Calcium Regulation of Pigment Transport In Vitro

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Abstract. Calcium has been implicated in the regulation of many cellular motility events. In this study we have examined the role of different Ca^{2+} concentrations on the in vitro transport of pigment within cultured chromatophores. Cells treated with Brij detergent for 1–2 min were stripped of their plasma membranes, leaving their cytoskeleton and associated pigment granules exposed to the external milieu. We found that retrograde pigment transport (aggregation) is induced upon addition of 1 mM $MgATP^{2-}$ with 10^{-7} M free Ca^{2+}, while an orthograde transport (redispersal) of pigment results from lowering the concentration of free Ca^{2+} to 10^{-8} M while maintaining 1 mM $MgATP^{2-}$. These Ca^{2+}-regulated movements are ATP dependent but are apparently independent of cAMP and insensitive to calmodulin inhibitors. The observations reported here provide novel evidence that the concentration of free Ca^{2+} acts to regulate the direction of intracellular organelle transport.

Most eukaryotic cells transport specific organelles to predefined cytoplasmic destinations in response to either internal or external stimuli. The centripetal translocation of endosomes toward the cell center, orthograde movement of secretory vesicles to the basal or apical plasma membrane, and the deliberate poleward segregation of chromosomes during anaphase A are all temporally distinct events initiated by specific signals. Although many of these processes can be induced through the external application of various hormones, ligands, or ions, the precise signals or triggers that initiate organelle movements in a specific direction are unknown.

Chromatophores provide convenient models for studying regulation of organelle transport for several reasons. Most importantly, pigment granule translocation occurs in two temporally distinct events: a retrograde transport that aggregates pigment to the cell center and a subsequent orthograde movement that disperses pigment to the cell periphery. These movements, depending on the type of chromatophore, may be very rapid (3–6 μm/s) and can be repeated dozens of times in rapid succession merely by introducing epinephrine to aggregate and caffeine to disperse (for reviews see Schliwa, 1981; McNiven and Porter, 1984).

It is known that, in most teleost chromatophores, pigment transport is dependent upon the presence of microtubules (Schliwa and Bereiter-Han, 1973; Beckerle and Porter, 1983) and that the intrinsic structural polarity of the microtubules confer directionality to translocating pigment granules. Aggregation recognizes, or interacts with, a plus to minus microtubule polarity and dispersion a minus to plus microtubule polarity (McNiven et al., 1984; McNiven and Porter, 1986). The cytoplasmic signals that initiate these directed movements remains undetermined. It is likely that calcium is somehow involved in this activation process because cultured erythrophores will not aggregate their pigment in calcium-free buffer and studies using the ionophore A23187 have shown that a minimum internal calcium concentration of 5×10^{-6} M is required to induce pigment aggregation (Ludy-Phelps and Porter, 1982). Whether calcium acts directly upon the motility machinery or is merely a single step in an elaborate cascade is unknown. The involvement of calcium in regulating cell motility is, of course, not unique to chromatophores. A rise in the concentration of cytoplasmic free calcium has long been known to stimulate contraction of skeletal (Ebashi et al., 1969) and smooth muscle (Adelstein, 1982), and is believed either to initiate or effect various nonmuscle motile events such as secretion (Malaisse, 1984), axoplasmic transport (Ochs et al., 1977), ciliary and flagellar wave form (Gibbons and Gibbons, 1980; Bessen et al., 1980), and, more recently, the onset of mitotic anaphase (Izant, 1983; Hepler, 1985; Wolniak and Bart, 1985).

There is also substantial evidence implicating the nucleotide cAMP in the regulation of pigment movements in chromatophores. Membrane-permeable forms of cAMP, exogenously added, can induce pigment dispersion in whole cells (Novales and Fujii, 1970; Abramowitz and Chavin, 1974) as does the pituitary hormone, melanocyte stimulating hormone, which has been demonstrated to increase cytoplasmic cAMP levels (Bagnara and Hadley, 1973). The observation that the addition, and subsequent removal, of cAMP to permeabilized melanophores stimulates a slow dispersion–aggregation cycle (Grundstrom et al., 1985; Rozdzial and Haimo, 1986*a*) further implicates cAMP as a transport regulator. Incubation of erythrophores in reagents that inhibit cyclic nucleotide phosphodiesterases (caffeine or theophylline) induces pigment dispersion. Finally, and most provocatively, a 57-kD granule-associated protein is reversibly phosphorylated by a cAMP-dependent protein kinase during pig-

ment dispersion (Lynch et al., 1986; Rozdzial and Haimo, 1986*b*). It is possible that both Ca^{2+} and cAMP work together to regulate pigment aggregation and dispersion, respectively.

In this study we have attempted to examine directly the regulatory effects of calcium and cAMP upon intracellular particle transport in vitro using a demembranated chromatophore model. We find that erythrophores cultured on glass coverslips, when treated with detergent and thus stripped of their plasma membranes, will rapidly aggregate their pigment in buffer solutions containing 10^{-7} M free calcium, 1 mM free Mg^{2+} and 1 mM $MgATP^{2-}$. Reducing the free calcium concentration 10-fold to 10^{-8} M while maintaining 1 mM $MgATP^{2-}$ reverses the direction of pigment transport from retrograde to orthograde (dispersion). Thus, in these chromatophores, an increase or decrease in calcium is responsible for initiating ATP-dependent pigment transport in either retrograde or orthograde directions, respectively.

Materials and Methods

Chromatophore Isolation

Erythrophores of the squirrel fish *Holocentrus* were cultured following the methods of Luby-Phelps and Porter (1980).

Reagents

All drugs and reagents were purchased from Sigma Chemical Co. (St. Louis, MO) except the following. Taxol was obtained upon request from the Division of Cancer Treatment, National Institutes of Health. Glycerol was from J. T. Baker Chemical Co. (Phillipsburg, NJ). The cAMP-dependent protein kinase inhibitor H-8 was purchased from Seikagaku Ltd., St. Petersburg, FL. Calmodulin antibodies were either purchased (Accurate Chemical & Scientific Corp., Westbury, NY), or obtained courtesy of Jan DeMay (Janssen Pharmaceutica, Beerse, Belgium). Glutaraldehyde, uranyl acetate, and osmium were purchased from Polysciences, Inc., (Warrington, PA). Tannic acid was obtained from Mallinckrodt Inc. (St. Louis, MO).

Cell Lysis and Reactivation

Chromatophores, cultured on 22-mm square carbon-coated, glow-discharged coverslips, were sealed with wax into a chamber that permitted rapid perfusion of liquids and high resolution light microscopic observations. Cells, before extraction, were first induced to aggregate or disperse their pigment by the addition of epinephrine or caffeine, respectively. The cells were next rinsed in buffers (modification from Schliwa and Van Blerkom, 1981) containing 60 mM Pipes, 25 mM Hepes, 2 mM EGTA, 10% wt/vol glycerol, 1 mM dithiotreitol, and 10^{-5} M taxol. The protease inhibitors used were; 0.2 mM phenolmethylsulfonylfluoride, 10 μg/ml Nā-p-tosyl-L-arginine methyl ester, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 0.2 TIU/ml aprotinin (trypsin inhibitory units). The final pH of the reactivation buffer was 7.25. The free Ca^{2+} and Mg^{2+} concentrations were controlled precisely through the use of a Ca^{+2}-EGTA buffering system designed by Brokaw (1986). Such a system conveniently allows one to change or maintain constant the desired concentrations of free Ca^{2+}, free Mg^{2+}, and $CaATP^{2-}$ or $MgATP^{2-}$ independently of one other.

The basic format for reactivation of pigment aggregation and then redispersion begins with a cell in the dispersed state and proceeds as follows. Healthy cultured erythrophores were induced to disperse their pigment in basal medium Eagle culture media with 1 mM caffeine. The cells were next rinsed for 1 min in calcium-free buffer containing 1 mM free Mg^{2+}. This buffer prevents an unwanted pigment aggregation during the extraction procedure. Rinsed cells were treated in the same buffer containing 0.5% Brij 58, then rinsed briefly in detergent-free buffer. Next, a rinse in buffer containing 10^{-7} M free Ca^{2+} and 1 mM free Mg^{2+} (buffer A)[1] followed by the same buffer with 1 mM $MgATP^{2-}$ induced pigment to aggregate. To redisperse the same cell we rinsed with buffer containing 10^{-8} M free Ca^{2+}, 1 mM free Mg^{2+} (buffer B), and 1 mM $MgATP^{2-}$.

The basic format for reactivation of pigment dispersion and then reaggregation begins with a cell in the aggregated state and proceeds as follows. Cultured cells were aggregated in culture medium containing epinephrine diluted at $1{:}10^7$ and then rinsed in buffer A. Detergent treatment was for 2 min in buffer A with 0.5% Brij 58. The cells were then rinsed with detergent-free buffer A then with buffer B. Reactivated dispersion was induced with buffer B plus 1 mM $MgATP^{2-}$. To reaggregate the same cell we rinsed with buffer A including 1 mM $MgATP^{2-}$.

