Intracellular Calcium and cAMP Regulate Directional Pigment Movements in Teleost Erythrophores

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Abstract. Teleost pigment cells (erythrophores and melanophores) are useful models for studying the regulation of rapid, microtubule-dependent organelle transport. Previous studies suggest that melanophores regulate the direction of pigment movements via changes in intracellular cAMP (Rozdzial and Haimo, 1986*a*; Sammak et al., 1992), whereas erythrophores may use calcium- (Ca^{2+}-) based regulation (Luby-Phelps and Porter, 1982; McNiven and Ward, 1988). Despite these observations, there have been no direct measurements in intact erythrophores or any cell type correlating changes of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) with organelle movements. Here we demonstrate that extracellular Ca^{2+} is necessary and that a Ca^{2+} influx via microinjection is sufficient to induce pigment aggregation in erythrophores, but not melanophores of squirrel fish. Using the Ca^{2+}-sensitive indicator, Fura-2, we demonstrate that $[Ca^{2+}]_i$ rises dramatically concomitant with aggregation of pigment granules in erythrophores, but not melanophores. In addition, we find that an erythrophore stimulated to aggregate pigment will immediately transmit a rise in $[Ca^{2+}]_i$ to neighboring cells, suggesting that these cells are electrically coupled. Surprisingly, we find that a fall in $[Ca^{2+}]_i$ is not sufficient to induce pigment dispersion in erythrophores, contrary to the findings obtained with the ionophore and lysed-cell models (Luby-Phelps and Porter, 1982; McNiven and Ward, 1988). We find that a rise in intracellular cAMP ($[cAMP]_i$) induces pigment dispersion, and that this dispersive stimulus can be overridden by an aggregation stimulus, suggesting that both high $[cAMP]_i$ and low $[Ca^{2+}]_i$ are necessary to produce pigment dispersion in erythrophores.

MICROTUBULE-based transport plays an important role in routing vesicular organelles within cells. This trafficking is accomplished by mechanochemical enzymes, or "microtubule motors," which carry vesicles along microtubules while hydrolyzing ATP. The ability of a motor to move an organelle may be altered by changes in either the association of that motor with specific organelles or its enzymatic activity. Little is understood about either of these proposed mechanisms.

Regulatory mechanisms for some of the actin-based myosin motors have been studied extensively and are known to be controlled by phosphorylation. It has been shown that heavy chain phosphorylation controls the ATPase activity of *Acanthamoeba* and *Dictyostelium* myosins I and several myosins II. Alternatively, other forms of myosin II from both muscle and nonmuscle sources are regulated by phosphorylation of their light chains by calcium- (Ca^{2+}-)[1] calmodulin stimulated myosin light chain kinase and protein kinase C (for a recent review on myosin phosphorylation, see Tan et al., 1992). In addition, calmodulin serves as a Ca^{2+}-sensitive regulatory light chain of avian brush border myosin I and several other newly discovered vertebrate myosins I (for a review see Cheney and Mooseker, 1992).

Regulation of microtubule-based motors and the vesicular movements they support are less defined, but are believed to use similar regulatory pathways to myosins, such as phosphorylation, and the second messengers, Ca^{2+} and cAMP. It is difficult to isolate and study the regulation of specific intracellular transport processes, because most cells in culture simultaneously transport different organelles in multiple directions. Fish chromatophores provide an ideal cell model for studying the regulation of directional, microtubule-based organelle trafficking (McNiven and Porter, 1984; Schliwa, 1987; Haimo and Rozdzial, 1989). Pigment granules in these cells exhibit graphic, temporally distinct movements, which are closely associated with and dependent upon (Schliwa and Bereiter-Hahn, 1973; Murphy and Tilney, 1974; Obika and Negishi, 1985) a dense radial array of microtubules. This transport is either completely retrograde (inward aggregation toward the "minus"-ends of microtubules at the cell center) or completely anterograde (outward dispersion toward the "plus"-ends at the cell periphery). Thus, the cellular

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1. *Abbreviations used in this paper*: BAPTA, 1,2-bis-(2-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid; BME, Basal Medium Eagle; 8-bromo cAMP, 8-bromoadenosine cAMP; but_2-cAMP, dibutyryl cAMP; Ca^{2+}, calcium; $[Ca^{2+}]_i$, concentration of free intracellular calcium; $[cAMP]_i$, concentration of free intracellular cAMP; K^+, potassium.

events that signal a specific directional organelle movement can be manipulated and studied.

Fish erythrophores and melanophores move pigment granules in similar ways. Squirrel fish erythrophores aggregate pigment smoothly and very rapidly (~10 μm/s), but disperse pigment more slowly with saltatory movements (Byers and Porter, 1977), while melanophores aggregate and disperse pigment at about one half of the erythrophore velocity. These velocities are comparable to that of fast axonal transport (0.5 μm/s–10 μm/s, Ochs, 1982). Interestingly, the excitability of erythrophores and melanophores can also be contrasted. Cultured erythrophores spontaneously aggregate pigment granules and can be stimulated to aggregate with either epinephrine, external potassium (K^+), direct mechanical stimulation, or by indirect mechanical stimulation through an adjacent cell. Melanophores do not aggregate pigment spontaneously and cannot be mechanically stimulated, but do aggregate pigment in response to K^+ and epinephrine. Caffeine and theophylline are effective dispersing stimuli in both types of chromatophores, and melanophore pigment can also be dispersed with IBMX (1-iso-3-butylmethylxanthine), a phosphodiesterase inhibitor.

Pigment dispersion and aggregation are thought to be mediated by kinesin- and dynein-like motors, respectively. This is supported by the observations that inhibitory kinesin antibodies block pigment dispersion, but not aggregation, in melanophores (Rodionov et al., 1991), and that dynein inhibitors block pigment aggregation in erythrophores (Beckerle and Porter, 1982) and permeabilized melanophores (Clark and Rosenbaum, 1982; Negishi et al., 1985; Rozdzial and Haimo, 1986*b*).

Of the possible pathways that may regulate pigment movements in chromatophores, those involving Ca^{2+} and cAMP have been the most widely studied. The evidence supporting the involvement of cAMP in orthograde dispersal of pigment in melanophores is extensive. Studies using lysed cell models suggest that cAMP stimulates pigment dispersion in *Tilapia* melanophores and *Carassius* xanthophores (Rozdzial and Haimo, 1986*a*; Palazzo et al., 1989), while direct dynamic measurements of intracellular cAMP ($[cAMP]_i$) show a cyclic rise and fall of this second messenger during pigment dispersion and aggregation in angelfish melanophores (Sammak et al., 1992).

The data supporting the role of Ca^{2+} in the regulation of pigment movement is less extensive than for cAMP, but equally convincing. Studies using ionophores (Luby-Phelps and Porter, 1982; Oshima et al., 1988) or lysed cell models (McNiven and Ward, 1988) have implicated a Ca^{2+}-based regulatory system in erythrophores. Raising $[Ca^{2+}]$ in these two models causes aggregation, and lowering $[Ca^{2+}]$ causes dispersion, suggesting that intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) is the exclusive regulatory signal for both aggregation and dispersion. Despite these observations, there have been no direct measurements in single, intact erythrophores, or any cell type, which correlates changes of $[Ca^{2+}]_i$ with organelle movements.

In this study, our goal was to define the role of Ca^{2+} in the regulation of pigment granule motion in intact erythrophores by directly altering $[Ca^{2+}]_i$ through microinjection, and by observing $[Ca^{2+}]_i$ dynamics in relation to pigment transport during aggregation and dispersion using the calcium sensitive probe, Fura-2. We first demonstrate that raising $[Ca^{2+}]_i$ is sufficient to stimulate pigment aggregation in erythrophores, but not melanophores. Second, a rise in $[Ca^{2+}]_i$ always occurs during pigment aggregation in erythrophores, but often does not occur and is not necessary in melanophores. Third, pharmacological studies indicate that pigment aggregation is induced by a rise in $[Ca^{2+}]_i$ in erythrophores and by a fall in $[cAMP]_i$ in melanophores. Fourth, pigment aggregating stimuli are transmitted through adjacent erythrophores via electrical coupling. Thus, a rise in $[Ca^{2+}]_i$ is a necessary and sufficient stimulus for pigment aggregation in erythrophores. To our knowledge, this is the first direct in vivo study demonstrating Ca^{2+}-induced retrograde microtubule-based organelle transport. In addition, we have explored the role of cAMP in erythrophore pigment granule dispersion by altering $[cAMP]_i$ with permeable cAMP analogs. We find that Ca^{2+} and cAMP act in opposition, since a fall in $[Ca^{2+}]_i$ and a rise in $[cAMP]_i$ are both necessary for erythrophore pigment dispersion.

