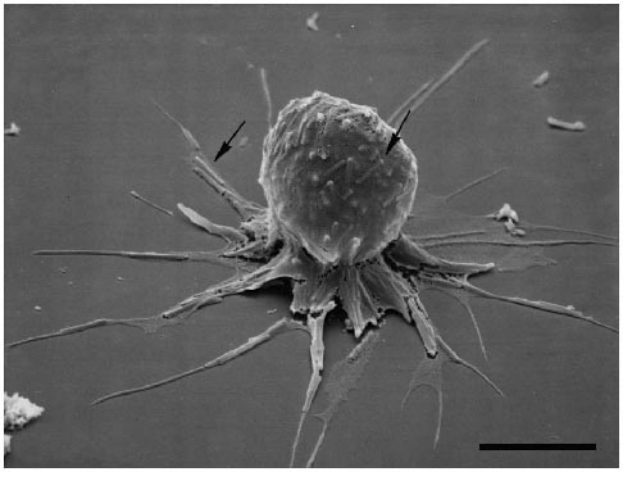

Fig. 1. Scanning electron micrograph of an isolated green sunfish RPE cell. During settling and attachment the apical projections of the isolated cells assume a radial arrangement. Cylindrical pigment granules (arrows) are visible beneath the plasma membrane. Bar, 10 μm.

Both sheets and dissociated RPE cells spontaneously dispersed their pigment granules after isolation, even when obtained from previously dark-adapted eyes (Garcia and Burnside, 1994; Bruenner and Burnside, 1986). To study the effects of altering requirements for intracellular and extracellular calcium levels ($[Ca^{2+}]_i$, $[Ca^{2+}]_o$) on pigment granule dispersion, cells were first triggered to aggregate pigment granules by the addition of 1 mM cAMP. Complete aggregation was usually achieved after 15 to 20 minutes. These previously aggregated RPE sheets or cells were incubated in the appropriate solutions for 15 to 30 minutes to deplete $[Ca^{2+}]_o$ or $[Ca^{2+}]_i$, then stimulated to disperse pigment granules by washing out cAMP with several changes of fresh cAMP-free medium for 30 minutes. Complete dispersion in RPE sheets and dissociated cells was achieved after 15 to 30 minutes.

To test effects of experimental treatments on pigment granule aggregation, RPE cells or sheets having dispersed pigment granules were incubated in the appropriate test solutions for 10 to 30 minutes, then stimulated to aggregate by addition of cAMP for 15 to 30 minutes. Most isolated cells retained the ability to move pigment granules for at least 48 hours when cultured under sterile conditions.

Effects of $[Ca^{2+}]_o$ or $[Ca^{2+}]_i$ on pigment granule dispersion

To explore the role of extracellular calcium in pigment granule dispersion, RPE sheets previously aggregated by cAMP treatment were triggered to disperse their pigment granules by washout of cAMP, using either control Ringer (2.4 μM free Ca^{2+}), calcium-free Ringer containing 1 mM EGTA (EGTA-Ringer, <1 nM free Ca^{2+}), or Ringer containing 1.8 mM free Ca^{2+}. Pigment granules dispersed in RPE sheets at all three calcium concentrations (Fig. 2). Pre-incubating the previously aggregated RPE sheets for 15 minutes in EGTA-Ringer plus cAMP before triggering dispersion by cAMP washout also had no effect on pigment migration; pigment granules dispersed normally in both control and EGTA-treated preparations (Fig. 3). These results indicate that extracellular calcium is not required for dispersion of pigment granules in RPE sheets. Similar results were obtained with dissociated, cultured RPE cells (data not shown).

Since 15 minute pre-incubations and 30 minute cAMP washout in 1 mM EGTA might be expected not only to deplete extracellular calcium, but also to lower intracellular cytosolic calcium, these experiments further suggested that changes in $[Ca^{2+}]_i$ might not be required for pigment granule dispersion. To test this more directly, previously aggregated RPE sheets were loaded with the calcium chelator, BAPTA. Sheets were incubated in 10 μM BAPTA-AM for 10 minutes in Ringer plus cAMP, then incubated in fresh Ringer/cAMP without BAPTA for 20 minutes. Preliminary experiments showed that this 20 minute recovery time after BAPTA loading was necessary to allow maximal pigment granule movements following the loading period. After BAPTA loading and recovery, pigment granules were stimulated to disperse by washing out cAMP with Ringer alone. All BAPTA solutions contained 0.25% DMSO as a carrier, and DMSO control treatments were included in all experiments. RPE cells loaded with BAPTA had no effect on pigment granule dispersion; sheets pre-incubated in BAPTA dispersed pigment granules equally as well as

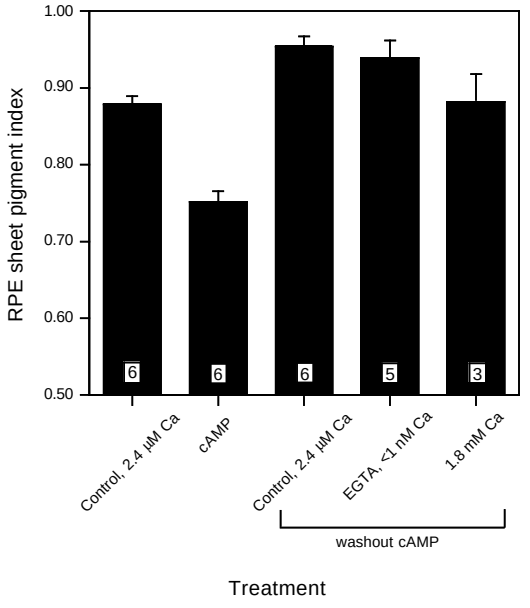

Fig. 2. Effect of depleting $[Ca^{2+}]_o$ on pigment granule dispersion in RPE sheets. Isolated RPE sheets were incubated in Ringer (2.4 μM free Ca^{2+}) for 15 minutes (Control, 2.4 μM Ca), stimulated to aggregate pigment granules by 1 mM cAMP in Ringer for 30 minutes (cAMP), then triggered to disperse pigment granules by a 30 minute cAMP washout in Ringer (Control, 2.4 μM Ca), calcium-free Ringer containing 1 mM EGTA (EGTA, <1 nM Ca), or Ringer containing 1.8 mM free calcium (1.8 mM Ca). Buffering of $[Ca^{2+}]_o$ by EGTA did not affect pigment granule dispersion. Numbers on bars represent *N*.