Assay for Involvement of Calmodulin in Pigment Transport

Erythrophores were tested for calmodulin function during pigment aggregation by exposing them to high concentrations of the calmodulin antagonists trifluoperazine, R24571, W-7, or polyclonal antibodies against calmodulin. All drugs and antibodies were used in the detergent buffer for 2 min, for 5 min after extraction, and were included in the reactivation rinse containing $MgATP^{2-}$. TFP and W-7 were used at 10^{-4} M; R24571 was used at three different concentrations, including saturation at 10^{-4} M. Because R24571 is highly hydrophobic it coated the glass slides enclosing the chamber, thus reducing the working concentration of drug exposed to the cells (Gietzen et al., 1981). To circumvent this, cells were cultured on plastic coverslips. Polyclonal antibodies to calmodulin were used at a 1:50 dilution. The ability of these antibodies to bind to mitotic spindle calmodulin was demonstrated by indirect immunofluorescence (not shown).

Assay for cAMP Involvement

Erythrophores, with pigment aggregated, were tested for involvement of cAMP during pigment dispersion by exposing cells to high concentrations of cAMP, phosphodiesterase, or cAMP-dependent protein kinase inhibitors as follows. cAMP, at a concentration of 1 mM in buffer B, was included after extraction and in the reactivation buffer B with $MgATP^{2-}$. Phosphodiesterase (3′:5′-cyclic nucleotide) treatment was carried out by lysing and rinsing cells and incubating them for 2 min in buffer A containing the phosphodiesterase activator calmodulin (5 U/ml; Sigma Chemical Co.). Next, a solution containing calmodulin plus 2×10^{-3} U/ml buffer of phosphodiesterase (mixed 15 min before use as directed) was added. Under these conditions 1 U of phosphodiesterase hydrolyses 1.0 μmol of 3′:5′-cAMP to 5′-AMP per minute (Sigma Chemical Co.). Incubation continued for 2–3 min followed by a rapid rinse in buffer B and the addition of 1 mM $MgATP^{2-}$. Protein kinase inhibitor (PKI; Sigma Chemical Co.) was then added to lysed cells at 0.5 mg/ml in buffer B and incubated for 5 min before redispersion. Inhibitor was present during reactivation with buffer B and $MgATP^{2-}$ as well. The cAMP-dependent PKI H-8 was added at a concentration of 2.5 mg/ml of buffer B during lysis, for 2–3 min after lysis, and was included in the reactivation rinse.

Video Microscopy

Brass chambers containing glass coverslips with cultured chromatophores were mounted on a Zeiss IM-35 microscope equipped with a Planapochromat, 63× Phase, oil immersion objective. All experiments were viewed and recorded with a Dage 66 vidicon camera and Panasonic time lapse 6050 recorder. Before recording, the image contrast was enhanced through adjustment of the gain and black levels. Photographs of selected tapes were taken with Technical Pan film using a Rembrandt #3500F Computer Graphics Film Recorder (Nise Inc., Cerretos, CA).

Scanning Electron Microscopy

Erythrophores, cultured on glass, were first rinsed in either buffer A or B then exposed, for varying times, to 0.5% Brij 58 in buffer A or B. The subsequent fixation procedure was as follows. Cells were fixed in 2% glutaraldehyde in Ca^{2+}, Mg^{2+}-free PBS (CMF-PBS) buffer (pH 7.2) for 30 min and then rinsed three times with CMF-PBS. The cells were postfixed in 1% OsO_4 in CMF-PBS for 5 min, rinsed with ddH_2O, and placed in 1% aqueous tannic acid for 30 min. After more ddH_2O rinses the cells were stained with 0.5% aqueous uranyl acetate for 30 min, dehydrated through a graded acetone series, critical point-dried (critical point drier, Ladd Research Industries, Inc., Burlington, VT), gold coated with a freeze drier-sputter coater (model No. E5300; Polaron Instruments, Inc., Hatfield, PA), and viewed with a JEOL JSM 35CF scanning electron microscope.

1. *Abbreviations used in this paper*: buffer A, 10^{-7} M free Ca^{2+}, 1 mM free Mg^{2+}; buffer B, 10^{-8} M free Ca^{2+}, 1 mM free Mg^{2+}; PKI, protease kinase inhibitor; TFP, trifluoperazine.

Results

Calcium-regulated Pigment Transport

To study the effects of Ca^{2+} and nucleotides on pigment transport, we treated cultured erythrophores with the detergent Brij 58 to expose cytoskeletons that could then be stimulated to transport pigment granules when bathed in buffers containing $MgATP^{2-}$. The scanning electron micrographs in Fig. 1 show that while the membrane of the untreated erythrophore (Fig. 1 *a*) is fully intact, obscuring the pigment granules from view, the plasma membrane of cells treated with 0.5% Brij 58 for 2–4 min (Fig. 1, *b–e*) are efficiently removed. The numerous spherical pigment granules and their associated microtubules are clearly visible (Fig. 1, *b*, *d*, and *e*). In these erythrophores the pigment granules (erythrosomes) are coated with a thin layer of dense material of unknown composition and not a conventional lipid bilayer as is the case with melanosomes. Therefore, detergent treatment appears to have little, if any, effect on the structural integrity of these granules.

To determine if pigment could be induced to move in extracted cytoskeletons, erythrophores were subjected to a series of steps which are depicted in Fig. 2. Cultured cells were rinsed in buffer A containing 0.5% Brij detergent for 2 min (see Materials and Methods). During extraction the plasma membrane visibly lyses and a small number (<5% estimated) of pigment granules are released into the surrounding buffer. Along with this lysis is a partial aggregation of pigment (Fig. 2 *b*) possibly due to the detergent-induced release of Ca^{2+} from intracellular stores. Extracted cells were next rinsed in buffer A without detergent and exhibited no further granule loss and no pigment movements. Cells left in this state for extended time periods (20–25-min incubations at room temperature) still remain functional. Upon addition of 1 mM $MgATP^{2-}$ to the buffer (Fig. 2, *c* and *d*) the pigment moves rapidly inward to the cell center. To induce pigment dispersion in these aggregated erythrophores (Fig. 2, *a'–d'*), cells were treated in a similar fashion but with lower free calcium in the buffer. After lysis and rinsing in buffer B (Fig. 2 *b'*), aggregated cells bathed in buffer B with 1 mM $MgATP^{2-}$ actively disperse pigment from their centers in a radial fashion along microtubules until the granules reach the ends of the tubules where they either stop or dissociate (Fig. 2, *c'* and *d'*). We were able to induce cells to perform as many as three translocation events. For example, the cell shown in Fig. 2, *a–d* was aggregated, then dispersed, and finally reaggregated for a second time (not shown).

The calcium/EGTA/EDTA buffering system used (Brokaw, 1986) in these experiments allowed us to define the concentrations of free and nucleotide-bound Ca^{2+} and Mg^{2+} in the reactivation solution. With this buffer we examined the response of lysed cells to different concentrations of free Ca^{2+}, $MgATP^{2-}$, $NaATP^{3-}$ and $CaATP^{2-}$ (Table I and Fig. 3). We find that, first, increasing $MgATP^{2-}$ concentrations in the buffer does not alter the concentration of free Ca^{2+} needed to induce pigment aggregation or dispersion; second, as expected, the velocities of pigment translocation are sensitive to the concentration of $MgATP^{2-}$ used. As the concentration of $MgATP^{2-}$ is increased from 0.01 to 10 mM the velocity of pigment transport increases from 0.2 to 1.5 μm/s for dispersion and 0.4 to 2.4 μm/s for aggregation (Table I, Fig. 3). The maximum velocities recorded were 80–100% of those observed in intact cells. It is interesting that these movements are smooth and nonsaltatory in nature in contrast to the saltatory movements seen during pigment dispersion in intact cells. Saltatory pigment motion does occur in reactivated cells exposed to Ca^{2+} concentrations midway between those needed to stimulate aggregation or dispersion. For example, 2.5×10^{-8} M free Ca^{2+} induces the pigment to saltate during dispersion, whereas 7.5×10^{-8} M free Ca^{2+} induces a saltatory aggregation, and 5.0×10^{-8} M free Ca^{2+} stimulates vigorous saltatory pigment movements without triggering either aggregation or dispersion. A similar phenomenon may have been observed by Stearns and Ochs (1982) who report that they were able to reactivate saltatory pigment motion in permeabilized erythrophores but not aggregation or dispersion.

Aggregation and Dispersion Have Different Requirements for Magnesium

One of the major advantages of a reactivated chromatophore system is that it allows one to separate orthograde and retrograde transport and examine the specific requirements for each event. For example, to study orthograde dispersion, intact aggregated cells are lysed and then rinsed before reactivation. It is during this rinsing period that cells can be exposed to, or deprived of, a particular drug, nucleotide, or ion. After such treatment, $MgATP^{2-}$ is added and the effects on pigment dispersion are observed. Using this format we have tested whether other agents such as Mg^{2+}, cAMP, and calmodulin also act with Ca^{2+} to regulate the direction of pigment transport.