Materials and Methods

Cell Culture

Chromatophores were cultured from squirrel fish (*Holocentrus ascensionus*) scales onto carbon-coated, glass coverslips by a modification of the collagenase isolation method of Luby-Phelps and Porter (1980). For each isolation, 10–12 scales were removed from the fish. Isolated cells were plated onto coverslips in a few drops of plating medium (Basal Medium Eagle [BME], GIBCO BRL, Gaithersburg, MD; supplemented with 1.88 *g* NaCl/l, 10% FBS, 1% penicillin-streptomycin) and allowed to adhere during overnight incubation at 26°C and 2.5% CO_2. By the following morning, cells had adhered to the coverslips. The dishes were rinsed and flooded with growth medium (plating medium, with 2% FBS and no antibiotics) and were ready for experiments the following day. Chromatophores could be maintained in culture under these conditions for ~5 d.

Microscopy and Stimulation of Pigment Granule Aggregation

For microscopic observation, cultured cells on coverslips were rinsed with serum-free BME, then with Ca^{2+}-free medium (Joklick's MEM, Sigma Chem. Co., St. Louis, MO; supplemented with 3.38 *g* NaCl/l) containing either 1 mM EGTA or 1 mM $CaCl_2$ for Ca^{2+}-free or Ca^{2+}-containing conditions, respectively. Coverslips were then mounted on chambers for microscopy, leaving them uncovered to allow for microinjection or the addition of agonist. The appropriate (Ca^{2+}-free of Ca^{2+}-containing) medium was added to the chambers. Video microscopy was performed with a Zeiss Axiovert 35 equipped with a Hammamatsu C2400 newvicon camera or XC-77 CCD with C2400-50 controller, with both time-lapse videotape and computer enhancement capabilities. Unless otherwise noted, images were captured via Image-1 software (Universal Imaging Corp., West Chester, PA).

Mechanical stimulation was produced by gently touching the cell with a microneedle. Cells were also induced to aggregate pharmacologically with 1 μM epinephrine (Parke Davis, Morris Plains, NJ). Unless otherwise noted, this was accomplished by adding 11 μl of 35 μM epinephrine (diluted fresh each day in either Ca^{2+}-free or Ca^{2+}-containing medium) to the bathing media (350 μl) during microscopic observation. For α-adrenoceptor pharmacological studies, α_1 and α_2 agonists and antagonists were obtained from Sigma Chem. Co. (St. Louis, MO). In all cases, the antagonist was added immediately before the experiment, followed by addition of the second receptor's agonist during microscopic observation. The final concentrations were 10^{-5} M antagonist (prazosin, α_1 antagonist; idazoxin, α_2 antagonist) and 10^{-7} M agonist (methoxamine, α_1 agonist; clonidine, α_2 agonist).

Microinjection

Cells were pressure microinjected with an IM-200 injection system (Narishege, Greenvale, NY). Unless otherwise indicated, the pipette concentration of $CaCl_2$ or the Ca^{2+}-chelator, BAPTA (1,2-bis(2-aminophen-

oxy)ethane-N,N,N',N'-tetraacetic acid; Molecular Probes, Eugene, OR), was 10 μM in microinjection buffer (10 mM KH_2PO_4, 75 mM KCL, pH 7.2). All injection solutions also contained about 200 μM FITC-dextran to control for successful injection. Samples were centrifuged for 20 min at 25,000 rpm in a TLA-100 micro-ultracentrifuge (Beckman, Palo Alto, CA) before use in order to prevent clogging of micropipettes. Micropipettes were pulled on a P-77 Brown-Flaming micropipette puller (Sutter Instrument Co., Novato, CA), then coated with hexamethyldisilazane (Pierce, Rockford, IL) vapor for 3–4 h to prevent cell debris from sticking to the pipettes. After injection, the cellular response was observed, after which the microscope was briefly switched to epifluorescence optics to determine if fluorescein had entered the cell. Assuming an injection of 5–10% cell volume, a rough estimate of the final intracellular concentration is 0.5–1.0 μM $CaCl_2$ or BAPTA.

Ratio Imaging

Cells were loaded with 5 μM membrane permeable Fura-2 AM (acetoxymethyl ester of Fura-2, Molecular Probes) in serum free BME (with an additional 1.88 *g* NaCl/l) supplemented with 0.1% BSA for 1 h at 26°C and 2.5% CO_2. When indicated, the cells were simultaneously loaded with 10 μM BAPTA-AM (acetoxymethyl ester of BAPTA, Molecular Probes). Coverslips were then rinsed and mounted in a chamber for microscopy. Epifluorescence imaging equipment and optics included a Zeiss 40 × Achrostigmat objective, either a Hammamatsu SIT camera, or a Hammamatsu XC-77 CCD (C2400-50 controller) with a C2400-68 intensifier. Image acquisition, ratio computations, and Ca^{2+} calibration were coordinated by Image-I/FL computer software (Universal Imaging Corp.). Fluorescence intensity measurements were alternately obtained with excitations wavelengths of 340 nm and 380 nm by the use of a computer controlled filter wheel (Lambda-10, Sutter Instrument Co.). The ratios of these measurements were then correlated to $[Ca^{2+}]_i$.

Fura-2 ratios were calibrated for $[Ca^{2+}]$ using an in vitro approach (Grynkiewicz et al., 1985). To calibrate, fluorescence intensity measurements were taken with 340 and 380 nm excitation of a MOPS solution (10 mM MOPS, 100 mM KCl, pH 7.4) containing 5 μM Fura-2 pentapotassium salt and either saturating (2 mM $CaCl_2$) or zero (1 mM EGTA) $[Ca^{2+}]$. Background fluorescence (obtained by measuring fluorescence of the MOPS solution containing no Fura-2) was subtracted from all calibration measurements. The K_d for Ca^{2+} binding of Fura-2 was assumed to be 224 nM. Many assumptions are made in any in vitro calibration (such as K_d of Fura-2 and the similarity of the cellular environment with the calibration solution), causing absolute measurements to be inaccurate. One side effect of this is that the color scales indicating $[Ca^{2+}]_i$ extend slightly into negative values. Despite these imperfections, qualitative conclusions and estimates of the magnitude of changes in $[Ca^{2+}]_i$ are valid.

Before any experimental perturbations, several ratio images were obtained from cells that were successfully loaded with Fura-2. After cells were stimulated to aggregate, images were obtained approximately every 2 s (limited by the speed of the filter changer). Phase observations were made before stimulation to aggregate pigment, shortly after stimulation to aggregate pigment, and various times from then on to assess the pigment distribution of the cells under study. In several experiments, we observed both ratio images and DIC images of the cells simultaneously. This was done by the use of a red filter in the path of the transmitted light source coupled with a dual camera system supplied by Fryer Co. (Carpentersville, IL). All of the red light (the DIC image) was diverted away from the intensified CCD camera to a newvicon camera.

Determination of the Effect of cAMP Analogs

During the investigation of the effect of permeable cAMP analogs on pigment dispersion, culture coverslips were mounted in an enclosed perfusion chamber, and immediately perfused with the K^+-medium. This medium was designed to produce a constant, but mild, threshold stimulus to maintain aggregation in most of the erythrophores present. For quantitation purposes, the distribution of pigment in each of the cells in several adjacent microscopic fields was determined and recorded as either tightly aggregated or partially dispersed (no fully dispersed cells were observed). Permeable or non-permeable cAMP (8-bromoadenosine cAMP [8-bromo cAMP], dibutyryl cAMP [but_2-cAMP], and cAMP were supplied by Sigma Chem. Co.) was then perfused into the chamber (1 mM in K^+-medium). Approximately 200 μl of fresh cAMP medium was perfused in every 5 min. After 30 min, the same cells were again observed, and the distribution of granules in each cell was determined to be completely dispersed or tightly aggregated (no partial pigment dispersals were observed). In each experiment, the percentage of cells that responded to cAMP by dispersing pigment was determined. Dynamic visualization of cAMP induced dispersion was performed using video microscopy by observing only one microscopic field for the duration of the experiment.

To investigate the effect of epinephrine on cAMP-dispersed cells, and to determine the reversibility of cAMP-induced dispersion, erythrophores were dispersed as described above. Epinephrine was then added to the cAMP-perfusion solution (1 μM final concentration) and perfused into the chamber. Epinephrine washout, followed by reversal of the cAMP-loading was then achieved by perfusion of K^+-medium, then adding 200 μl of fresh K^+-medium every 5 min for 20–30 min.