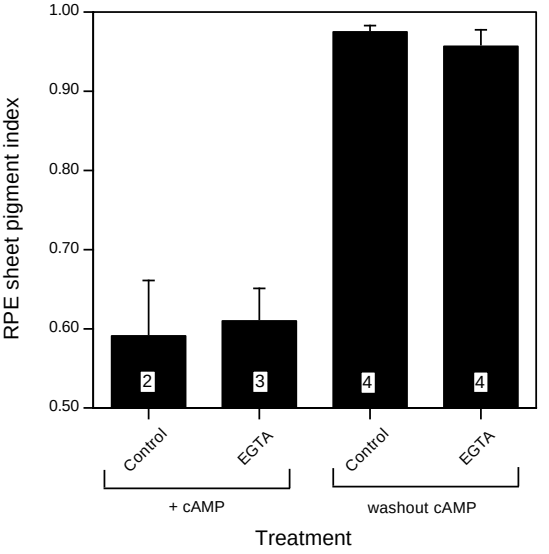

Fig. 3. Effect of a 15 minute incubation in calcium-free medium on pigment granule dispersion in RPE sheets. Previously aggregated RPE sheets were pre-incubated for 15 minutes in either Ringer + cAMP (Control + cAMP) or calcium-free Ringer plus 1 mM EGTA and cAMP (EGTA + cAMP), and triggered to disperse by cAMP washout in Ringer (Control, washout cAMP) or calcium-free Ringer plus 1 mM EGTA (EGTA, washout cAMP), and fixed after 30 minutes. Depletion of $[Ca^{2+}]_o$ by a 15 minute incubation in EGTA-Ringer did not affect dispersion of pigment granules.

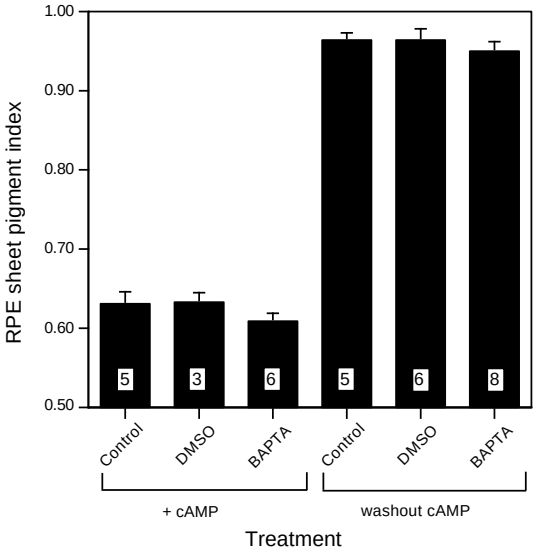

Fig. 4. Effect of buffering $[Ca^{2+}]_i$ on pigment granule dispersion in RPE sheets. Previously aggregated RPE sheets were pre-incubated in 10 μM BAPTA-AM in Ringer plus cAMP (BAPTA + cAMP), 0.25% DMSO in Ringer plus cAMP (DMSO+cAMP), or Ringer +cAMP alone (Control+cAMP) for 10 minutes, given a recovery period of 20 minutes in Ringer plus cAMP, then triggered to disperse by cAMP washout in Ringer alone. RPE sheets were fixed after the 20 minute recovery period (left three columns) or 30 minutes after cAMP washout in Ringer; the right three columns show the PI for sheets pre-incubated in the indicated medium. BAPTA and DMSO-treated sheets dispersed pigment granules equally as well as controls despite buffering of $[Ca^{2+}]_i$.

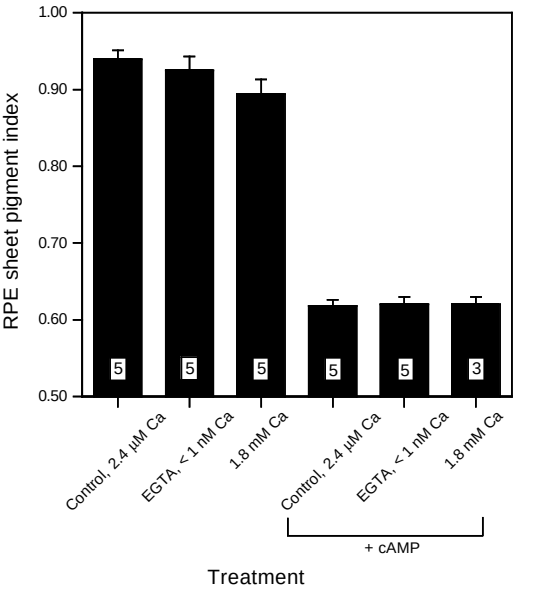

Fig. 5. Effect of depleting $[Ca^{2+}]_o$ on pigment granule aggregation in RPE sheets. Dispersed RPE sheets were preincubated for 30 minutes in control Ringer (Control, 2.4 μM Ca), calcium-free Ringer containing 1 mM EGTA (EGTA, <1 nM Ca) or Ringer containing 1.8 mM free Ca^{2+} (1.8 mM Ca). RPE sheets were then stimulated to aggregate by the addition of 1 mM cAMP. Pigment granules aggregated equally well in all three free calcium concentrations.

controls (Fig. 4). Such results corroborate the EGTA experiments, suggesting that buffering of $[Ca^{2+}]_i$ does not affect RPE pigment granule dispersion.