We have examined the effects that varying concentrations of Mg^{2+}, Ca^{2+}, and ATP have upon pigment transport to determine whether aggregation and dispersion require either $MgATP^{2-}$ or $CaATP^{2-}$. As plotted in the graph in Fig. 3, the velocities of pigment transport increase linearly as the concentrations of both $MgATP^{2-}$ and $CaATP^{2-}$ are increased. Maximum velocities for pigment dispersion are obtained at 10^{-4} M $MgATP^{2-}$ and 10^{-8} M $CaATP^{2-}$, while aggregation is fastest at 10^{-4} mM $MgATP^{2-}$ and 10^{-7} M $CaATP^{2-}$. To discriminate which metal/ATP complex is being used by the motility machinery we bathed extracted cells in Mg^{2+}-free buffer so that any added ATP would exist either as $CaATP^{2-}$, $NaATP^{3-}$, or in the uncomplexed form (Table I*). Under these reactivation conditions (in buffer B) pigment does not disperse but remains motionless even when the $CaATP^{2-}$ concentration is raised to a maximum (10^{-6} M), indicating that cells must have $MgATP^{2-}$ present in order to disperse pigment. The $MgATP^{2-}$ requirements for aggregation are less absolute. Cells can aggregate pigment in Mg^{2+}-free buffer in the presence of 0.5 mM $NaATP^{3-}$ and 10^{-5} M $CaATP^{2-}$ (Table I) but at a reduced rate (0.4–0.6 μm/s). We do not know whether the aggregation mechanism uses $CaATP^{2-}$ or $NaATP^{3-}$ under these Mg^{2+}-free conditions.

$MgATP^{2-}$ also effects pigment granule–cytoskeletal interactions and stimulates granule release into the surrounding buffer. This release is more evident in dispersing than in aggregating cells and is maximized as the concentration of $MgATP^{2-}$ is increased, at which point granules translocate for only a short time along microtubules before detaching. It is unlikely that the release is a result of a cytoskeletal solubilization by salt or detergent since granules are not liberated from lysed cells treated with 50 mM NaCl or 0.5%

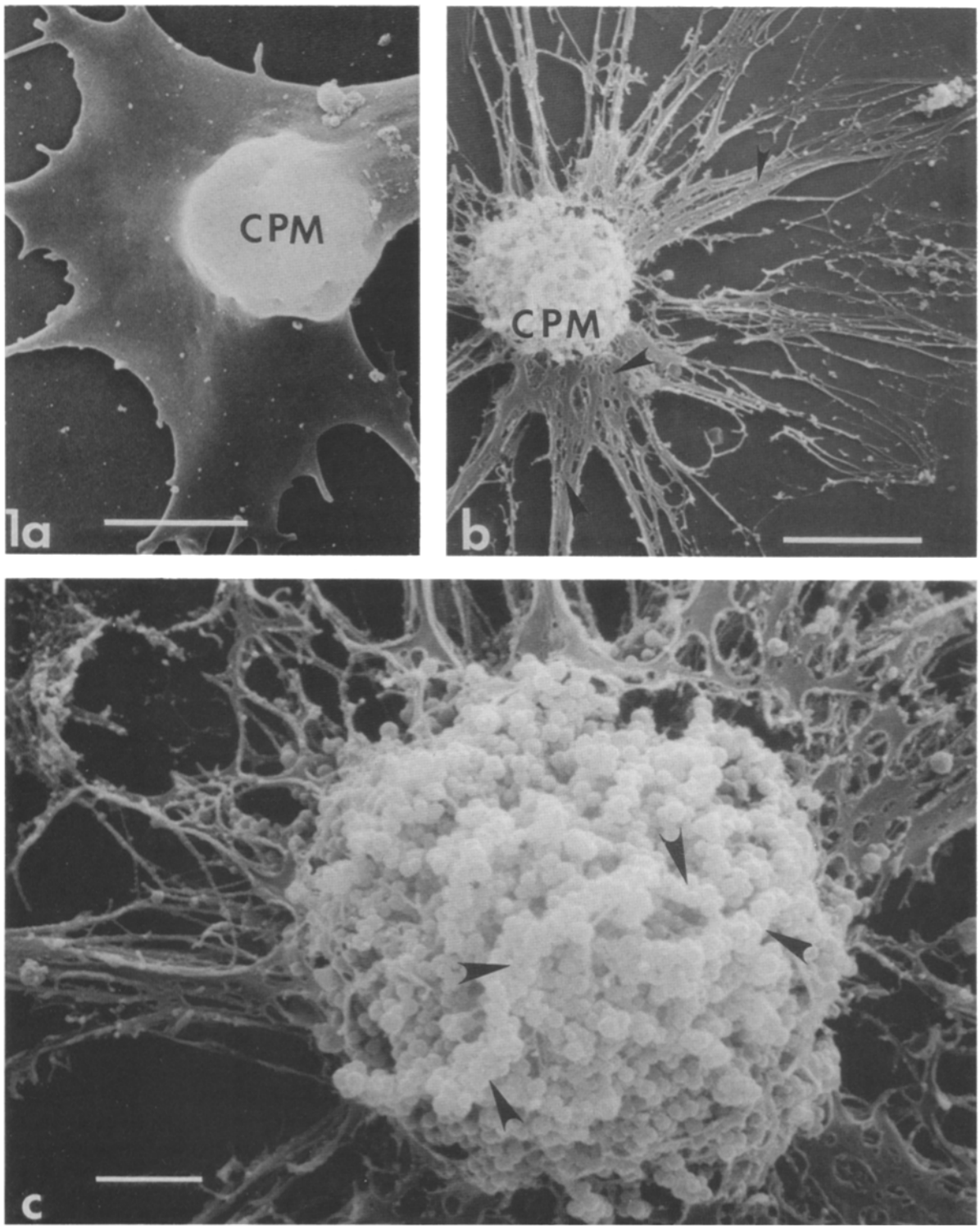
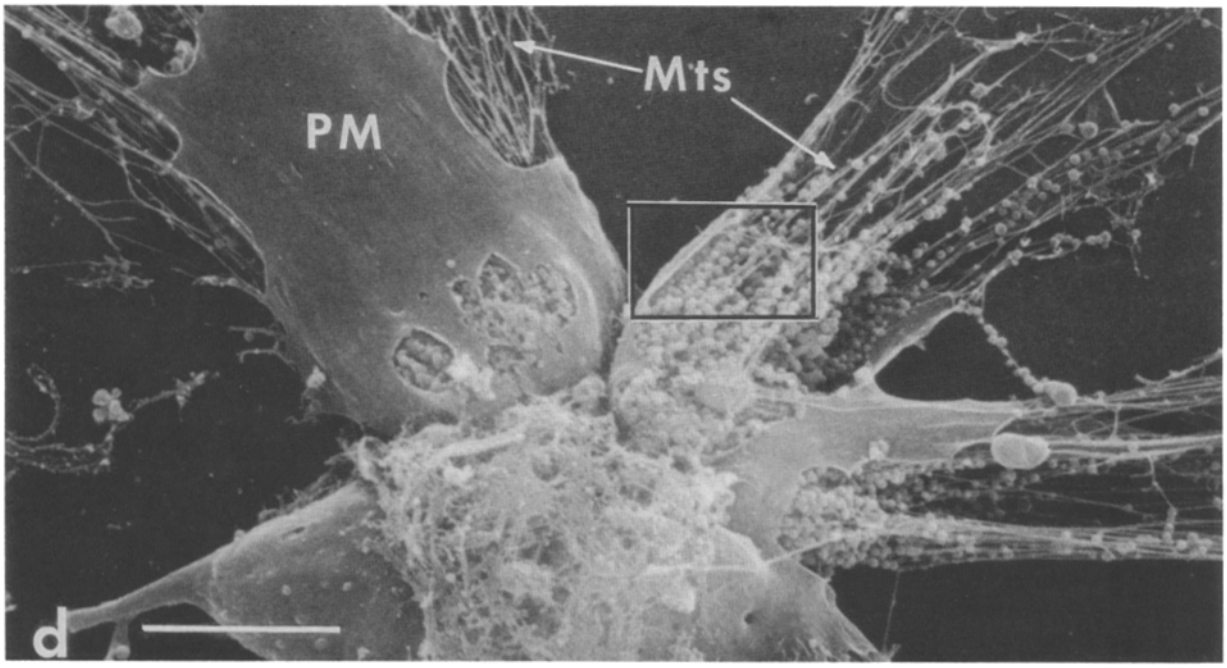


Figure 1. Detergent treatment removes the plasma membrane while leaving granule–cytoskeletal associations intact. A series of scanning electron micrographs of cultured erythrophores subjected to various detergent treatments. (*a*) A cell that was fixed after rinsing with reactivation buffer without detergent. The intact plasma membrane blankets the central pigment mass (*CPM*), obscuring the pigment granules from view. (*b*) An erythrophore with aggregated pigment, after exposure to 0.5% Brij 58 for 4 min, has little membrane remaining. Some residual membrane is left along microtubule bundles (*arrows*) which extend outward from the (*CPM*). This exposed pigment mass (*c*) is comprised of thousands of small spherical pigment granules which, in many instances, appear to wind around microtubules in a helical fashion (*arrows*). (*d*) An erythrophore, partially dispersed, which was treated for only 1 min in 0.5% Brij. A greater portion of the plasma membrane (*PM*) remains between openings through which many microtubules protrude (*MTs*; *arrows*). (*e*) A higher magnification image of the boxed region in *d*. The plasma membrane (*PM*) has receded leaving the pigment granules and associated microtubules (*Mts*) fully exposed. Bars: (*a*, *b*, and *d*) 5 μm; (*c*) 1 μm; (*e*) 0.5 μm.