Results

Erythrophores, But Not Melanophores Require Extracellular Ca^{2+} for Aggregation

As mentioned earlier, erythrophores and melanophores are stimulated by similar agonists, but differ in several characteristics of pigment transport. To test the extracellular Ca^{2+} requirement for pigment granule aggregation in erythrophores and melanophores, these cells were challenged to aggregate pigment with 1 μM epinephrine under Ca^{2+}-free and Ca^{2+}-containing conditions. As shown in Fig. 1, erythrophores absolutely required extracellular Ca^{2+} for pigment granule aggregation in response to epinephrine, while melanophores aggregated in response to epinephrine regardless of the presence or absence of extracellular Ca^{2+}. All chromatophores

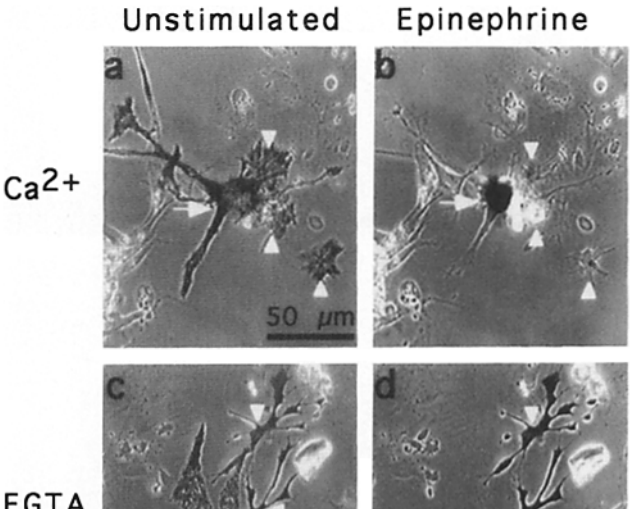

Figure 1. Erythrophores require extracellular Ca^{2+} to aggregate pigment, but melanophores do not. Erythrophores (*arrowheads*) and melanophores (*arrows*) were visualized in medium containing either 1 mM $CaCl_2$ (*a* and *b*) or 1 mM EGTA (*c* and *d*). The cells were then stimulated to aggregate with 1 μM epinephrine as described in Materials and Methods. Images of the cells were captured several minutes later after the melanophores had substantially aggregated (the erythrophores aggregated immediately after stimulation). Note that the erythrophores required extracellular Ca^{2+} (*b*), while the melanophores aggregated regardless of the presence or absence of Ca^{2+} (*b* and *d*).

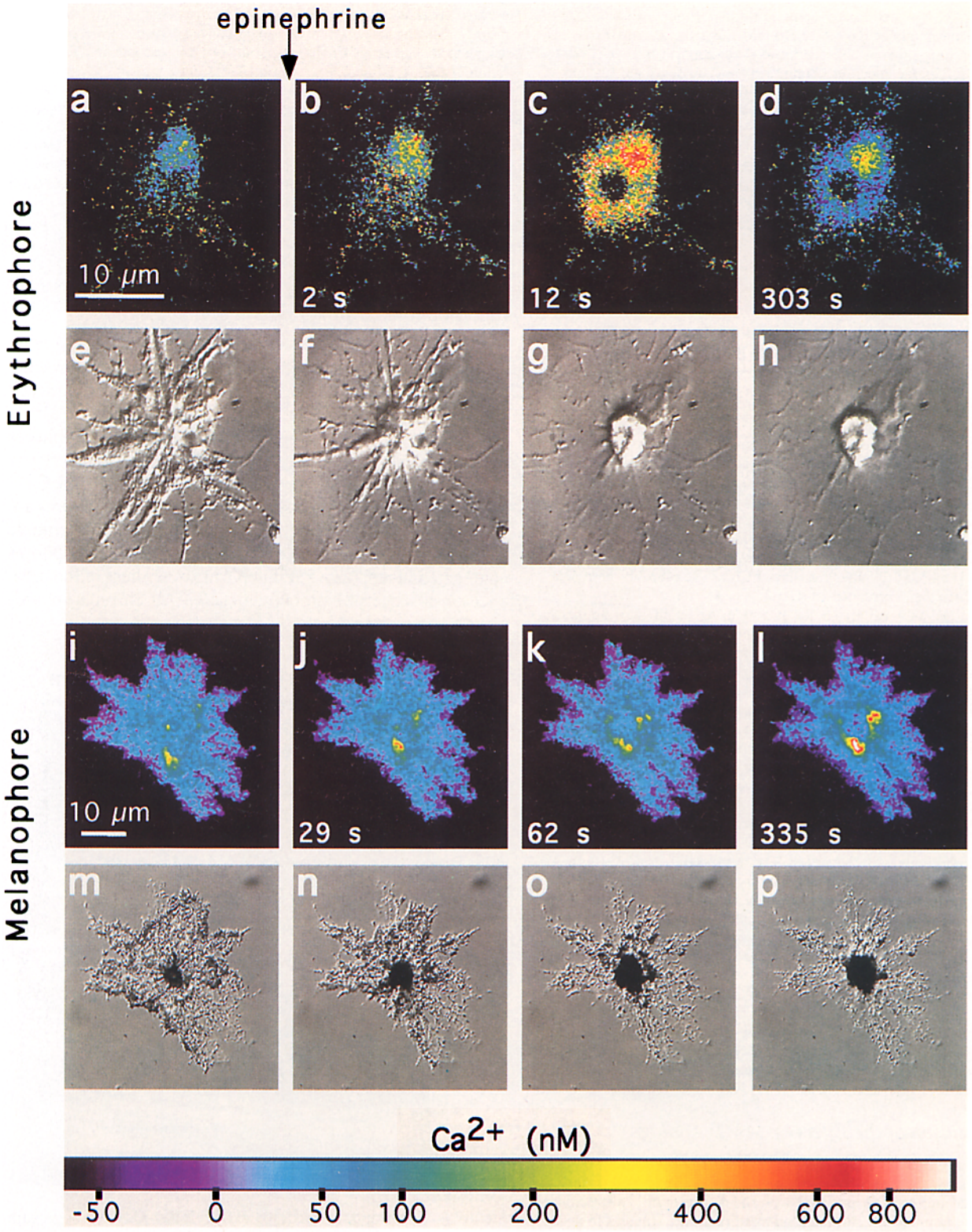

Figure 2. Pigment aggregation is accompanied by a rise in $[Ca^{2+}]_i$ in erythrophores, but not melanophores. The images in *a–p* are paired, simultaneous pseudocolor ratio images (*a–d*, *i–l*) and DIC images (*e–h*, *m–p*) of an erythrophore (*a–h*) and a melanophore (*i–p*). The left column of images depicts cells before stimulation. The cells were then stimulated to aggregate pigment with 1 μM epinephrine as described in Materials and Methods, with the time after stimulation indicated on the images. The color bar delineates the pseudocolor representation of $[Ca^{2+}]_i$. Note that the erythrophore images are magnified more than those of the melanophore, causing stippled ratio images due to lower light intensity. All the studies used for data collection were done at the magnification level of the melanophore, in order to assure a strong fluorescence signal throughout the cell. Note that $[Ca^{2+}]_i$ increased dramatically in the erythrophore during aggregation (*b* and *c*), but returned to nearly basal levels while the pigment remained aggregated (*d* and *h*). In contrast, the epinephrine-stimulated melanophore aggregated pigment without a rise in $[Ca^{2+}]_i$ (*j–l*).

observed in Ca^{2+}-containing conditions (n = 81 erythrophores, 8 melanophores) aggregated upon the addition of epinephrine (Fig. 1 *b*), whereas all melanophores, but no erythrophores aggregated when stimulated with epinephrine under Ca^{2+}-free conditions (Fig. 1 *d*) (n = 93 erythrophores, 6 melanophores). In addition, erythrophores aggregated pigment spontaneously and could be induced mechanically to aggregate pigment only when Ca^{2+} was present (data not shown), suggesting that aggregation requires an influx of Ca^{2+} from the extracellular environment. Unlike erythrophores, melanophores do not exhibit periodic spontaneous aggregations, nor can they be mechanically stimulated to aggregate under any condition. These observations clearly demonstrate a difference between erythrophore and melanophore regulation. One cannot exclude, however, that perturbing extracellular Ca^{2+} concentrations changes many actors, including ion permeabilities and membrane excitability. These complicating issues demand further exploration to delineate the role of Ca^{2+} in pigment aggregation.