Effects of $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ on pigment granule aggregation

To examine calcium effects on pigment granule aggregation, RPE sheets having dispersed pigment granules were pre-incubated in EGTA-Ringer (<1 nM free Ca^{2+}), control Ringer (2.4 μM free Ca^{2+}), or Ringer containing 1.8 mM free Ca^{2+} for 30 minutes, then triggered to aggregate by the addition of cAMP. Pigment granule aggregation occurred in all three treatments (Fig. 5). Similar results were obtained using dissociated RPE cells (data not shown). These observations indicate that extracellular calcium is not required for pigment granule aggregation. Buffering intracellular calcium using BAPTA also had no effect on pigment granule aggregation. After 10 minutes of loading of 10 μM BAPTA and 20 minutes of recovery in medium lacking BAPTA, cells aggregated pigment granules normally when exposed to cAMP (Fig. 6).

$[Ca^{2+}]_i$ measurements

Experiments using EGTA and BAPTA to deplete $[Ca^{2+}]_o$ and buffer $[Ca^{2+}]_i$ strongly suggested that calcium does not play a necessary role in the regulation of pigment granule movements. To examine calcium fluxes in RPE directly, we used the calcium indicator dyes fura-2 and fura-pe3 with isolated RPE cells to monitor $[Ca^{2+}]_i$ during pigment granule

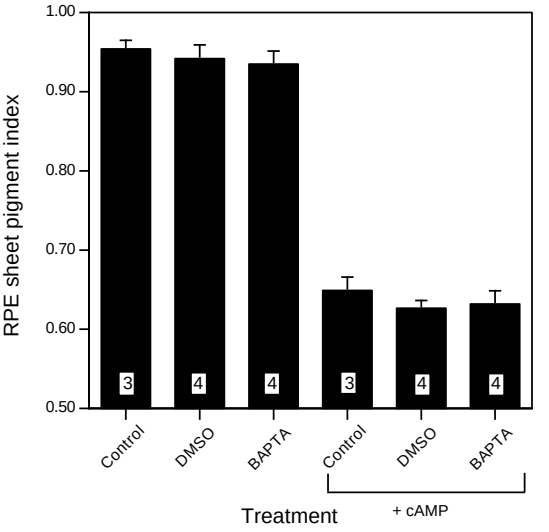

Fig. 6. Effect of buffering $[Ca^{2+}]_i$ on pigment granule aggregation in RPE sheets. Dispersed RPE sheets were incubated for 10 minutes in 10 μM BAPTA-AM in 0.25% DMSO (BAPTA), 0.25% DMSO alone (DMSO), or Ringer alone (Control), then incubated in Ringer alone for a 20 minute recovery period (left three columns). RPE sheets were then triggered to aggregate pigment granules by addition of cAMP (right three columns). Buffering of $[Ca^{2+}]_i$ by BAPTA-AM did not affect pigment granule aggregation.

aggregation and dispersion, and to test the buffering of $[Ca^{2+}]_i$ in BAPTA-loaded cells. Isolated RPE cells were microinjected with fura-2 while in the dispersed or aggregated states, then

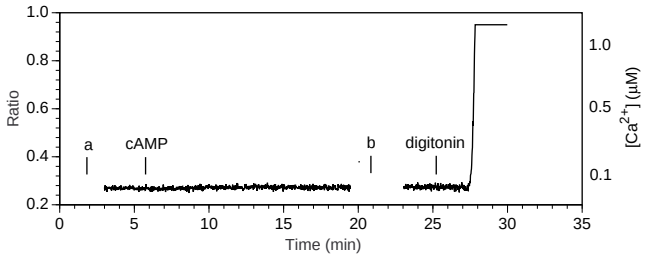

Fig. 7. Fura-2 fluorescence ratio (357 nm/385 nm) of an isolated RPE cell during pigment granule aggregation. The cell was photographed after microinjection of fura-2, before cAMP application (a), and after aggregation was complete (b). Digitonin (10 μM) was added at the time indicated to permeabilize the cell and elicit a rise in $[Ca^{2+}]_i$. Pigment granule aggregation was not associated with a change in $[Ca^{2+}]_i$ over basal levels.

stimulated to aggregate pigment granules by adding cAMP to the medium, or stimulated to disperse pigment granules by washing out cAMP with Ringer. For these experiments, cells were incubated in Ringer containing 1.8 mM Ca^{2+} to enhance wound healing after injection (Swezey and Epel, 1989; Rehder et al., 1992). Estimated basal $[Ca^{2+}]_i$ in RPE from measurements of injected cells was 129 ± 22 nM (mean and s.e.m., n=19). In some experiments, the calculated basal $[Ca^{2+}]_i$ was a negative number because of inaccuracies in converting fluorescence ratios to calcium concentration based on in vitro calibration values (see Materials and Methods).