Mts
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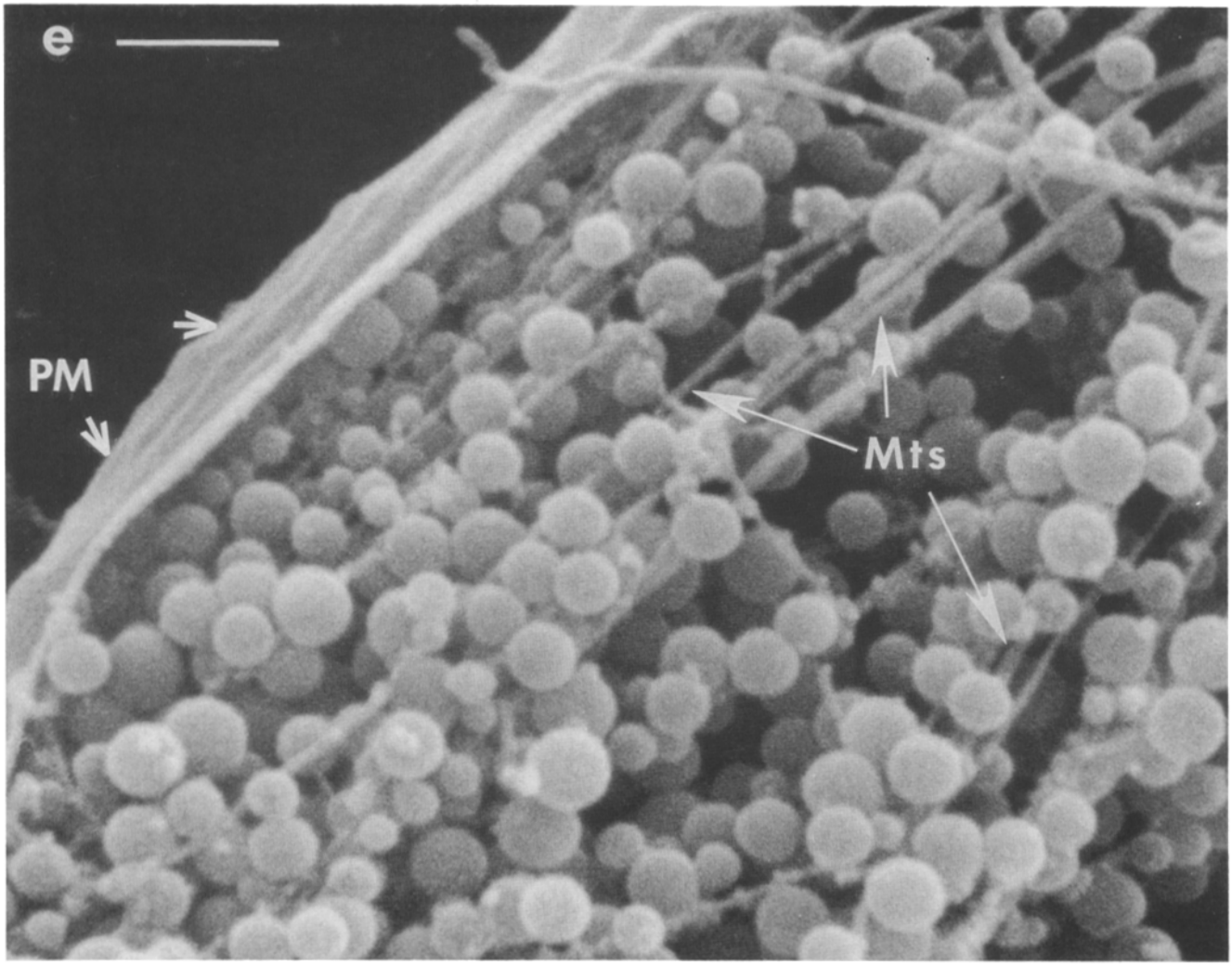
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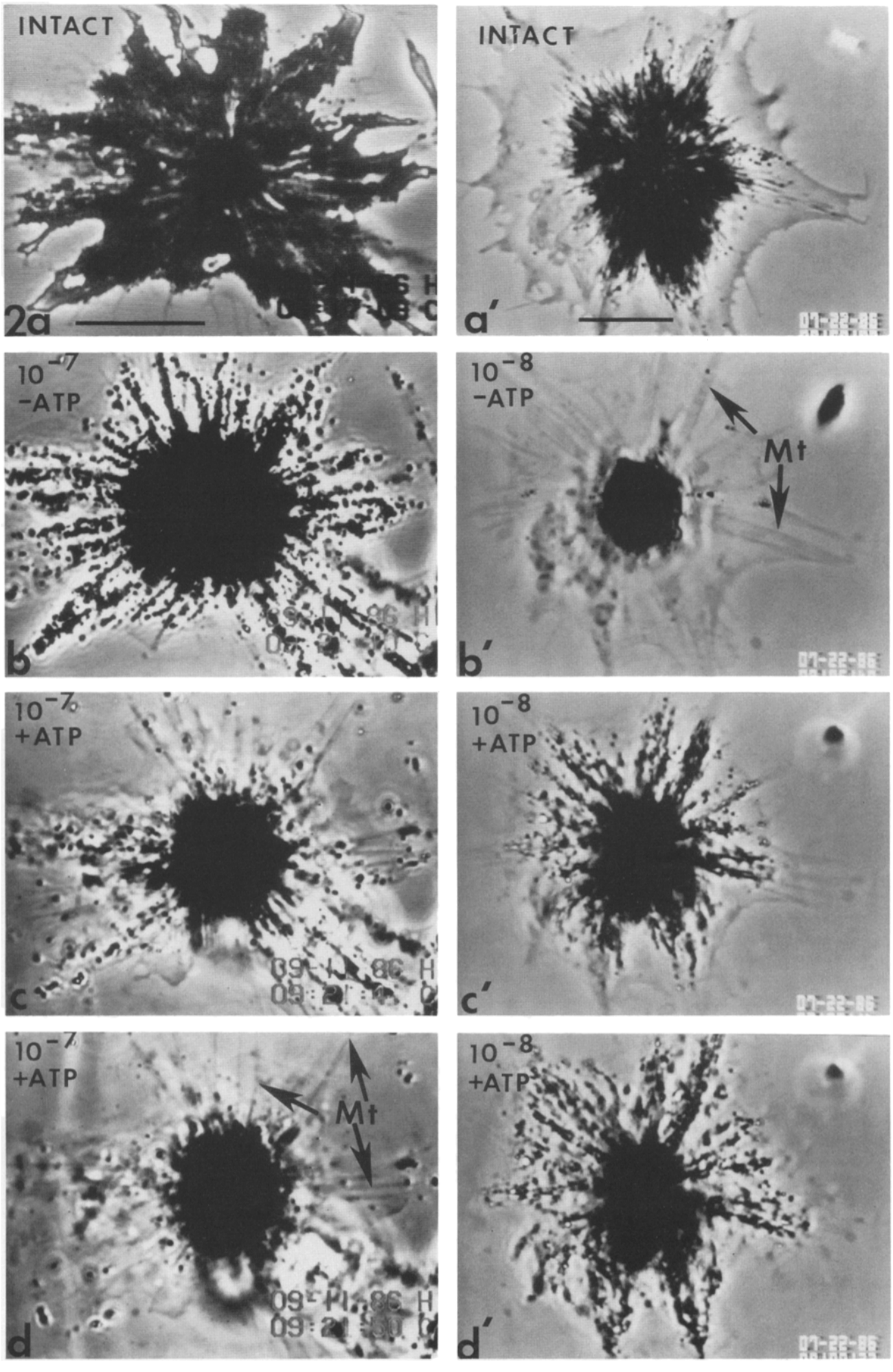
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Table I. Concentrations of Ions and Nucleotides Required for Reactivation of Pigment Transport

Free Ca^{2+}	$CaATP^{2-}$	$MgATP^{2-}$	$NaATP^{3-}$	Free Mg^{2+}	Rate
	M	*mM*	*mM*	*mM*	*μm/s*
Dispersion					
Intact cell	?	?	?	?	1.6
10^{-8}*	2.0×10^{-8}	0	0.01	0.0	0.0
10^{-8}*	1.0×10^{-6}	0	0.5	0.0	0.0
10^{-8}	4.0×10^{-11}	0.01	–	1.0	0.2
10^{-8}	4.0×10^{-10}	0.1	–	1.0	0.6
10^{-8}	2.0×10^{-9}	0.5	–	1.0	1.1
10^{-8}	4.0×10^{-9}	1.0	–	1.0	1.2
10^{-8}	2.0×10^{-8}	5.0	–	1.0	1.4
10^{-8}	4.0×10^{-8}	10.0	–	1.0	1.5
Saltation					
5×10^{-8}	5.0×10^{-8}	1.0	–	1.0	Saltation
Aggregation					
Intact cell	?	?	?	?	3.1
10^{-7}*	2.0×10^{-7}	0.0	0.01	0.0	0.4
10^{-7}*	1.0×10^{-5}	0.0	0.5	0.0	0.6
10^{-7}	4.0×10^{-10}	0.01	–	1.0	0.4
10^{-7}	4.0×10^{-9}	0.1	–	1.0	1.4
10^{-7}	2.0×10^{-8}	0.5	–	1.0	1.6
10^{-7}	4.0×10^{-8}	1.0	–	1.0	1.8
10^{-7}	2.0×10^{-7}	5.0	–	1.0	2.0
10^{-7}	4.0×10^{-7}	10.0	–	1.0	2.4

A minimum number of five cells were examined at each free Ca^{2+}, $MgATP^{2-}$ concentration.
* Mg^{2+}-free conditions.