Microinjection of Ca^{2+} Induces Aggregation in Erythrophores, But Not Melanophores

Because extracellular Ca^{2+} was found to be necessary for pigment granule aggregation in erythrophores, we wanted to determine whether an influx of Ca^{2+} is sufficient to induce aggregation. This was accomplished by microinjecting either Ca^{2+} or Ca^{2+}-chelator into chromatophores in the presence of Ca^{2+}-free medium, and observing the effects on pigment movement (see Materials and Methods).

We found that 90% of erythrophores aggregated when injected with Ca^{2+} (10 μM pipette concentration; n = 20), but none aggregated when injected with the Ca^{2+}-chelator, BAPTA (10 μM pipette concentration; n = 12). We used BAPTA-containing microinjection buffer as a control, because erythrophores injected with buffer alone were induced to aggregate, presumably due to trace amounts of Ca^{2+} in the buffer, which could further trigger a Ca^{2+}-induced Ca^{2+} release. These results illustrate that a rise in $[Ca^{2+}]_i$ is a sufficient stimulus for aggregation in erythrophores. We also found that only 2 out of 11 melanophores aggregated pigment after injection of Ca^{2+}, suggesting that Ca^{2+} is not a sufficient signal for pigment aggregation in melanophores. This is consistent with the findings of Sammak et al. (1992) in other teleost melanophores. The two melanophores which did aggregate upon Ca^{2+} injection could have done so due to the microinjection process stimulating a component of a Ca^{2+}-independent regulatory pathway. Though no BAPTA-injected melanophores aggregated pigment (n = 6), 2 out of

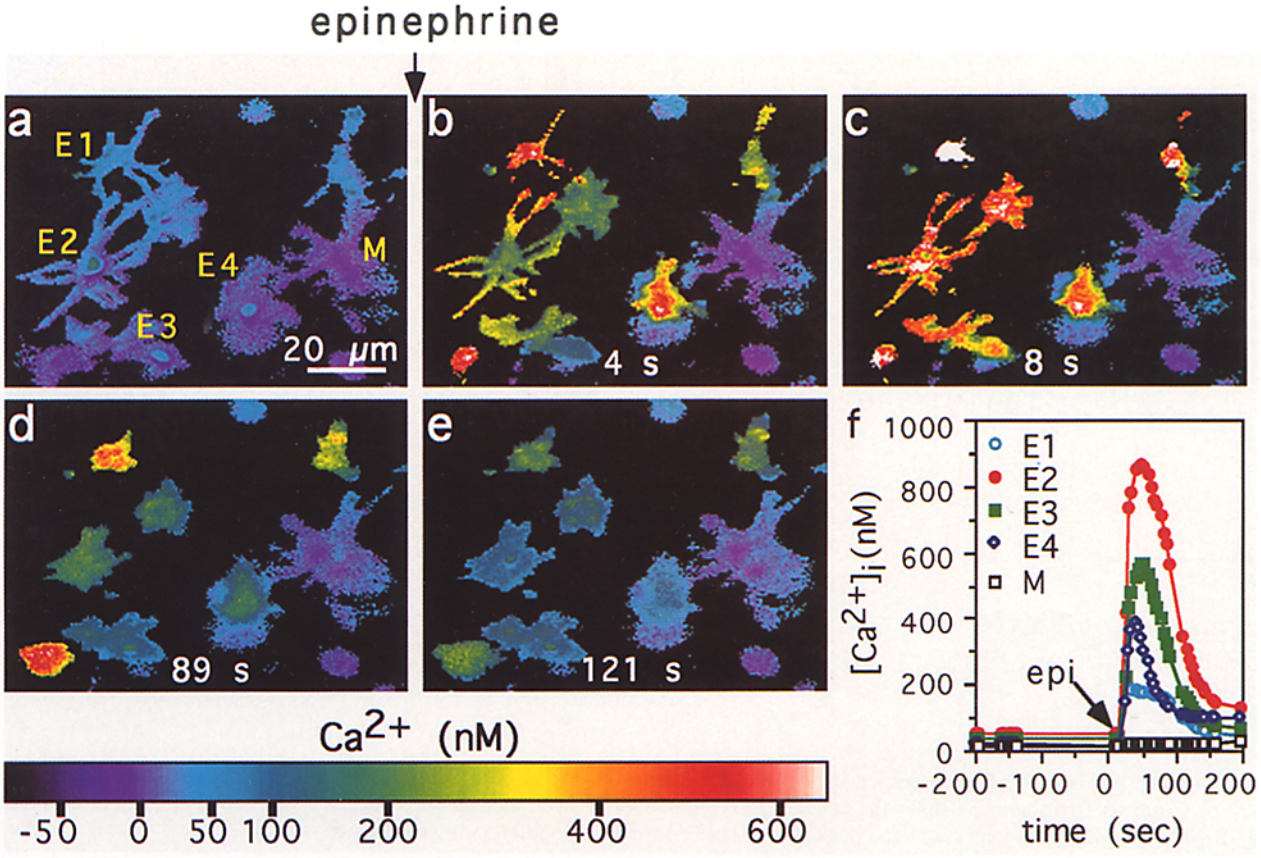

Figure 3. Quantitative summary of Fura-2-measured $[Ca^{2+}]_i$ changes during pigment aggregation in several chromatophores. Chromatophores were loaded with Fura-2 AM, then observed while in Ca^{2+}-containing medium. Ratio images (*a–e*) and a graphical representation of $[Ca^{2+}]_i$ (*f*) are shown for four neighboring erythrophores (labeled *E1–E4* on the first ratio image) and a melanophore (*M*). Note that three other erythrophores in the same field of view are not represented on the graph in *f*. The cells were stimulated with epinephrine at the indicated time. Note that all erythrophores exhibited a dramatic, early increase (10–20-fold rise) in $[Ca^{2+}]_i$ during pigment aggregation (*b–c*), then fell to nearly basal levels, during which time the pigment remained aggregated (*e*). In contrast, the melanophore aggregated pigment without a rise in $[Ca^{2+}]_i$.

7 EGTA-injected melanophores (10 μM pipette concentration) aggregated pigment, further illustrating that this microinjection-induced aggregation is Ca^{2+}-independent.

Intracellular Ca^{2+} Increases Dramatically during Pigment Aggregation in Erythrophores, But Not Melanophores

Fig. 2 illustrates $[Ca^{2+}]_i$ changes during aggregation in both erythrophores and melanophores as measured by ratio imaging of Fura-2 fluorescence (see Materials and Methods). Nearly simultaneous ratio and DIC images were obtained with the use of a dual camera system, allowing correlation of $[Ca^{2+}]_i$ changes with pigment movement. Timelapse images of a single erythrophore (*a–h*) graphically illustrate the rise in $[Ca^{2+}]_i$ that occurred during epinephrine-stimulated pigment aggregation. In contrast, the melanophore shown (*i–p*) aggregated pigment without a significant rise in $[Ca^{2+}]_i$. The images and graph in Fig. 3 further illustrate the difference in Ca^{2+} signaling by showing $[Ca^{2+}]_i$ in a melanophore with several neighboring erythrophores simultaneously treated with epinephrine. While all of these cells aggregated, $[Ca^{2+}]_i$ rose only in the erythrophores.

Fig. 4 summarizes the rise in $[Ca^{2+}]_i$ during pigment aggregation in over 80 erythrophores and 12 melanophores. The values shown for resting chromatophores are average $[Ca^{2+}]_i$ per cell, while all other values are peak $[Ca^{2+}]_i$ obtained during spontaneous, epinephrine-induced or mechanically induced pigment aggregation. Erythrophores, but not melanophores exhibited dramatic increases in $[Ca^{2+}]_i$ during aggregation (Fig. 4 *a*). The magnitude of this rise was at least threefold in 84% of spontaneously aggregating cells, 97% of epinephrine-treated cells, and 100% of mechanically stimulated cells. Greater than 10-fold increases were seen during 37% of spontaneous, 71% of epinephrine-induced, and 91% of mechanically induced pigment aggregations. In addition, erythrophores that were stimulated with epinephrine in the absence of extracellular Ca^{2+} did not experience a rise in $[Ca^{2+}]_i$ and did not aggregate pigment ($n = 6$), further supporting the idea that a $[Ca^{2+}]_i$ rise is necessary for pigment aggregation. Melanophores (Fig. 4 *b*), on the other hand, did not exhibit $[Ca^{2+}]_i$ increases during pigment granule aggregation (83% displayed less than a threefold rise in $[Ca^{2+}]_i$; 42% experienced no $[Ca^{2+}]_i$ increase). Furthermore, melanophores loaded with the Ca^{2+}-chelator, BAPTA-AM, were able to aggregate with no demonstrable increase in $[Ca^{2+}]_i$ as measured by Fura-2 ratio imaging ($n = 3$).