Fig. 7 shows the fluorescence ratio of a cell that was injected while in the dispersed state, then stimulated to aggregate pigment granules by addition of cAMP. The cell was photographed after microinjection and prior to the addition of cAMP (Fig. 8A) at the time indicated in Fig. 7. The cell was photographed again after aggregation was complete (Fig. 8B). Ratio measurements show that no change in basal $[Ca^{2+}]_i$ could be detected during pigment granule aggregation triggered by cAMP (Fig. 7). After aggregation was complete, 10 μM digitonin was added to demonstrate that the ratio measuring system was able to detect increases in calcium caused by cell lysis and entry of extracellular calcium. A pattern similar to that shown in Fig. 7 was observed in 12 of 12 injected cells that subsequently aggregated pigment granules; aggregation in these cells took place at the same rate as uninjected cells.

We estimated the volume of fura-2 injected into cells to be 4.2×10^{-15} to 3.4×10^{-14} l by measuring the volume of a droplet injected into oil. Assuming a spherical average volume of 6.2×10^{-12} l for RPE cells based on diameters of 20 to 25 μm (see Fig. 1), and given a fura-2 concentration of 5.4 mM in the pipette, we calculate the final concentration of fura-2 in injected cells to be approximately 16 μM. This fura-2 concentration in injected RPE cells would probably have not been sufficient to buffer a transient change in $[Ca^{2+}]_i$, since cytosolic fura-2 concentrations less than 100 μM generally minimize calcium buffering effects (Williams and Fay, 1990). In any event, pigment granule aggregation took place in the absence of any detectable change in $[Ca^{2+}]_i$.

We also tested whether $[Ca^{2+}]_i$ changed during dispersion of pigment granules. Cells were first incubated in cAMP to aggregate pigment granules, then microinjected with fura-2. Cells were stimulated to disperse by washing out cAMP in Ringer containing 1.8 mM calcium. In some cells, we observed a slow increase in ratio during dispersion (Fig. 9A). However,

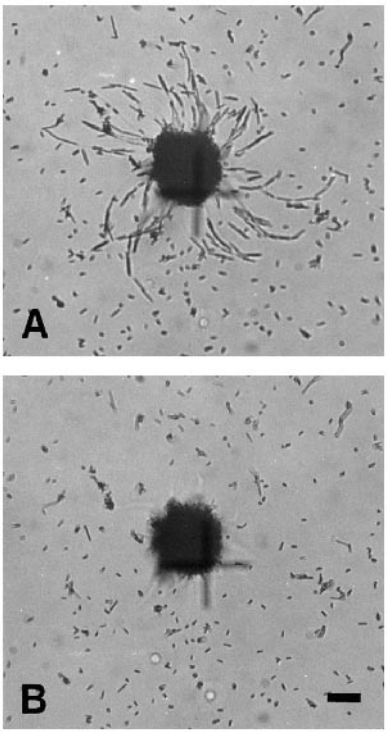

Fig. 8. Photomicrographs of microinjected cell from Fig. 7. (A) After microinjection, before application of cAMP. Pigment granules remained dispersed after puncture of the cell with a microneedle during injection. (B) After pigment granule aggregation. Bar, 10 μm.

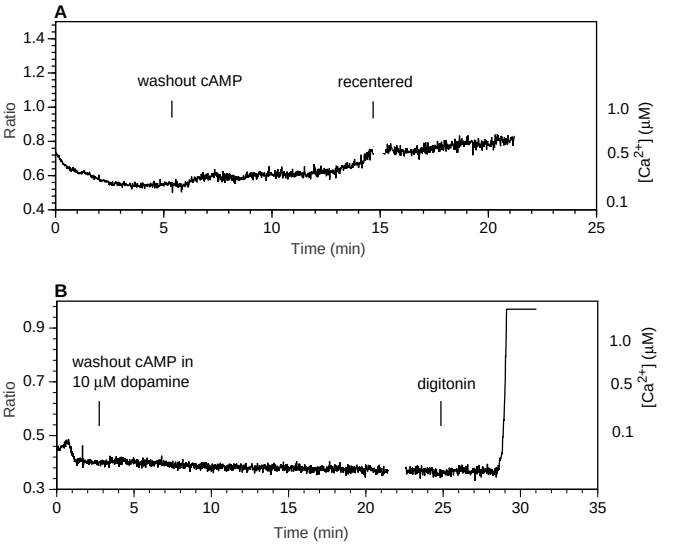

Fig. 9. fura-2 fluorescence ratios of microinjected RPE cells during pigment granule dispersion. Cells were previously aggregated with cAMP prior to microinjection. (A) Fluorescence ratio (340 nm/385 nm) of microinjected RPE cell triggered to disperse pigment granules by cAMP washout using several changes of Ringer (1.8 mM free Ca^{2+}) without cAMP. Basal $[Ca^{2+}]_i$ in this cell was higher than normally observed, and drifted somewhat over time. The cell was recentered under the apparatus at the time indicated. Pigment granules were partly dispersed at the end of the experiment. (B) Microinjected RPE cell triggered to disperse in medium containing 10 μM dopamine. Digitonin was added at the time indicated.

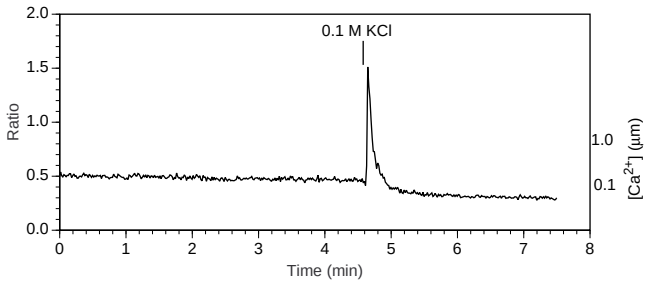

Fig. 10. Calcium transient elicited by application of 0.1 M KCl; 357 nm/385 nm ratio. KCl caused a calcium spike, indicating that the fura dye and the detection system were functional.

since the basal $[Ca^{2+}]_i$ was usually higher than average in such cells, it is likely that these cells were adversely affected by the injection procedure, and the gradual increase in the ratio reflected their inability to maintain constant $[Ca^{2+}]_i$. Despite the failure in some cases to maintain intracellular calcium homeostasis, four of four cells dispersed pigment granules without an appreciable change in $[Ca^{2+}]_i$. We also triggered dispersion of pigment granules in medium containing 10 μM dopamine, since dopamine appears to enhance the extent of dispersion of pigment granules into apical projections (Dearry and Burnside 1989; Garcia and Burnside, 1994). The presence of dopamine during washout of cAMP did not affect basal $[Ca^{2+}]_i$ (Fig. 9B; observed in three of three aggregated cells injected and dispersed in dopamine).