Brij 58 for 15 min (not shown). Instead release appears to be directly dependent upon the levels of $MgATP^{2-}$ in the reactivation buffer. This notion is illustrated in Fig. 4 which shows the effect that different concentrations of $MgATP^{2-}$ has upon the release of pigment granules from two different cells. The cell in Fig. 4, *a–d* was lysed in Mg^{2+}-free buffer which not only reduced granule release but induced the subsequent sticking of many granules to microtubule bundles. When Mg^{2+}-free conditions are maintained during reactivation with $NaATP^{3-}$, instead of $MgATP^{2-}$, granules remain static and their release is prevented. It is only after the addition of $MgATP^{2-}$ that the granules disperse and eventually release. In comparison, a cell stimulated to disperse pigment with buffer B containing 10 mM $MgATP^{2-}$ (Fig. 4, *a′–d′*) rapidly releases granules into the surrounding buffer, leaving the microtubule bundles naked.

We have observed that the granules released during reactivation with $MgATP^{2-}$ maintain their capacity to bind to another cell's microtubules and translocate. This is shown in Fig. 5, which depicts two adjacent erythrophores with overlapping microtubule bundles. After addition of buffer A plus 1 mM $MgATP^{2-}$, aggregation occurs and induces some of the granules to release from one cell and bind to the intruding microtubule bundle of the adjacent cell. These bound granules subsequently translocate retrograde along the microtubules into the adjacent cell center at 1–2 μm/s (Fig. 5, *d–e*). Thus, granules released from a cell are still competent to move along foreign microtubules from adjacent cells. An interesting contrast to this exchange of motile granules from cell to cell is seen when the cells themselves are pulled together during reactivation with $MgATP^{2-}$. Fig. 6 shows three cultured erythrophores that lie adjacent to one another and also have interdigitating microtubule bundles. When these cells were treated with detergent and then reactivated with buffer A plus 1 mM $MgATP^{2-}$ the pigment aggregated normally into the cells' respective centers. By the end of aggregation the cells start to move toward each other at a rate of 0.5–1.0 μm/s and eventually cluster together to form a single, seemingly amorphous, mass of pigment (Fig. 6, *c–e*). We have observed this phenomena in six different cells during

Figure 2. The direction of pigment movement in lysed erythrophores is controlled by the concentration of added Ca^{2+}. A series of phase contrast, video-enhanced light micrographs of two different cultured erythrophores that have been lysed and reactivated with $MgATP^{2-}$ to either aggregate (*a–d*), or disperse (*a′–d′*) pigment. The Ca^{2+} concentrations and presence or absence of $MgATP^{2-}$ for each step are noted in the upper left of each panel. (*a*) An intact erythrophore with its pigment dispersed that has been rinsed with Ca^{2+}-free reactivation buffer. (*b*) The same cell after a 2-min exposure to detergent, rinsed in buffer A. The stationary pigment granules are radially disposed along microtubules. (*c* and *d*) Addition of 1 mM $MgATP^{2-}$ to the buffer stimulates a rapid pigment aggregation into the cell center at a rate of 2 μm/s. Numerous phase-dense microtubule bundles (*arrows*) can be seen protruding out from under the plasma membrane remnant. The second cell (*a′–d′*) was aggregated with epinephrine (*a′*), then treated and reactivated to induce a pigment dispersion. (*b′*) After a 2-min detergent treatment the cell was rinsed in buffer B. The addition of 1 mM $MgATP^{2-}$ stimulates the pigment to translocate outward along microtubules to the cell periphery (*c′–d′*). Bars, 10 μM.

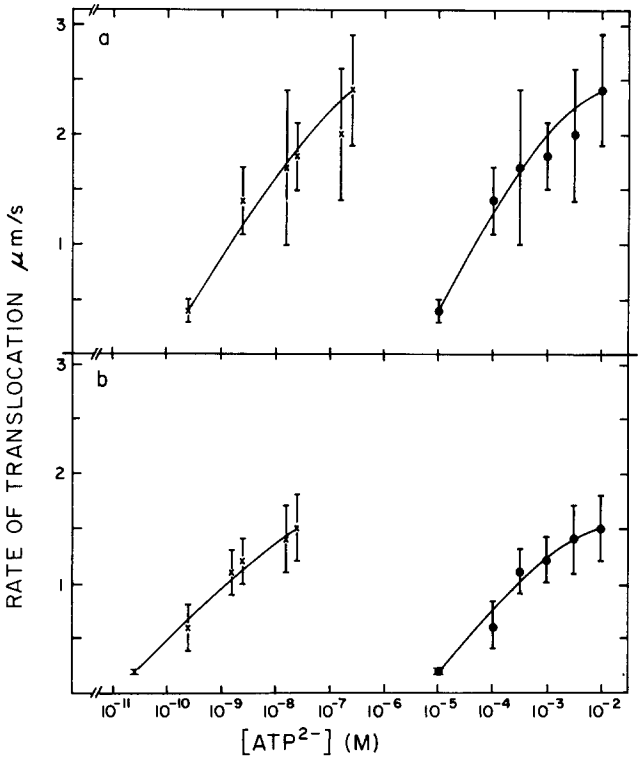


Figure 3. Velocities of pigment transport in vitro. Graphs depicting the velocities of pigment translocation during reactivated aggregation (*a*), and dispersion (*b*), in response to varying concentrations of $MgATP^{2-}$ (•) and $CaATP^{2-}$ (×) in the buffer. The bars show the standard deviation of ten different rate measurements for each $MgATP^{2-}$ concentration used. Measurements were taken from two to four different cells for each concentration tested.

three different reactivation trials. Whether these events indicate that a force-generating mechanism resides on the microtubules or granules (or both) is considered in the discussion.