These results indicate that while melanophores do not require, nor experience a $[Ca^{2+}]_i$ rise during pigment granule aggregation, erythrophores do exhibit and require a dramatic

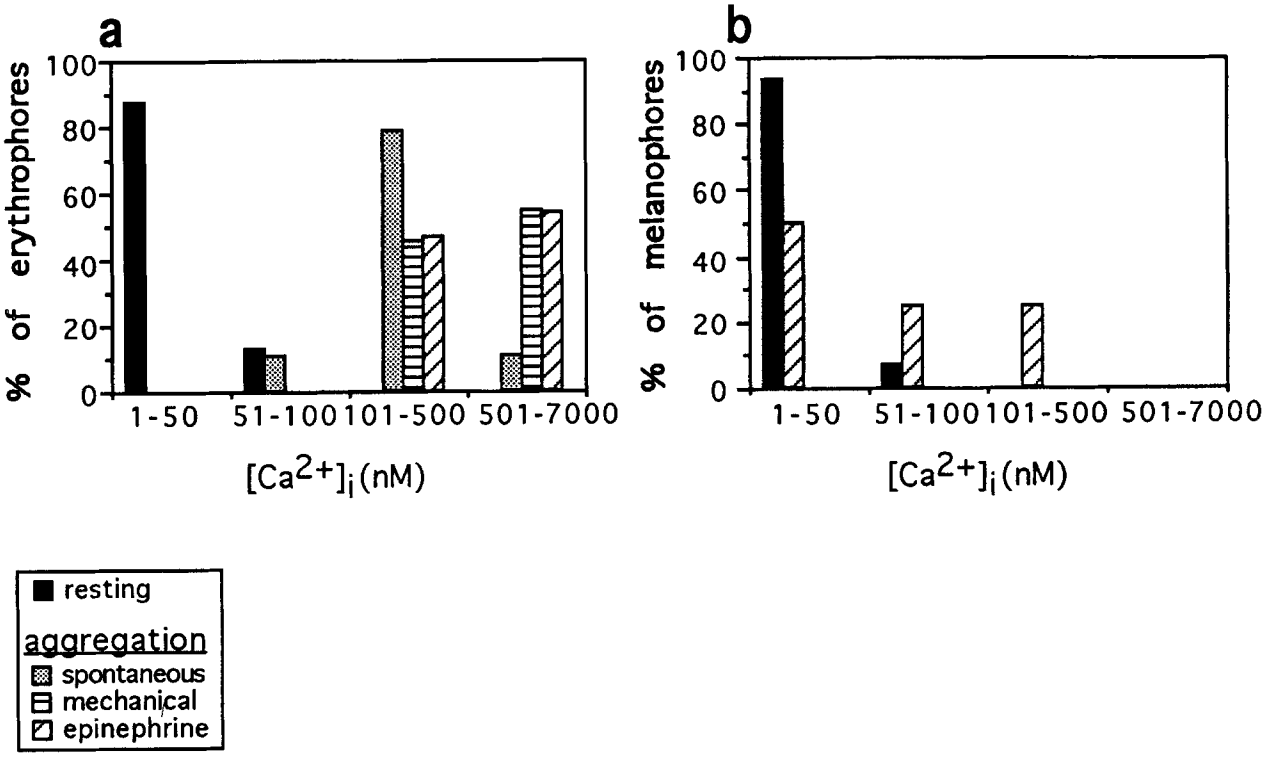

Figure 4. Pigment aggregation is accompanied by a dramatic increase in $[Ca^{2+}]_i$ over resting levels in erythrophores, but not melanophores. The frequency distributions display the percent of erythrophores (*a*) and melanophores (*b*) exhibiting various $[Ca^{2+}]_i$ levels under resting, dispersive conditions (*solid bars*) and peak $[Ca^{2+}]_i$ levels during aggregation (*stippled* and *striped* bars). The data is a summary of several experiments of the type shown in Figs. 3 and 4. The resting $[Ca^{2+}]_i$ level for each cell was determined by averaging several of the $[Ca^{2+}]_i$ measurements obtained before stimulation. The data summarizes resting $[Ca^{2+}]_i$ for 80 erythrophores, and 15 melanophores and peak $[Ca^{2+}]_i$ during pigment aggregation for 71 erythrophores (spontaneous: $n = 19$; mechanical: $n = 11$; epinephrine: $n = 41$) and 12 melanophores. Note that during pigment aggregation, $[Ca^{2+}]_i$ rose dramatically in erythrophores (*a*), but not melanophores (*b*). Note also that all resting erythrophores with $[Ca^{2+}]_i$ of less than 50 nM contained dispersed pigment, with higher concentrations inducing pigment aggregation.

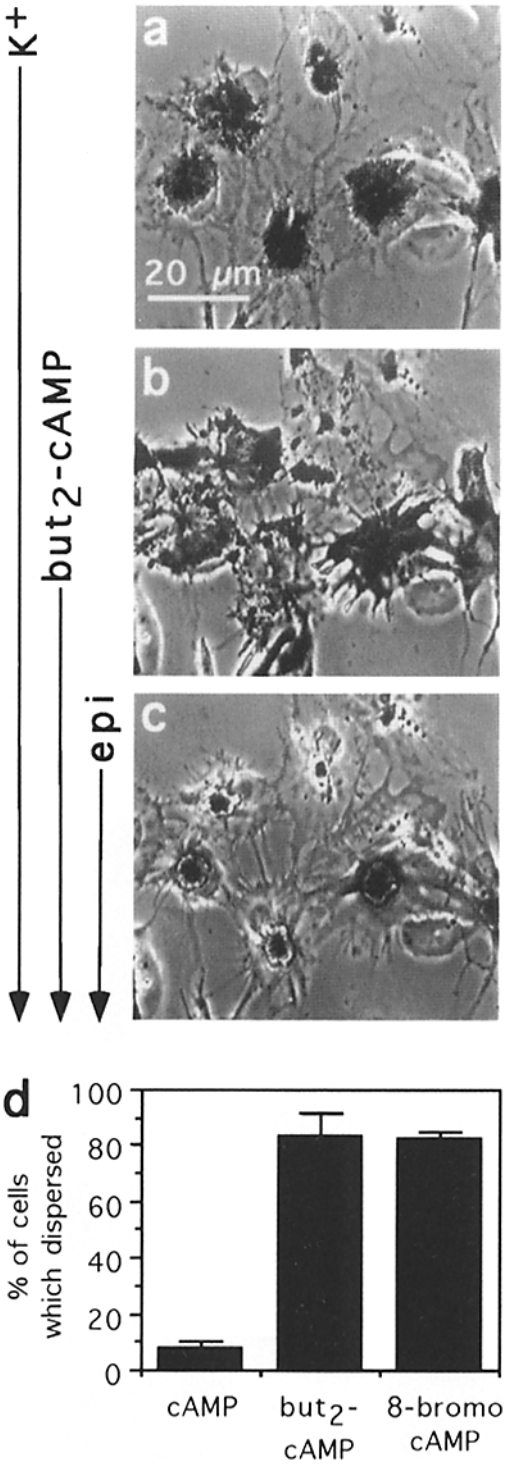

Figure 5. Membrane-permeable cAMP analogs cause pigment dispersion in erythrophores, which can be overridden by aggregating stimuli. Cells were aggregated with K^+-supplemented media (*a*), then perfused with cell-permeable, but$_2$-cAMP (*b*) while maintaining the extracellular [K^+] as described in Materials and Methods. Cells are shown 30 min after perfusion (*b*). Note that the but$_2$-cAMP caused a complete dispersion with some cells appearing hyperdispersed. Epinephrine (1 μM) in media containing both supplemented K^+ and dibutyryl cAMP was then applied to the dispersed cells (*c*). This caused an immediate aggregation, demonstrating that the cAMP dispersive signal can be overridden by Ca^{2+}-mediated aggregation. The graph in *d* illustrates the ability of two different permeable cAMP analogs to cause pigment dispersion in erythrophores within 30 min of perfusion of the analog. Non-permeable cAMP was perfused as a control. This data summarizes two independent experiments per cAMP analog as described in Materials and Methods. Note that 84 (±8)% of erythrophores treated with but$_2$-cAMP (n = 83, 78) and 82(±3)% of erythrophores treated with 8-bromo cAMP (n = 57, 47) completely disperse pigment within 30 min. In contrast, only 8(±2)% of erythrophores treated with non-permeable cAMP (n = 40, 35) responded by dispersing pigment. Error bars indicate SD.

$[Ca^{2+}]_i$ rise during pigment granule aggregation resulting from three different stimuli.