As a supplementary check that our detection system was able to measure changes in calcium, we measured $[Ca^{2+}]_i$ during depolarization of the cell by 0.1 M KCl (Fig. 10). The calcium spike elicited by KCl is clearly above basal $[Ca^{2+}]_i$, which suggests that the lack of any change in 340/385 ratio during pigment granule movements was not due to lack of sensitivity of our measuring system. This transient increase in $[Ca^{2+}]_i$ had no effect on pigment granule position.

To be sure that the BAPTA used to buffer $[Ca^{2+}]_i$ in RPE sheets during pigment granule dispersion and aggregation (Figs 4, 6) was effective, we measured $[Ca^{2+}]_i$ in BAPTA-treated isolated RPE cells during treatment with the calcium ionophore, ionomycin. For these experiments, cells were loaded with the acetyl methoxy ester of fura-pe3, a newly introduced dye that is less easily sequestered or extruded from the cell. Fura-loaded cells were incubated in HEPES-buffered Ringer containing 50 μM calcium, and treated with 5 μM ionomycin. Ionomycin added to control cells elicited an increase in $[Ca^{2+}]_i$ within seconds (Fig. 11A). In contrast, in BAPTA-loaded cells, $[Ca^{2+}]_i$ remained at basal levels for a period of minutes (Fig. 11B). These results suggest that BAPTA was effective in buffering a physiological increase in $[Ca^{2+}]_i$.

We also used fura-pe3-AM to examine the effect of elevated $[Ca^{2+}]_i$ on pigment granule dispersion and aggregation. Cells with aggregated pigment granules were stimulated to disperse and then aggregate granules by washout and addition of cAMP in HEPES-buffered Ringer containing 50 μM calcium (Fig. 12A). Triggering pigment movements by cAMP washout or cAMP addition elicited no change in $[Ca^{2+}]_i$, as observed in previous experiments. Ionomycin was added, causing an increase in $[Ca^{2+}]_i$, then pigment granules were triggered to redisperse by cAMP washout. Despite sustained elevated $[Ca^{2+}]_i$, pigment granules dispersed normally (Fig. 12A, observed in two of two cells monitored).

A similar experiment was done to examine the effect of elevated $[Ca^{2+}]_i$ on pigment granule aggregation (Fig. 12B). Aggregation and dispersion of pigment granules in medium containing 50 μM calcium caused no detectable change in $[Ca^{2+}]_i$, as described previously. Addition of ionomycin to dispersed cells increased $[Ca^{2+}]_i$, but did not interfere with subsequent reaggregation of pigment granules (Fig. 12B, observed in seven of seven cells measured). Interestingly, addition of cAMP caused a transient increase over the elevated $[Ca^{2+}]_i$ induced by ionomycin (Fig. 12B). Treatment with ionomycin followed by forskolin did not produce the transient increase (data not shown), suggesting that it may due to interaction of cAMP with an extracellular receptor. In any event, the experiments illustrated in Fig. 12 demonstrate that elevated $[Ca^{2+}]_i$ had no effect on pigment granule migration in either direction.

The ionomycin experiments also demonstrated the effect of elevated $[Ca^{2+}]_i$ on the maintenance of the dispersed or aggregated states. Treatment of aggregated or dispersed cells with ionomycin and subsequent elevation of $[Ca^{2+}]_i$ (Figs 11, 12) did not trigger aggregation or dispersion. Likewise, treatment with 0.1 M KCl (Fig. 10) had no effect on the position of pigment granules. In addition, puncture of dispersed or aggregated cells with a microneedle during microinjection did not trigger pigment granules to move, even though the puncture causes a transient rise in $[Ca^{2+}]_i$ (C. King-Smith, unpublished data; see also Steinhardt et al., 1994). Together these observations show that a calcium increase is not sufficient to stimulate pigment granule motility.

Calcium imaging with fura-pe3

The ratio measurements described above were obtained from photometric measurements that averaged fluorescence in the cell body and proximal portions of apical projections. However, since many calcium signaling events are very localized, we could not be certain that we were not missing a localized change in $[Ca^{2+}]_i$ that was obscured by the averaged whole-cell ratio measurements. We therefore used a calcium imaging system to evaluate the spatial distribution of $[Ca^{2+}]_i$ in subregions of RPE cells, particularly the apical projections, where pigment movements were taking place.