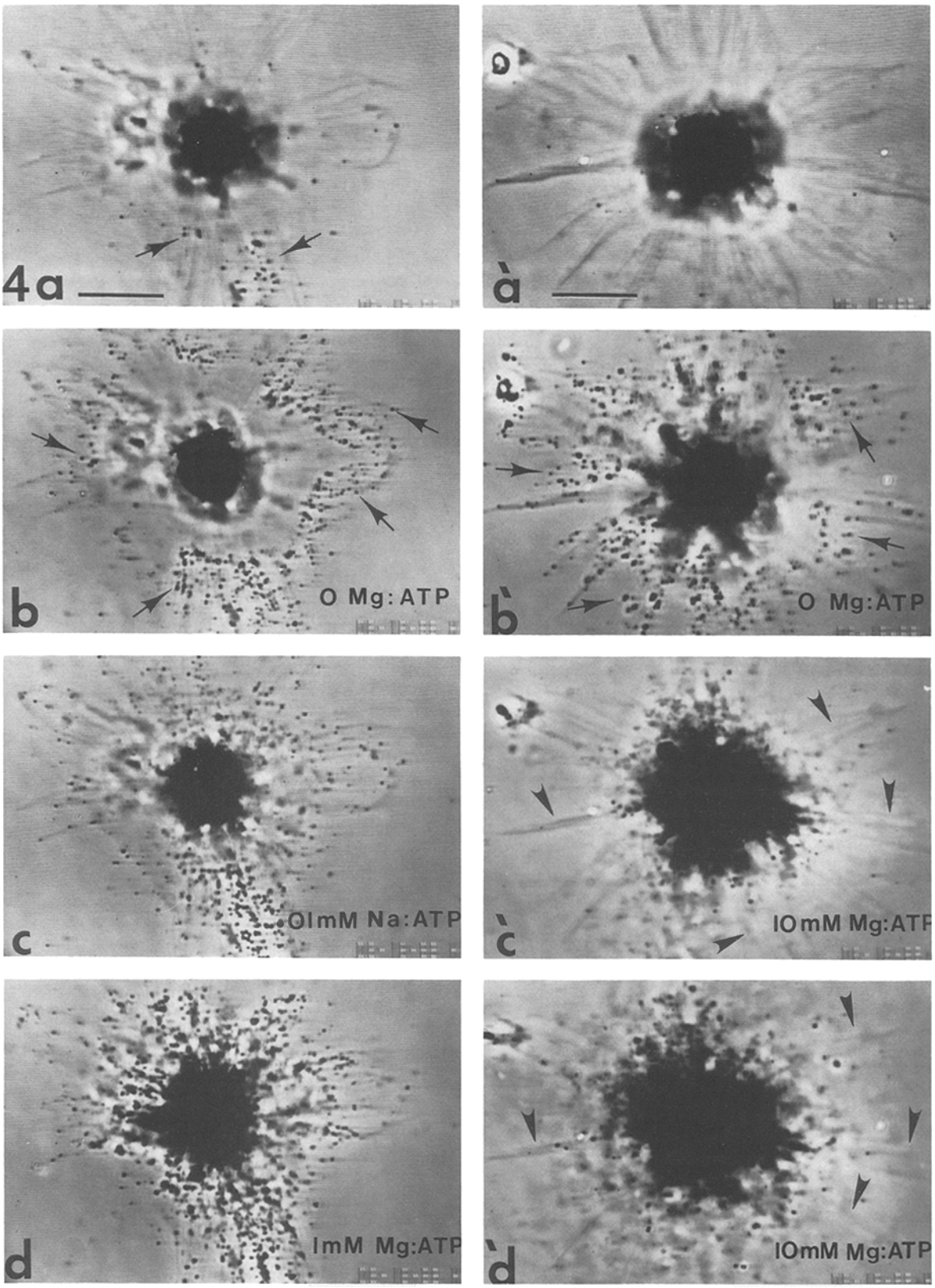
Calmodulin and Pigment Granule Movement

The data presented above indicate that the motility machinery within erythrophores is regulated by changes in the concentration of Ca^{2+}. How might Ca^{2+} exert its effects? There are several possibilities, the strongest being the involvement of the Ca^{2+}-binding protein calmodulin which is known to regulate many Ca^{2+}-initiated cell functions (Cheung, 1980; Means et al., 1982). To test for the involvement of calmodulin in the regulation of pigment transport we exposed reactivated cells to several calmodulin antagonists including trifluoperazine (TFP), R24571, and W-7. These drugs have been shown to strongly inhibit calmodulin-controlled functions such as activation of myosin light chain kinase (Sheterline, 1980), 5′-3′phosphodiesterase (Levin and Weiss, 1976), and Ca^{2+}-transport ATPase (Gietzen et al., 1981). When lysed cells are exposed to these drugs or to polyclonal antibodies against calmodulin (in buffer A with $MgATP^{2-}$), pigment aggregates normally and no inhibitory effects are observed (Table II). These results support earlier studies which show that the microinjection of either TFP or two different polyclonal calmodulin antibodies into erythrophores did not alter pigment aggregation (Beckerle, 1982). We do find that R24571 will completely prevent aggregation when used at saturation (10^{-4} M), a concentration up to 100 times that was previously shown to inhibit calmodulin function in other systems (Gietzen et al., 1981).

Involvement of cAMP in Pigment Dispersion

As mentioned earlier, there is substantial evidence implicating cAMP in the induction of pigment dispersion in chromatophores (Grundstrom et al., 1985; Lynch et al., 1986; Rozdzial and Haimo, 1986*a*, *b*). From these observations, and our Ca^{2+} findings reported here, it seemed likely that both Ca^{2+} and cAMP work together to regulate pigment aggregation and dispersion in erythrophores as predicted by Luby-Phelps and Porter (1982). Therefore we tested for cAMP-regulated pigment movements using heavily extracted, aggregated erythrophores with fully exposed cytoskeletons as shown in Fig. 1, *b* and *c*. The extraction was followed by prolonged rinsing with a large volume of buffer (5 min with 5 ml) to remove any endogenous cAMP as well as the membrane-bound adenylate cyclase. After rinsing, the cells were washed with buffer B plus 1 mM $MgATP^{2-}$, which induced a normal pigment dispersion. To address the possibility that some cAMP was retained in the extracted erythrophores, we incubated them with high concentrations of 3′:5′-cyclic nucleotide phosphodiesterase in the presence of its calmodulin activator (see Materials and Methods). The measured concentrations of cAMP in cells is extremely low, brain having the most cAMP at 0.2–1.5 nM/g wet wt (Schmidt et al., 1972; Eichelman et al., 1976). Thus, we were able to add ~1,000 times the concentration of phosphodiesterase needed to hydrolyse a comparable amount of cAMP in erythrophores. Again, we find that addition of buffer B with $MgATP^{2-}$ stimulates a normal pigment dispersion. Including 1 mM cAMP to buffer B with $MgATP^{2-}$ after phosphodiesterase incubation does not hasten the onset of dispersion nor increase the velocity of pigment transport. An additional test for cAMP-dependent transport was performed, not by altering cAMP concentrations, but by attempting to inhibit the cAMP-dependent protein kinase. Lysed cells, in the aggregated state, were incubated in high concentrations of either "protein kinase inhibitor" (PKI) or H-8 (see Materials and Methods). The PKI is known to displace the regulatory subunit from the catalytic subunit of the cAMP-dependent protein kinase and inhibit its activity in a variety of different

Figure 4. Pigment granule–microtubule associations are affected by $MgATP^{2-}$. The phase-contrast video micrograph series of two different erythrophores demonstrates the effects $MgATP^{2-}$ has upon pigment granule–cytoskeletal interactions. The first cell is shown in *a–d*, the second cell in *a′–d′*. (*a*) An erythrophore just after detergent treatment and rinsing with buffer containing 10^{-8} M Ca^{2+}, no Mg^{2+}, and no ATP. A few pigment granules have fallen from the central pigment mass and attach to peripheral microtubule bundles (*arrows*). 1 min later many more granules have become associated with the microtubules (*b*). The clear region about the cell center in which no granules adhere is the plasma membrane remnant. When 0.01 mM $NaATP^{3-}$ is present in the buffer in the absence of Mg^{2+} (*c*), the granules remain fixed to the microtubules. Although there is some slight oscillatory motion, no net translocation occurs. Raising the level of $MgATP^{2-}$



from 0 to 1 mM (*d*) stimulates orthograde pigment transport outward along the microtubules accompanied by moderate granule release. (*a'–d'*) The second cell was lysed under the same conditions as the first in buffer with 10^{-8} M Ca^{2+} containing no Mg^{2+} or ATP. At the time shown, few, if any pigment granules adhere to peripheral microtubules (*a'*). Over a 1-min time span, some granules release from the cell center and stick to microtubules (*arrows*, *b'*). Addition of 10 mM $MgATP^{2-}$ initiates granule translocation followed by an excessive granule release into the surrounding buffer (*c'* and *d'*). The spontaneity of this high $MgATP^{2-}$–stimulated release greatly reduces any net orthograde translocation and leaves the peripheral microtubule bundles naked. Bars, 10 μm.