α-Adrenoceptor Pharmacology of Chromatophores

To further illustrate the contrasting second messenger pathways used during pigment aggregation in erythrophores and melanophores, we studied the effects of both α_1- and α_2-specific agonists on pigment distribution in both of these cell types. Similar studies have been done in two other melanophore models (Thaler and Haimo, 1992; Sammak et al., 1992). Stimulation of α_1-adrenergic receptors causes an increase in $[Ca^{2+}]_i$, whereas α_2 stimulation induces a fall in $[cAMP]_i$ (Exton, 1985). Because these agents are not completely specific, we applied each agonist while blocking the opposing receptor. We found that increasing $[Ca^{2+}]_i$ with an α_1 agonist (10^{-7} M methoxamine) while blocking a fall in $[cAMP]_i$ with an α_2 antagonist (10^{-5} M idazoxin) caused only 12% of melanophores to aggregate pigment (n = 17), whereas all observed erythrophores aggregated pigment (n > 30). In contrast, decreasing $[cAMP]_i$ without inducing a rise in $[Ca^{2+}]_i$ by α_2 stimulation (10^{-7} M clonidine) in the presence of an α_1 blocker (10^{-5} M prazosin) caused all observed melanophores to aggregate pigment (n = 33), while >90% of erythrophores did not aggregate pigment (n > 30). Measurements of $[Ca^{2+}]_i$ by Fura-2 ratio imaging demonstrated that $[Ca^{2+}]_i$ did not increase in melanophores during this α_2-specific stimulation. These pharmacological studies illustrate that pigment aggregation is dependent upon a rise in $[Ca^{2+}]_i$ in erythrophores, but not melanophores, while a fall in $[cAMP]_i$ causes pigment aggregation in melanophores. Thus, these results, in conjunction with those of Sammak et al. (1992), demonstrate that melanophores from two different species of fish use similar second messenger cascades to regulate pigment transport.

Cell Permeable cAMP Analogs Induce Dispersion in Aggregated Erythrophores

Results derived from both the ionophore (Luby-Phelps and Porter, 1982) and lysed cell models (McNiven and Ward, 1988) discussed earlier suggest that $[Ca^{2+}]_i$ alone determines the distribution of pigment granules in squirrel fish erythrophores, because raising or lowering $[Ca^{2+}]$ in the bathing buffer is sufficient to induce both aggregation and dispersion, respectively. Using direct, dynamic $[Ca^{2+}]_i$ measurements we were surprised to find that after the addition of epinephrine to erythrophores, $[Ca^{2+}]_i$ initially rose causing rapid pigment aggregation, then dropped off while the pigment granules remain aggregated (Figs. 2 *d* and 3). In addition we observed that microinjection of the Ca^{2+}-chelator, BAPTA (0.9 mM pipette concentration), in the presence of

extracellular Ca^{2+} did not induce pigment dispersion in 88% of injected cells (n = 41), even though $[Ca^{2+}]_i$ was low, as demonstrated by Fura-2 imaging (data not shown). These results suggest that there is another messenger involved in signaling dispersion. We investigated the possible role of cAMP, because it is involved in signaling pigment movements in melanophores, and because past studies in erythrophores have implicated cAMP in dispersion (Byers and Porter, 1977).

To study the process of dispersion more closely, erythrophores were held in a partially aggregated state using K^+-enriched medium (see Materials and Methods). Erythrophores were observed while permeable cAMP analogs (either but_2-cAMP or 8-bromo cAMP) were perfused into the chamber with K^+-enriched conditions maintained throughout the experiment (Fig. 5, *a* and *b*). Erythrophores were also observed during perfusion of non-permeable cAMP to control for extracellular cAMP effects. Within 30 min of perfusion with permeant cAMP, erythrophore pigment was fully dispersed (Fig. 5 *b*). Observation of many cells (two independent experiments per analog) revealed that erythrophores treated with but_2-cAMP or 8-bromo cAMP completely disperse pigment within 30 min (Fig. 5 *d*), with many of these cells appearing hyperdispersed with central clearing (Fig. 5 *b*). In contrast, only a minority of erythrophores treated with non-permeable cAMP responded by dispersing pigment (Fig. 5 *d*). The permeable cAMP-loading was reversible, demonstrated by reaggregation within 20–30 min of perfusion with K^+-enriched medium after washout of cAMP (data not shown).

Permeable cAMP analog-induced dispersion could be overridden by the aggregation stimulus, epinephrine. After 30 min of perfusion with permeable cAMP analogs, the addition of epinephrine (in permeable cAMP-containing, high-K^+-medium) resulted in immediate, tight pigment aggregation in all observed cells (Fig. 5 *c*). Replacing the epinephrine with high-K^+ medium caused a redispersion, demonstrating that these cells were still loaded with high levels of cAMP. These results suggest that both high $[cAMP]_i$ and low $[Ca^{2+}]_i$ are necessary for dispersion and that high $[Ca^{2+}]_i$ alone is a sufficient stimulus for aggregation, and can override dispersive signals.

Aggregation Stimuli and $[Ca^{2+}]_i$ Increases Are Rapidly Transmitted to Adjoining Erythrophores

A mechanically stimulated erythrophore rapidly transmits the aggregation stimuli to neighboring cells, causing simultaneous aggregation of large groups of erythrophores. Fig. 6 illustrates a small group of cells in which we performed

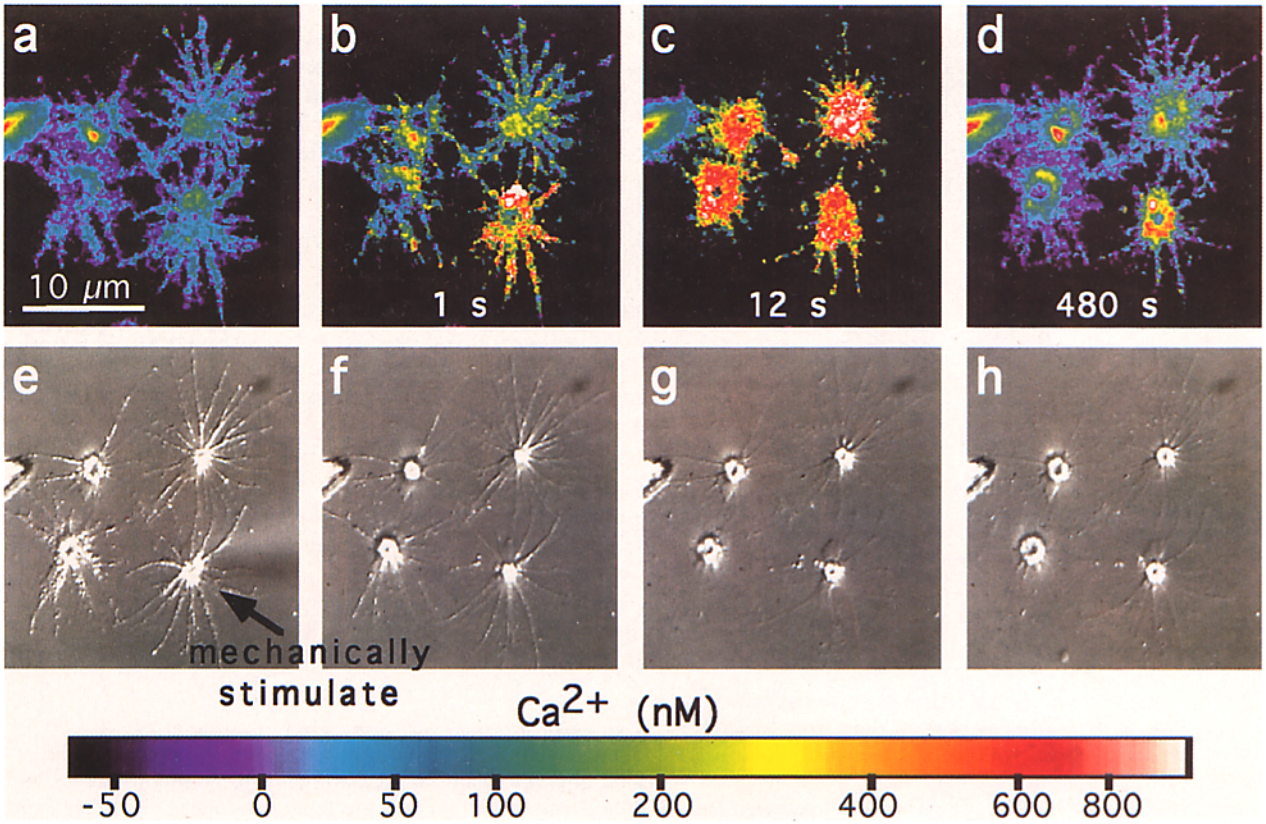

Figure 6. Erythrophores rapidly transmit a Ca^{2+}-based aggregation stimulus to adjoining erythrophores. Erythrophores were loaded with Fura-2 AM, then imaged while in Ca^{2+}-containing medium. The paired images are simultaneous pseudocolor ratio (*a–d*) and DIC (*e–h*) images of coupled erythrophores. The lower right cell (*arrow*) was mechanically stimulated to aggregate pigment after the first pair of images were acquired (*a* and *e*). The color bar delineates the pseudocolor representation of $[Ca^{2+}]_i$. Note that both the stimulus to aggregate pigment (*f–h*) and the rise in $[Ca^{2+}]_i$ (*b–c*) were immediately transmitted to the adjacent erythrophores. This calcium signaling requires physical contact by adjacent cells and is not perpetuated by melanophores or fibroblasts (data not shown).