Cells used for calcium imaging experiments were treated in the same manner as those used in photometric calcium ratio experiments; previously aggregated RPE cells were stimulated to disperse by washing out cAMP in medium containing dopamine, or previously dispersed cells were stimulated to aggregate pigment granules by addition of cAMP while measuring fluorescence ratios. In experiments using the fluorescence imaging system, autofluorescence from non-loaded cells was not detectable. Emission ratios were calculated in particular areas of the cell including small regions centered on the bases of apical projections near the cell body (Fig. 13). No differences were observed among ratio measurements of the cell body and measurements within apical projections in cells before or after triggering dispersion of pigment granules by cAMP washout (Fig. 13). Similarly, cells triggered to aggregate by addition of cAMP showed no differences in fluorescence ratio among subregions of the cell, or between the

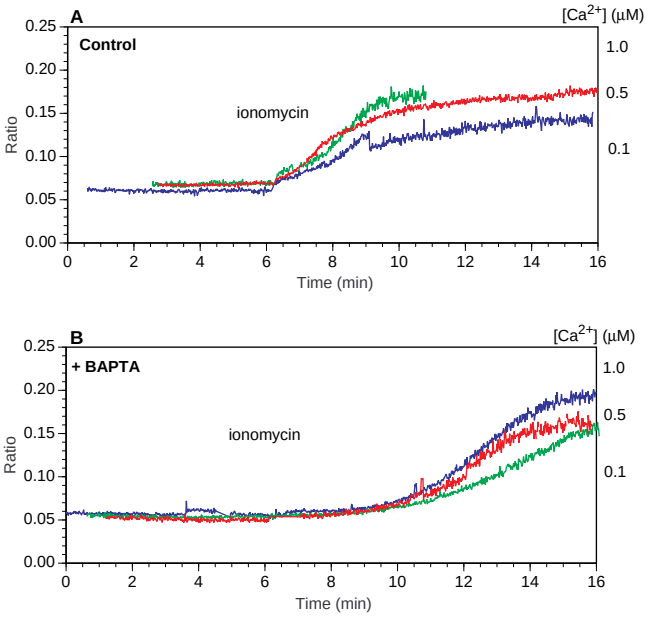

Fig. 11. Effect of 5 μM ionomycin on $[Ca^{2+}]_i$ in isolated RPE cells loaded with fura-pe3-AM, with or without BAPTA; 350 nm/385 nm ratio. Medium contained 50 μM calcium. (A) Control cells. (B) BAPTA-loaded cells.

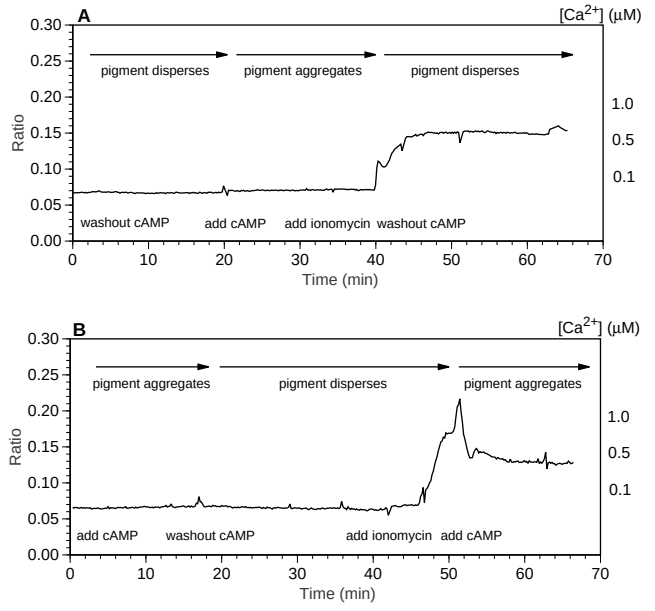

Fig. 12. Elevated $[Ca^{2+}]_i$ induced by 5 μM ionomycin did not affect pigment granule movements (350 nm/385 nm ratio). Cells were loaded with fura-pe3-AM and incubated in medium containing 50 μM calcium. (A) Dispersion, aggregation, and redispersion of pigment granules in the presence of ionomycin. (B) Aggregation, dispersion, and re-aggregation in the presence of ionomycin.

aggregated and dispersed states (not shown). These results support the idea that aggregation and dispersion of pigment granules are not associated with localized changes in $[Ca^{2+}]_i$ within the apical projections.

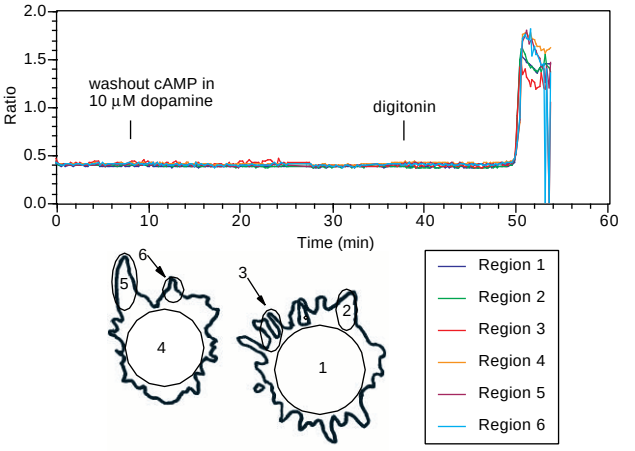

Fig. 13. Fluorescence ratios (340 nm/380 nm) of subregions in two isolated RPE cells during pigment granule dispersion in 10 μM dopamine; results of an imaging experiment using fura-pe3-AM. Outlines of cells were traced from the video screen; regions measured within cells are numbered. Fluorescence intensity of distal portions of the apical projections was too low to be measured, so proximal regions of projections were monitored. Ratios are similar among cell bodies and apical projections of both cells before, during, and after pigment granule dispersion.