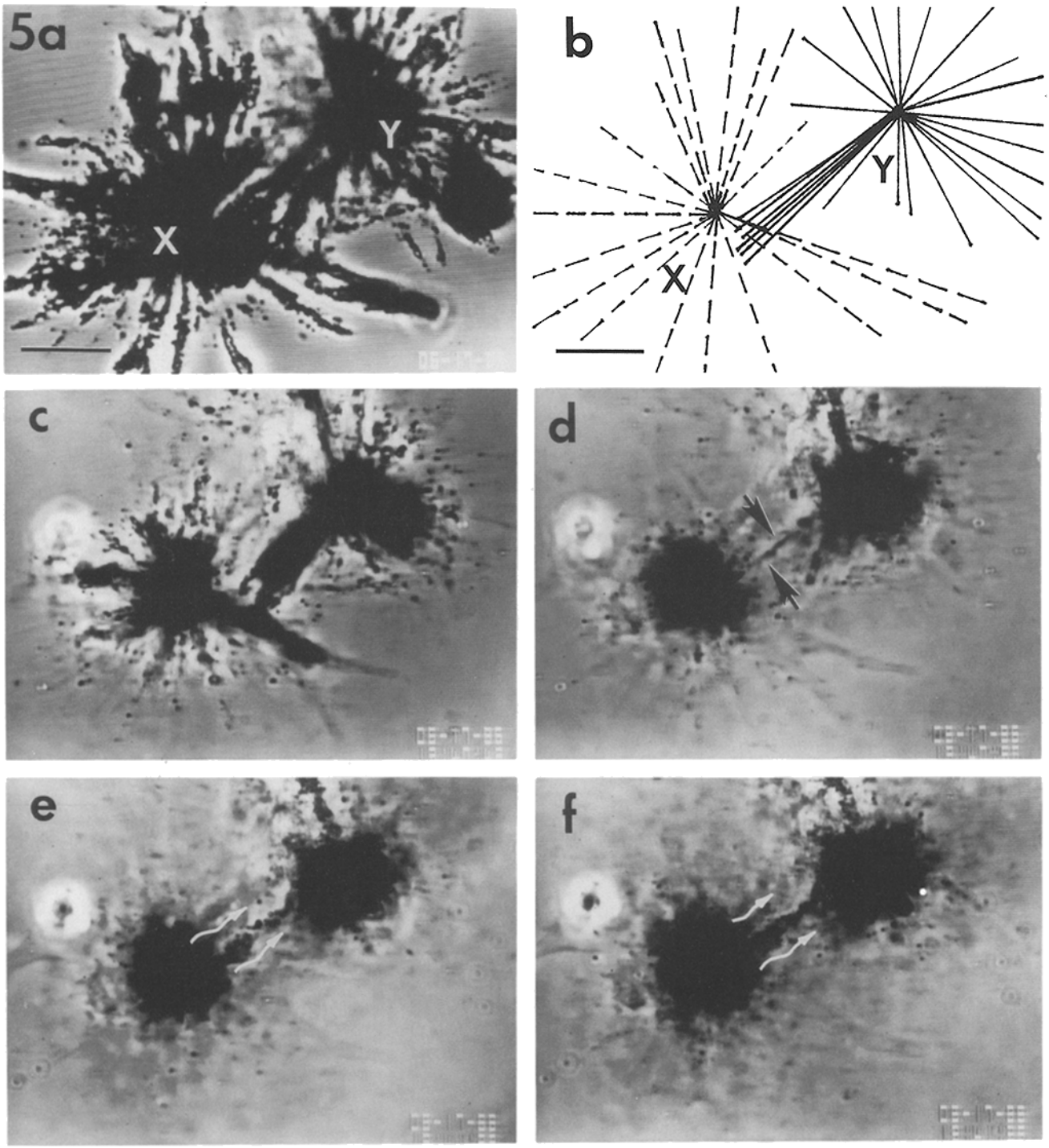


Figure 5. Pigment granules released from one cell are gathered up and transported along an adjacent cell's microtubules. A phase-contrast, video-enhanced series of two cultured erythrophores induced to aggregate pigment during reactivation. (*a*) Two cells (*X* and *Y*) which were attached and spread side by side. The cells have been lysed and rinsed in buffer A. A large microtubule bundle from cell Y extends into the central pigment mass of the adjacent cell X. The disposition of the cellular microtubule arrays are illustrated in a schematic drawing (*b*). The microtubules (which are greatly reduced in number from a real cell) are depicted as broken and solid lines for cell X and Y, respectively. Addition of 1 mM $MgATP^{2-}$ to buffer A induces each cell to aggregate pigment to its center (*c* and *d*). The invading microtubule bundle from cell Y can be seen as a phase-dense line (*arrows*) extending toward the pigment mass of cell X (*d*). Several seconds after aggregation, pigment granules release from cell X, attach to the intruding microtubule bundle, and translocate into the center of the adjacent cell (*e* and *f*). Wavy arrows indicate direction of transport. This phenomena may last up to 15 min. Bars, 10 μm.

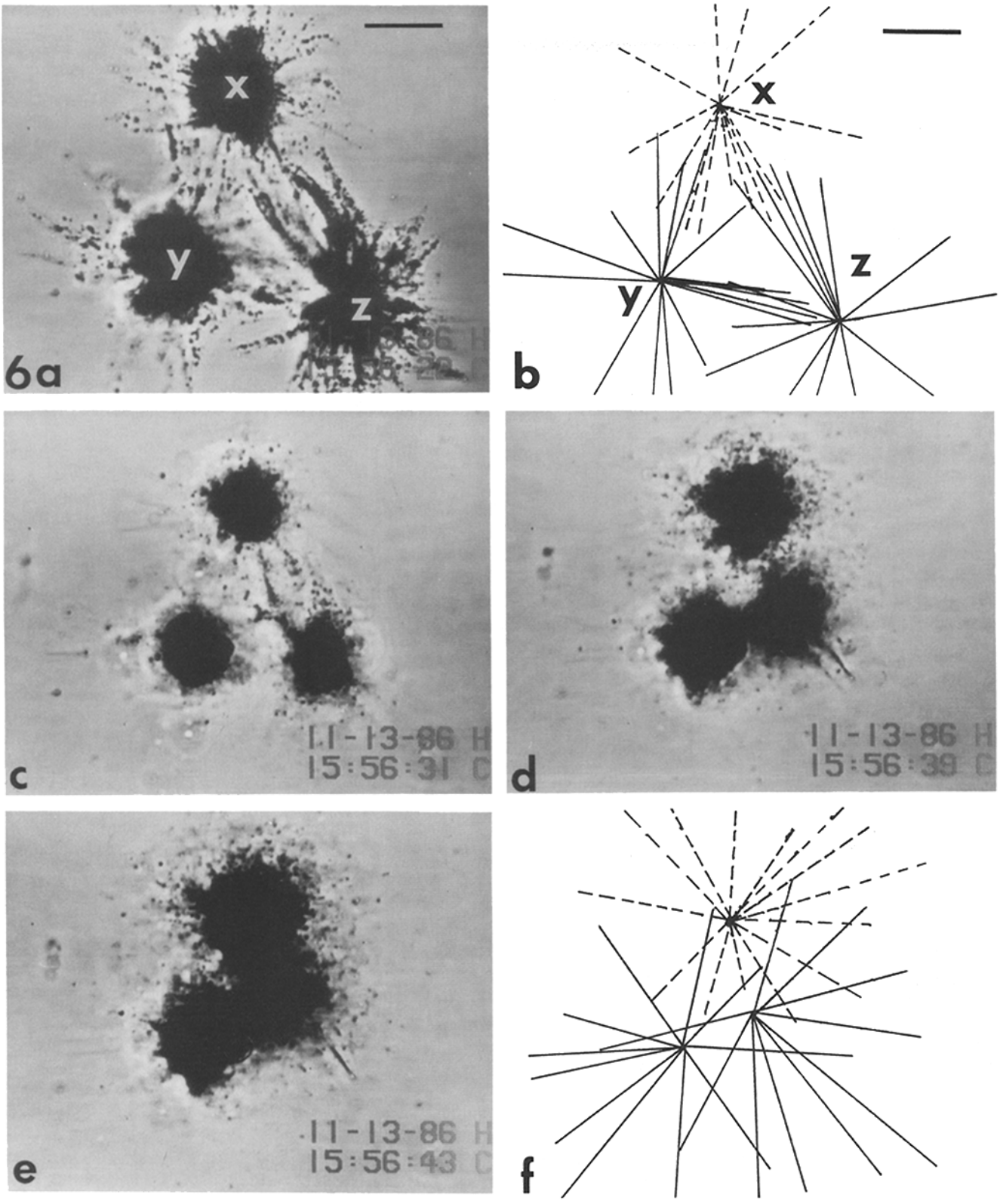


Figure 6. Reactivated erythrophores exert pulling forces upon each other. The phase microscopy series depicts three different erythrophores labeled *x*, *y*, and *z*. (*a*) Cells, with pigment dispersed, were lysed with 0.5% Brij detergent and rinsed with buffer. The drawing (*b*) is a simplified schematic showing the position of the three microtubule asters and the arrangement of some of their microtubules as they exist in *a*. Note that each cell extends microtubules that interdigitate with those of the other cells. Upon additiion of 1 mM $MgATP^{2-}$ to buffer A the pigment aggregates inward as expected (*c*). By the end of the aggregation process the three cells are seen actively moving toward each other until they eventually coalesce to form a single massive pigment clump (*c–e*). The drawings in *f* are taken from *e* and show the repositioning of the three asters. Bars, 10 μm.

Table II. Sensitivity of Reactivated Pigment Movements to Calmodulin and cAMP Probes

Drug test	Aggregation	Dispersion
Calmodulin		
TFP (10^{-4} M)	+	+
T24571 (2×10^{-5} M)	+	+
T24571 (5×10^{-5} M)	+,−	ND
T24571 (10^{-4} M)	−	ND
W-7 (10^{-4} M)	+	ND
No. 1 calmodulin antibody (1:50)	+	ND
No. 2 calmodulin antibody (1:50)	+	ND
cAMP		
cAMP PKI (0.5 mg/ml)	ND	+
H-8 (2.5 mg/ml)	ND	+
Phosphodiesterase (2×10^{-3} U/ml) with added activator	ND	+

Minimum of 10 cells were examined for each drug test except for the drug H-8, in which four cells were examined. ND, not done. +, normal pigment transport; −, inhibition of pigment transport.

tissue types (Walsh et al., 1970; Hofmann et al., 1977); H-8 has also been shown to be a potent inhibitor of cyclic nucleotide-dependent protein kinases (Hidaka et al., 1984). We find that the PKI, even when used at 10-fold higher concentrations than needed for maximal kinase inhibition in vitro (Hofmann et al., 1977), has no effect on the pigment dispersion. Likewise, high concentrations of H-8 are ineffective (Table II). Because the varied treatments described above all have little or no effect, we assume that pigment dispersion in these cells is not controlled by cAMP.

Discussion

Calcium-regulated Pigment Transport

We have developed an in vitro reactivated erythrophore system that provides several advantages for studying directed organelle transport. First, the cells are removed from the scale stroma, maintained in culture, and are thereby completely isolated from the influence of surrounding nerve fibers and epithelial cells which may either effect motility or degrade the quality of optical observations. Second, the erythrophores are not merely permeabilized but stripped of most of their membrane, thereby facilitating full access of exogenous reagents, ions, and nucleotides to the motility machinery. Although scanning electron microscopy of treated cells confirms the effects of detergent (Fig. 1), the visible opening of the plasma membrane and the subsequent release of pigment granules into the surrounding buffer conveniently demonstrates that a cell has been lysed. Therefore, we are confident that each cell under observation is fully exposed to the external milieu without the need for time consuming procedures that test diffusion of antibodies or colloids, such as ferritin or gold, through the plasma membrane.

From the observations reported here it appears that these erythrophores control the directed transport of their pigment granules simply by raising or lowering the concentration of their cytoplasmic free calcium to induce pigment aggregation or dispersion, respectively. The saltatory pigment motion exhibited by intact cells during dispersion may reflect their inability to lower instantaneously cytoplasmic Ca^{2+} levels. Such bidirectional movements could represent single or dual motors switching from orthograde to retrograde and back again. Because the Ca^{2+} levels in reactivated cells are exchanged rapidly and completely through massive quantities of buffer, the extracted cells are not normally exposed to intermediate ion concentrations, thereby eliminating saltation. How intact erythrophores regulate their internal calcium levels is unknown although it is probably controlled by a form of the smooth endoplasmic reticulum. This reticulum, as viewed by the electron microscope in both whole mount and thick-sectioned preparations (Luby-Phelps and Porter, 1982; McNiven, M. A., and K. R. Porter, unpublished observations), surrounds the cell center and extends outward toward the cell cortices parallel to microtubule bundles. It is attractive to find in the structure and function of this reticulum a parallel to the vesicular membrane systems observed in dividing sea urchin eggs (Silver et al., 1980; Petzelt and Hafner, 1986) and barley cells (Hepler et al., 1981), which are believed to regulate Ca^{2+} levels during mitosis.