simultaneous ratio (*a–d*) and DIC (*e–h*) imaging to demonstrate the coupling of both aggregation and $[Ca^{2+}]_i$ increases. The cell at the lower right was mechanically stimulated after the first pair of images was obtained. The $[Ca^{2+}]_i$ increased rapidly in the mechanically stimulated cell, while initiating a $[Ca^{2+}]_i$ transient and aggregation in the neighboring cells (Fig. 6, *b* and *f*). This coupling phenomenon is not limited to immediate neighbors of a stimulated cell, but can travel through many cells (data not shown). The absence of any significant lag (<1 s) between cell responses suggests that the coupling is most likely due to a membrane action potential, rather than diffusion of soluble signals through gap junctions.

Discussion

Antagonistic Regulation of Pigment Transport by cAMP and Ca^{2+}

Past study of the regulation of pigment movement in fish chromatophores has implicated both cAMP and Ca^{2+} as second messengers (Rozdzial and Haimo, 1986*a*; Palazzo et al., 1989; Sammak et al., 1992; Luby-Phelps and Porter, 1982; Oshima et al., 1988; McNiven and Ward, 1988). It has become clear, however, that two types of chromatophores, melanophores and erythrophores, use contrasting regulatory schemes. Several different observations strongly suggest that the melanophore is regulated exclusively by altered levels of cAMP. First, studies using permeabilized cell models demonstrate that pigment dispersion and aggregation can be induced by the inclusion or removal of cAMP, respectively (Rozdzial and Haimo, 1986*a*). Second, direct measurements using quantitative fluorescent probes and dynamic, ratio imaging techniques show a rise and fall of $[cAMP]_i$ during pigment dispersion and aggregation, respectively, with no requirement for a rise in $[Ca^{2+}]_i$ (Sammak et al., 1992). Third, as demonstrated here, neither Ca^{2+}-free media nor microinjection of Ca^{2+} has an effect on melanosome translocation. Fourth, BAPTA-loaded melanophores aggregate pigment with no measured increase in $[Ca^{2+}]_i$ (present study and Sammak et al., 1992). Fifth, our study of α_1- and α_2-adrenoceptor stimulation indicates that a fall in $[cAMP]_i$ without a rise in $[Ca^{2+}]_i$ is sufficient for melanosome aggregation. An isolated study has provided indirect and contrasting data that $[Ca^{2+}]_i$ is also necessary for pigment aggregation in *Tilapia* melanophores, based on pharmacological manipulations of α_1 and α_2 adrenoceptors (Thaler and Haimo, 1992). Although a third type of melanophore was used, this pharmacological study examined melanophores in situ, innervated by numerous contacting neurons, which may have produced findings which differ from the two studies using isolated melanophores.

In contrast, Ca^{2+} has been implicated as the sole regulator of pigment movements in erythrophores. The ionophore studies of Luby-Phelps and Porter (1982) and lysed cell model of McNiven and Ward (1988) demonstrated that pigment aggregation resulted when free $[Ca^{2+}]$ was raised to a threshold level (ionophore: $\geq 10^{-6}$ M Ca^{2+}; lysed-cell: $\geq 10^{-7}$ M Ca^{2+}). Pigment dispersion was induced when free $[Ca^{2+}]$ was reduced (ionophore: $\leq 10^{-7}$ M Ca^{2+}; lysed-cell: $\leq 10^{-8}$ M Ca^{2+}). These experimental models, however, may not portray realistic regulatory events. In this study, using isolated, intact erythrophores, we first demonstrate that Ca^{2+} is absolutely necessary for pigment aggregation by blocking this event in the absence of extracellular Ca^{2+} (Fig. 1). Second, we demonstrate through microinjection of Ca^{2+} that an influx of this ion provides a sufficient signal to cause pigment aggregation. Third, using ratio imaging and the Ca^{2+}-sensitive probe, Fura-2, we determine that a rise in $[Ca^{2+}]_i$ always occurs during pigment aggregation in erythrophores (Figs. 2–4), whether it is a spontaneous or stimulated event. Resting erythrophores with dispersed pigment have low $[Ca^{2+}]_i$ ($3.1\ (\pm 1.8) \times 10^{-8}$ M). Upon pigment aggregation, $[Ca^{2+}]_i$ rises dramatically (ranging from 3- to greater than 100-fold increases) with an apparent threshold of $\sim 10^{-7}$ M Ca^{2+} (Fig. 4 *a*), consistent with the predictions derived from the lysed cell model (McNiven and Ward, 1988).

We were surprised to find that after epinephrine-induced stimulation of erythrophores, $[Ca^{2+}]_i$ fell to nearly basal levels, while the pigment remained aggregated (Figs. 2 and 3). In addition, microinjection of BAPTA did not induce pigment dispersion in erythrophores. These observations are not consistent with the previously suggested model in which lowering $[Ca^{2+}]_i$ is sufficient to induce pigment dispersion. Thus, we hypothesized that there is a second signal involved in dispersion, which the ionophore and lysed cell models failed to demonstrate. We chose to study the role of cAMP in erythrophore pigment dispersion, because cAMP is involved in pigment regulation in melanophores, and because a past study in squirrel fish erythrophores has implicated the involvement of cAMP in this cell (Byers and Porter, 1977).

We find that the permeable cAMP analogs, but_2-cAMP and 8-bromo cAMP, induce pigment dispersion (Fig. 5) in erythrophores while under a mild aggregation stimulus (K^+-enriched medium), whereas non-permeable cAMP is unable to induce pigment dispersion. This cAMP-based dispersive stimulus can be overridden by Ca^{2+}-induced aggregation, illustrated by the addition of epinephrine to erythrophores which had been dispersed with permeable cAMP. We know that epinephrine is not inducing a rapid loss of cAMP, because rinsing away the epinephrine with K^+-enriched medium causes a return to the completely dispersed state.

From the observations described here, we postulate that antagonistic regulation of pigment motility by Ca^{2+} and cAMP in erythrophores could occur as depicted in Fig. 7. Cultured erythrophores are normally dispersed until a stimulus, such as epinephrine, binds to a receptor in the cell membrane (Fig. 7 *a*). This event or other stimuli (mechanical stimuli or stimuli from a neighboring cell) trigger an influx of Ca^{2+} from the extracellular environment. The Ca^{2+} spike could then be enhanced by release from intracellular stores in a Ca^{2+}- or IP_3-dependent manner. The mechanism by which Ca^{2+} might regulate a microtubule motor is undefined, but several possibilities may be considered. Ca^{2+} could act to turn off an anterograde motor, or to turn on a retrograde motor. This signaling could occur by direct binding of Ca^{2+} to the motor itself, or indirectly by Ca^{2+} acting as a second messenger signaling downstream events such as activation of a protein kinase or a protein phosphatase. Stimulated pigment dispersion could occur by the activation of adenylate cyclase coupled with Ca^{2+} sequestration allowing a cAMP-dependent protein kinase to activate the anterograde motor (Fig. 7 *b*). Possible mechanisms by which Ca^{2+} and cAMP evoke their effects will be discussed later.