DISCUSSION

Calcium independence of pigment granule movements in teleost RPE in vitro

Our observations indicate that calcium is neither necessary nor sufficient for triggering pigment granule motility in RPE cells in vitro; rather, addition or removal of cAMP alone is sufficient to elicit pigment granule movements. Neither extracellular nor intracellular calcium was required for triggering or sustaining RPE pigment granule migration in isolated RPE. Pre-treatment of RPE cells with EGTA-Ringer (free calcium <1 nM) for 15 to 30 minutes had no effect on pigment granule aggregation or dispersion triggered by cAMP application or washout. Although this pre-treatment with EGTA-Ringer might be expected to deplete cytosolic free calcium, it did not inhibit RPE pigment granule aggregation or dispersion. Buffering $[Ca^{2+}]_i$ with BAPTA likewise had no effect on aggregation or dispersion. No change in $[Ca^{2+}]_i$ from basal levels was detected when pigment granules were stimulated to aggregate using cAMP, or triggered to disperse by cAMP washout with or without dopamine. Furthermore, experimental elevation of $[Ca^{2+}]_i$ with ionomycin had no affect on aggregation or dispersion, or on the maintenance of the aggregated or dispersed states.

These findings do not rule out the possibility that during light regulation of pigment migration in vivo, calcium signaling events might play a role proximal to changes in intracellular cAMP concentration ($[cAMP]_i$). Although intraocular injections of cAMP can induce pigment granule aggregation (Burnside et al., 1982) it is not yet clear whether extracellular cAMP is the endogenous dark signal responsible for triggering pigment granule aggregation in vivo. Exogenous cAMP may fortuitously increase $[cAMP]_i$ via organic anion transporters in the plasma membrane of the RPE cell (Garcia and Burnside,

1994). Thus, we may have bypassed calcium-requiring steps in the endogenous signaling pathway by which light regulates pigment granule migration in vivo. Nonetheless, our studies demonstrate that changing $[cAMP]_i$ alone is sufficient to produce aggregation or dispersion of pigment granules in the absence of calcium changes.

Previous studies have reported contrasting effects of altering $[Ca^{2+}]_o$ on RPE pigment granule migration. Extracellular calcium was reported to be required for RPE pigment granule aggregation in frog eyecups (Snyder and Zadunaisky, 1976), but inhibitory to pigment aggregation in teleost RPE-retina (Dearry and Burnside, 1984). In both these studies, the presence of the neural retina made it impossible to ascertain the site of calcium action. An insensitivity of pigment granule motility to $[Ca^{2+}]_o$ was also noted in a previous study of isolated RPE cells (Bruenner and Burnside, 1986), thus prompting us to undertake the present analysis.

The basal $[Ca^{2+}]_i$ that we estimated for green sunfish RPE was 129 nM. This value is within the range reported for RPE from other species using fura-2 or quin-2 calcium dyes (90 nM, Osborne et al., 1993; to 276 nM, Friedman et al., 1988). Basal $[Ca^{2+}]_i$ was not influenced by dopamine in RPE cells, although dopamine induces pigment granule dispersion in teleost RPE both in vivo and in vitro (Dearry and Burnside, 1988). Although dopamine has been reported to block voltage-dependent calcium channels and inhibit K^+-induced $[Ca^{2+}]_i$ increases in frog pituitary melanotrophs, it did not alter basal $[Ca^{2+}]_i$ in those cells (Desrues et al., 1993).

Although calcium is not required for regulating pigment granule migration, several studies suggest that it plays other signaling roles in RPE. Along with other ions, calcium is transported through the RPE in the choroid-to-retina direction; the driving force being the Na^+/K^+-ATPase at the apical membrane (Miller and Steinberg, 1977). Also, RPE has been shown to possess both low and high affinity carrier-mediated calcium transport systems (Salceda, 1989) and a Na^+/Ca^{2+} exchanger (Salceda, 1989; Fijisawa et al., 1993). L-type calcium channels have been detected in rat RPE cells (Ueda and Steinberg, 1993; Strauss and Weinrich, 1994), consistent with our observation of a KCl-triggered calcium spike in isolated fish RPE cells.

Regulation of pigment granule movements by calcium in other cell types

Extracellular calcium has been implicated in regulation of pigment granule motility in several other types of pigmented cells. For example, extracellular calcium was shown to be required for pigment granule migration in retinular cells of invertebrate photoreceptors, including those of flies (Kirschfeld and Vogt, 1980; Howard, 1984), meal moths (Weyrauther, 1989) and wharf crabs (King-Smith and Cronin, 1996). Although extracellular calcium was not required for pigment granule migration in crayfish retinular cells, pigment movements were affected by raising $[Na^+]_o$ or $[K^+]_o$, which may have affected release of calcium from internal stores (Frixione and Arechiga, 1981). Extracellular calcium was required for spontaneous aggregation of pigment granules in squirrelfish erythrophores in vitro (Luby-Phelps and Porter, 1982; Kotz and McNiven 1994). Pigment granule movements were triggered in chromatophores of several species by experimentally altering $[Ca^{2+}]_i$ by the use of ionophores (Luby-Phelps and Porter, 1982; Oshima et al., 1988), cell puncture (Kotz and McNiven, 1994), or permeabilized cell preparations (Stearns and Ochs, 1982; McNiven and Ward, 1988; Fujii et al., 1991).

On the other hand, other studies suggest that regulatory mechanisms vary among chromatophore type and species. In melanophores of the cichlid fish, *Tilapia*, aggregation required concomitant increase in calcium and decrease in cAMP (Thaler and Haimo, 1992). In frog melanophores, pigment granule dispersion was activated either by cAMP-dependent protein kinase A or by calcium-dependent protein kinase C (Sugden and Rowe, 1992). In angelfish melanophores, cAMP was shown to be necessary and sufficient for triggering pigment granule aggregation by epinephrine, even though epinephrine induced a transient rise in $[Ca^{2+}]_i$ (Sammak et al., 1992). In squirrelfish, regulation of pigment granule migration in melanophores differs from that in erythrophores: cAMP alone was sufficient for pigment granule migration in melanophores, but both calcium and cAMP were required for erythrophores (Kotz and McNiven, 1994).