From the findings presented here it does not appear that erythrophores use calmodulin to convey a calcium signal to their pigment-moving mechanism since we are unable to inhibit pigment aggregation with a number of calmodulin inhibitory drugs used at prescribed concentrations (Table II). Because extremely high concentrations of the calmodulin antagonist R24571 completely prevent aggregation (perhaps nonspecifically), we are presently searching for the involvement of calcium-binding proteins other than calmodulin. It will be of interest to learn whether calcium exerts its influence through the activation of a protein kinase, phosphatase, or by binding directly to the motors as is the case of the contractile protein spasmin (Routledge et al., 1976). We are also examining the effects of calcium on dispersion with the purpose of finding out whether calcium inactivates the dispersing motor during aggregation or if it is perpetually on and simply overwhelmed by a more powerful aggregating mechanism. The latter possibility is strengthened by the observation that erythrophores, inhibited from aggregating pigment with the drug R24571, disperse many of their granules (data not shown). Thus, it appears that if the aggregating motor is inhibited, a limited dispersion may occur by default even in high concentrations of free calcium. The fact that the orthograde transport of axoplasmic vesicles, in vitro, is insensitive to calcium (Schnapp et al., 1985; Allen et al., 1985; Gilbert et al., 1985) may also support this.

Surprisingly, we find that none of the assays used to test for involvement of cAMP have any effect on pigment dispersion (Table II). This conflicts with an earlier study (Byers and Porter, 1977) which reports that squirrel fish erythrophores disperse pigment when exposed to membrane permeable forms of cAMP or methylxanthines such as caffeine and theophylline, which indirectly raise cAMP levels via the inhibition of phosphodiesterases. In our hands, medium containing 5 mM cAMP does not stimulate intact erythrophores to disperse pigment any better than does drug-free media. This suggests that the methylxanthines may act nonspecifically and stimulate pigment dispersion through means other than increasing cAMP. It is known that in addition to inhibiting phosphodiesterase both caffeine and theophylline effect the membrane potential of nerve cells (Kuba and Nishi, 1976), the functioning of membrane calcium pumps, and alter the capacity of the smooth endoplasmic reticulum to se-

quester and release calcium (Weber, 1968; Stephenson, 1981). We realize that our results do not completely rule out cAMP involvement since drug studies need to be interpreted with caution. It is possible that remnants of plasma membrane that remain after extraction contain enough functional adenylate cyclase to raise the cAMP concentration to a level sufficient for dispersion. Another possibility is that a substantial percentage of the cAMP-dependent protein kinase within the erythrophore is structurally bound to microtubules, as it is in nerve cells (Vallee et al., 1981), making it less likely to be extracted by detergents or inhibited by drugs. Because of these possibilities we are continuing to test for the involvement of cyclic nucleotides in regulating pigment dispersion. However, the fact that none of the varied treatments alters reactivated dispersion does suggest that pigment movement in these erythrophores is not under an antagonistic cAMP/Ca^{2+} control mechanism. It is unlikely that heavily extracted erythrophores (Fig. 1), continually flushed with massive quantitities of buffer, could generate enough cAMP to induce pigment dispersion. Indeed, it has been shown that reactivated melanophores, which are gently permeabilized and have most of their membranes intact, still require the addition of exogenous cAMP to stimulate dispersion (Rosdzial and Haimo, 1986*a*). It also seems unlikely that an erythrophore could repeatedly manufacture and degrade cAMP in rapid succession (every 2–3 s) to induce the characteristic rhythmic pigment pulsations. This may explain why xanthrophores and melanophores, which are sensitive to cAMP, transport pigment more slowly (0.1–1.0 μm/s) and do not undergo rhythmic pulsations.

ATP and Magnesium

The observation that pigment in lysed erythrophores is motionless before the addition of ATP indicates that the nucleotide is required for both aggregation and dispersion. This result supports previous reports that demonstrate permeabilized melanophores require ATP to aggregate pigment (Clark and Rosenbaum, 1984; Rozdzial and Haimo, 1986*a*), yet contrasts with earlier studies that demonstrate an inhibition of dispersion in chromatophores exposed to various metabolic inhibitors of ATP production (Junqueira et al., 1974; Luby and Porter, 1980). From these earlier studies it was postulated that aggregation did not require ATP since it used potential energy stored during the previous ATP-requiring dispersion process. In view of the findings presented here, and elsewhere, it is possible that intact erythrophores are able to aggregate pigment in the presence of metabolic poisons due to the initial effects of energy depletion upon ATP-dependent Ca^{2+} pumps, the final result being a rise in intracellular Ca^{2+} levels and a subsequent aggregation of pigment. This proposal is supported by the fact that intact erythrophores with dispersed pigment do not aggregate their pigment granules when treated with metabolic poisons in calcium-free media (McNiven, M. A., unpublished observations).

By altering the concentrations of $MgATP^{2-}$ and $CaATP^{2-}$ in the reactivation buffer we have gained insight into some of the specific effects that ATP has on pigment motion. First, it is apparent that pigment aggregation and dispersion have different requirements for $MgATP^{2-}$ since a slow pigment aggregation does occur in vitro in buffer with $NaATP^{3-}$ without Mg^{2+} and $MgATP^{2-}$ (Fig. 6; Table I) while dispersion does not. We assume that the retarded aggregation exhibited under these Mg^{2+}-free conditions indicates that the retrograde ATPase is able to use either $CaATP^{2-}$ or $NaATP^{3-}$ but with a reduced efficiency. Alternatively, additional force-generating ATPases that require Mg^{2+} may be induced into a rigorlike state in the absence of Mg^{2+} to produce a resistance, thereby slowing aggregation. A similar phenomenon may occur during reactivation of cilia or flagella in Mg^{2+}-free buffer. In this instance, the dynein ATPase activity of flagella in Mg^{2+}-free buffer remains relatively high at 50–80% of that observed in the presence of Mg^{2+}, although microtubule sliding (disintegration) and flagellar beat are greatly reduced (Gibbons and Gibbons, 1972; Zanetti et al., 1979). Second, we find that changes in the $MgATP^{2-}$ concentration effect not only the velocity of pigment transport (Table I) but also the ability of the granules to bind to the erythrophore microtubules after detergent treatment. Granule release is initiated upon exposure to 0.5–1.0 mM $MgATP^{2-}$ and maximized at 10 mM $MgATP^{2-}$ (Fig. 4). Apparently, conditions of our reactivation system (1 mM $MgATP^{2-}$ and 1 mM free Mg^{2+}) provide a balanced state between granule binding and release, thereby enabling the granules to translocate. The effects of $MgATP^{2-}$ on granule binding and release to microtubules parallels those seen for kinesin, which remains bound to microtubules until exposed to $MgATP^{2-}$ (Schnapp, B. J., personal communication). Dynein also exhibits a rigorlike binding to microtubules in demembranated flagella that are reactivated in Mg^{2+}-free buffer (Gibbons and Gibbons, 1978). Whether pigment granules are released from microtubules during transport in vivo is not known. Pigment does appear to swirl and percholate about the cell center as if free after each aggregation. It will be interesting to examine erythrophores fixed or frozen during the reactivation process to observe the physical connections between granules and microtubules and how they are affected by $MgATP^{2-}$.

Evidence for Microtubule- vs. Granule-associated Translocators

It has been predicted that organelle-associated motility motors bind to the surface lattice of a microtubule and cleave ATP to generate a motive force (Veslop, 1975; Pratt, 1980; Vale et al., 1985). However the fact that adjacent erythrophores with overlapping microtubules pull together upon reactivation (Fig. 6) provides an alternative model. Because some cells cluster together after their pigment has fully aggregated, it appears that a motive force is generated where microtubules from each cell interdigitate, suggesting that a retrograde motor resides on the microtubules themselves. There is a precedent for this prediction found within the giant fresh water protozoan *Reticulomyxa* which undergoes a similar phenomenon when reactivated in vitro (Koonce et al., 1987). Normally, in the presence of $MgATP^{2-}$, organelles within the protozoan translocate rapidly along the cell's microtubule bundles which extend for great lengths. However, if the bundles are severed into free-ended segments and are then exposed to $MgATP^{2-}$, they actively slide apart and subsequently disintegrate, not unlike the ATP-induced sliding of trypsin-treated flagella originally reported by Summers and Gibbons (1971). Thus, it appears that both erythrophore and *Reticulomyxa* exhibit ATP-dependent microtubule sliding. Despite these observations it remains difficult to

conceptualize how a motor residing upon microtubules could be used to generate a retrograde force utilized during pigment aggregation. This arrangement dictates that the structural end of the translocator molecule would be in contact with the microtubule while the enzymatic or "business" end would interact with the pigment granule surface, an unprecedented arrangement. We are optimistic that future studies using the reactivated erythrophore model described here will be insightful.

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