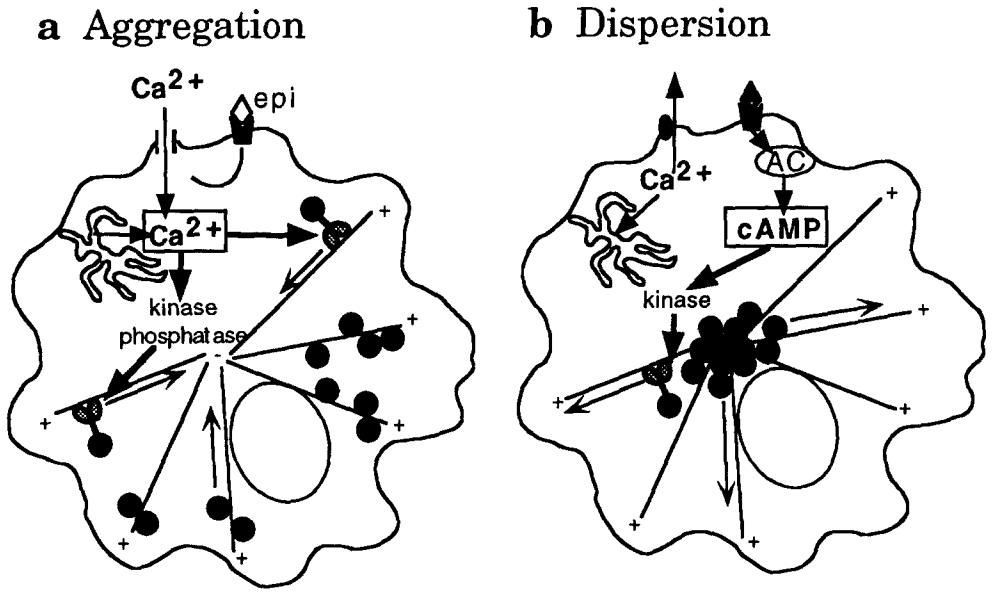

Figure 7. Ca^{2+} and cAMP act in opposition to regulate pigment aggregation and dispersion in erythrophores. The model shows potential mechanisms by which Ca^{2+} and cAMP regulate erythrophore pigment distribution. In the case of aggregation (*a*), epinephrine receptor binding causes influx of Ca^{2+} into the cell from the extracellular space. This may also cause a Ca^{2+}-induced Ca^{2+}-release or an IP_3-mediated release from intracellular stores, thus enhancing the Ca^{2+} transient. Ca^{2+} may then interact with a microtubule motor by direct binding or indirectly via a kinase or phosphatase. During a dispersive stimulus (*b*), a cAMP-dependent protein kinase may activate the anterograde motor, while Ca^{2+} is sequestered into stores or pumped out of the cell, allowing the anterograde motor to function without opposition. *epi*, epinephrine; *AC*, adenylate cyclase.

There are two ways that cAMP could participate in cyclic regulation of pigment aggregation and dispersion. First cAMP could rise and fall along with directional pigment transport. Cycles of atropine-induced pigment dispersion and epinephrine-stimulated pigment aggregation in *Pterophyllum* melanophores are accompanied by a rise and fall in cAMP, respectively (Sammak et al., 1992). Second cAMP could remain high, while another signal transiently overrides this dispersive stimulus. In erythrophores, we have demonstrated that while cAMP remains high enough to induce dispersion in K^+-enriched medium, epinephrine is able to cause an overriding Ca^{2+}-mediated pigment aggregation. Such a mechanism could explain how the erythrophore is able to undergo rapid cycles of aggregation and dispersion, possibly without the need for rapid manufacture and degradation of cAMP. Likewise, epinephrine-stimulated pigment aggregation could lead to both a Ca^{2+}-induced pigment aggregation and a drop in cAMP, which would prevent dispersion once $[Ca^{2+}]_i$ has returned to basal levels.

Differences between Lysed Models and Intact Cells

The finding that Ca^{2+} is not the only regulatory signal necessary for bidirectional pigment movement in squirrel fish erythrophores was unexpected considering the results obtained using ionophore and lysed cell models (Luby-Phelps and Porter, 1982; McNiven and Ward, 1988). It is possible that a regulatory element lost or altered in the model systems results in a constitutively active cAMP cascade, allowing dispersion in the absence of cAMP. It is known that components of cAMP-dependent protein kinase bind to cytoskeletal components, such as microtubule-associated proteins, including MAP-2 and to neurofilaments (Theurkauf and Vallee, 1982; Miller et al., 1982; Lohmann et al., 1984; Trinczek and Schwoch, 1990). In addition, MAP-2 is believed to comigrate with pigment granules in squirrel fish erythrophores (Stearns and Binder, 1987). Thus, the kinase could remain bound in the model systems, while other regulatory components of the cascade are washed away, resulting in uncoupling of the cAMP regulatory pathway. Whatever the reason, the model systems clearly contrast with the intact cell studies by suggesting that Ca^{2+} is the exclusive regulator. The possibility that lysed cell models may differ from whole cells is reviewed elegantly by Swanson (1993) in a recent review of lysed cell models of motility.

How Do Ca^{2+} and cAMP Activate Pigment Movements?

While this is the first direct demonstration of $[Ca^{2+}]_i$ changes accompanying the initiation of microtubule-based organelle transport in single cells, Ca^{2+} has been implicated in regulating other microtubule-based motility, such as flagellar beat and wave form (Holwill and McGregor, 1976; Hyams and Borisy, 1978; Gibbons and Gibbons, 1980), ciliary beat frequency in respiratory tract epithelial cells (Sanderson et al., 1990), and in vitro dynein-based ciliary gliding (Mori and Miki-Noumura, 1992). As discussed earlier, Ca^{2+}-based regulation in erythrophores might occur directly by Ca^{2+} or Ca^{2+}-binding protein interactions with the motors, or indirectly by activation of Ca^{2+}-dependent protein kinases or phosphatases (Fig. 7 *a*).

To our knowledge, no observations have been made that microtubule motors are Ca^{2+}-binding proteins, but Ca^{2+}-dependent calmodulin binding and regulation of actin-based and microtubule-based motors has been detected. Calmodulin regulates both avian brush border myosin I (Conzelman and Mooseker, 1987; Collins et al., 1990) and mammalian myosin I (Barylko et al., 1992) and has been recently shown to bind and inhibit the ATPase activity of purified bovine brain kinesin in a Ca^{2+}-dependent manner (Matthies et al., 1993). The latter observation would agree with the scenario we see in erythrophores, namely Ca^{2+}-dependent retrograde transport. The same paper demonstrated that this inhibition of ATPase activity does not occur if kinesin is phosphorylated by cAMP-dependent protein kinase. Thus, Ca^{2+}/

calmodulin could also function through activation of a protein phosphatase which would enable the binding of Ca^{2+}/calmodulin to exert its inhibitory effect on kinesin. Previous studies in *Tilapia* melanophores have implicated the Ca^{2+}-regulated phosphatase, calcineurin in pigment aggregation (Thaler and Haimo, 1990). Though calmodulin-independent Ca^{2+}-based regulation in erythrophores is supported by the observation that calmodulin inhibitors do not inhibit aggregation in the lysed erythrophore model (McNiven and Ward, 1988), the involvement of calmodulin can not be ruled out at this point. Ca^{2+} could also function by activating a protein kinase, as suggested by a recent finding that Ca^{2+} ionophore decreases in vivo phosphorylation of kinesin light chains in chick sympathetic neurons (Hollenbeck, 1993*a*).

The role of cAMP may be less complicated than that of Ca^{2+}, due to more limited possibilities as a second messenger. The downstream effect of cAMP is most likely an activation of a cAMP-dependent protein kinase, which acts on one or both motors (Fig. 7 *b*). There is strong evidence that cAMP-dependent protein kinase activity activates anterograde motors. Previous studies have revealed that pigment dispersion and aggregation in *Tilapia* melanophores, are regulated by cAMP-dependent protein phosphorylation and dephosphorylation, respectively (Rozdzial and Haimo, 1986*b*). In addition, a protein associated with pigment granules from goldfish xanthophores is phosphorylated in a cAMP-dependent manner accompanying dispersion (Lynch et al., 1986; Palazzo et al., 1989). As mentioned above, phosphorylation of purified bovine brain kinesin by cAMP-dependent protein kinase prevents calmodulin inhibition of kinesin ATPase activity (Matthies et al., 1993). Hollenbeck has recently demonstrated that cAMP causes less retrograde, and more anterograde transport of a specific class of phase-dense organelles in cultured sympathetic neurons (1993*b*), though he also observed that cAMP-inducing agents did not change the phosphorylation state of kinesin in vivo (1993*a*). An in vitro observation that cAMP-dependent phosphorylation of kinesin causes this motor to dissociate from synaptic vesicles (Sato-Yoshitake et al., 1992) is the opposite effect that we would expect based on observations in chromatophores, purified bovine brain kinesin, and sympathetic neurons, but they clearly demonstrate that cAMP can play a role in the regulation of motor function.

In closing, the discussions presented here and by others indicate that Ca^{2+} plays an important role in regulating pigment transport in erythrophores. The molecular mechanism by which Ca^{2+} effects organelle motility in these and other neuronal cells will prove interesting.

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