Different types of pigmented cells depend on different cytoskeletal mechanisms of force production for pigment granule migration. Migration is microtubule-dependent in teleost melanophores (see Haimo and Thaler, 1994, for review). In RPE cells, on the other hand, pigment granule movements appear to be actin-dependent (Burnside et al., 1983; King-Smith et al., 1994, and unpublished data). Therefore, the regulatory mechanisms of chromatophores and RPE may not be directly comparable.

cAMP in RPE

In our studies, RPE pigment granule migration was triggered by application and washout of exogenous cAMP. Exogenous cAMP has also been shown to affect the basal membrane resistance and electroretinogram of chick RPE (Nao-i et al., 1990) and fluid and ion transport in frog RPE (Miller and Farber, 1984; Hughes et al., 1984). The route of entry of cAMP into RPE cells may be via organic anion transporters, since drugs that block such transporters also blocked cAMP-induced pigment granule aggregation, but did not block forskolin-induced aggregation (Garcia and Burnside, 1994). RPE from both human and bullfrog possess proton-lactate co-transport systems (la Cour et al., 1994; Lin et al., 1994); possibly cAMP enters cells through similar co-transport pathways.

In RPE sheets, Garcia and Burnside (1994) found that a two to three log-unit increase in extracellular cAMP concentration was required to produce full pigment granule aggregation from the dispersed state. In angelfish melanophores, Sammak et al. (1992) observed that a two- to threefold decrease in intracellular cAMP was required to produce full pigment granule aggregation from the dispersed state. The high extracellular concentration necessary to stimulate pigment granule movement in RPE could reflect inefficient entry of cAMP into RPE cells. In addition, RPE may have high phosphodiesterase activity (Garcia and Burnside, 1994) or high phosphatase activity. Application of okadaic acid, a phosphatase inhibitor, mimicked the effect of cAMP in RPE sheets and isolated cells, and stimulated pigment granule aggregation (D. Garcia, unpublished observations).

cAMP-dependent, calcium-independent regulation

Although the action of calcium and cAMP are usually coupled in cells, other examples of cAMP effects that are independent of calcium have been reported. Calcium and cAMP most often exist as 'synarchic' messengers (Rasmussen, 1981); that is, they act together in a variety of ways. For instance, calcium can control cAMP action or metabolism by increasing or decreasing adenylate cyclase activity, or by increasing cAMP phosphodiesterase activity. Cyclic AMP can influence calcium metabolism in cells by increasing calcium uptake into the endoplasmic reticulum (ER), by increasing or decreasing plasma membrane permeability to calcium or by affecting the activity of calcium pumps in the ER membrane (see Rasmussen, 1981, for review). Although not observed as frequently, calcium-independent, cAMP effects have been reported in other cell types besides RPE. Cyclic AMP is necessary and sufficient to stimulate pigment granule motility in melanophores from some fish species (Sammak et al., 1992; Kotz and McNiven, 1994). Another example is the control of water permeability of amphibian bladder epithelial cells (see Rasmussen, 1981; Hays et al., 1993, for reviews) where fusion of aggrephores containing water channels with bladder epithelial cell membranes appears to be solely under the control of cAMP.

The motile events of pigment granule migration in RPE cells and aggrephore fusion in bladder epithelial cells both depend upon the actin cytoskeleton. The cAMP-stimulated fusion of membrane vesicles in bladder epithelial cells requires depolymerization of actin filaments at the base of apical microvilli (Hays et al., 1993, review). Cytochalasin studies suggest that in teleost RPE cells, the actin cytoskeleton mediates both pigment granule dispersion (Burnside et al., 1983) and pigment granule aggregation (King-Smith et al., 1994, and unpublished data), although how bidirectional motility is mediated by actin filaments is not clear. Thus, pigment granule translocation in RPE cells appears to be mediated by an actin-dependent process that is not regulated by calcium. This is a surprising finding, since in vertebrates most known examples of actin-dependent force production occur via myosin motors that are calcium-regulated. Although force production via actin and myosin motile systems is most commonly associated with filament sliding, there is growing evidence that actin-myosin systems may also translocate organelles. Organelles have been observed to move along actin filaments in squid axoplasm (Kuznetsov et al., 1992), and myosin has been identified on axoplasmic organelles and Golgi-derived vesicles from epithelial cells (Bearer et al., 1993; Fath and Burgess, 1993).

Recent studies have revealed numerous classes of myosins, representing a large superfamily of related proteins (see Mooseker, 1993; Titus, 1993, for reviews). Some of the novel myosin classes have been associated with vesicle motility, including members of the myosin V and myosin VI classes (Cheney et al., 1993; Mermall et al., 1994; Govidan et al., 1995). Although the regulatory mechanisms of most of the novel myosins are not known, many have sites for calmodulin binding, and therefore may be calcium-regulated (Mooseker, 1993; Titus, 1993; reviews). One exception is myosin II, which is regulated by phosphorylation of the myosin light chain (Tan et al., 1992, review); although most commonly phosphorylated by calcium-dependent myosin light chain kinase, some cell types exhibit calcium-independent myosin light chain phosphorylation (Matsumara et al., 1982, 1983; Deery and Heath, 1994). Conceivably, regulation of a myosin in RPE responsible for pigment granule translocation could also be achieved by calcium-independent phosphorylation. Although the details of the cytoskeletal and regulatory mechanisms remain to be elucidated, it seems likely that pigment granule translocation in RPE cells is an example of an actin-dependent motile process that is regulated by cAMP rather than by calcium.

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