Regulation of Organelle Transport in Melanophores by Calcineurin

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Abstract. Previous studies have shown that pigment granule dispersion and aggregation in melanophores of the African cichlid, *Tilapia mossambica*, are regulated by protein phosphorylation and dephosphorylation, respectively (Rozdzial, M. M., and L. T. Haimo. 1986. *Cell.* 47:1061–1070). The present studies suggest that calcineurin, a Ca^{2+}/calmodulin-stimulated phosphatase, is the endogenous phosphatase that mediates pigment aggregation in melanophores. Aggregation, but not dispersion, is inhibited by okadaic acid at concentrations consistent with an inhibition of calcineurin activity. Inhibition of aggregation in melanophores that have been BAPTA loaded or treated with calmodulin antagonists implicate Ca^{2+} and calmodulin, respectively, in this process. Moreover, addition of calcineurin rescues aggregation in lysed melanophores which are otherwise incapable of aggregating pigment. Immunoblotting with an anticalcineurin IgG reveals that calcineurin is a component of the dermis, which contains the melanophores, and indirect immunofluorescence localizes calcineurin specifically to the melanophores. Finally, this antibody, which inhibits calcineurin's phosphatase activity (Tash, J. S., M. Krinks, J. Patel, R. L. Means, C. B. Klee, and A. R. Means. 1988. *J. Cell Biol.* 106:1625–1633), inhibits aggregation but has no effect on pigment granule dispersion. Together these studies indicate that retrograde transport of pigment granules to the melanophore cell center depends upon the participation of calcineurin.

All eukaryotic cells are capable of organelle transport. Because bidirectional organelle translocations can occur along single microtubules (Hayden and Allen, 1984; Allen et al., 1985; Koonce and Schliwa, 1985), which in most cells are arranged with uniform polarity (Euteneuer and McIntosh, 1981), a different motor protein may generate transport along microtubules in each direction (Vale et al., 1985*a*). Recent studies suggest that kinesin may be responsible for transport of organelles to the plus ends of microtubules (Vale et al., 1985*a*,*b*; Brady, 1985; Schroer et al., 1988), referred to as orthograde movements, while cytoplasmic dynein (Paschal et al., 1987; Lye et al., 1987; Vallee et al., 1988) may drive organelles towards the minus ends of the microtubules (retrograde movements) (Schnapp and Reese, 1989; Schroer et al., 1989). Alternatively, a single motor may be differentially regulated to generate bidirectional organelle movements along microtubules (Euteneuer et al., 1988, 1989).

To regulate the direction in which organelles move, cells must be able to control the interaction of the motor proteins with the organelle, with the microtubule, or with other factors (Schroer et al., 1988, 1989), or be able to adjust the activity of the motors. The mechanisms by which these regulatory processes occur are not well understood and can be difficult to study since, in most cells, organelles move in both directions simultaneously.

Chromatophores have been used as model cell types to study both organelle transport and its regulation (Schliwa, 1984). They are especially suited for studying regulation of organelle translocations since the organelle (pigment granule) movements are coordinated both temporally and spatially; that is, the pigment moves either away from (dispersion) or towards (aggregation) the cell center in response to an appropriate stimulus (Bagnara and Hadley, 1973). This coordination allows one to investigate the regulatory mechanisms for each direction of transport without being hampered by regulatory signals associated with the opposite direction.

There is strong evidence that cAMP-dependent protein phosphorylation regulates the direction of pigment granule transport in two classes of chromatophores. Xanthophores (Lynch et al., 1986*a*,*b*) and melanophores (Rozdzial and Haimo, 1986*b*) undergo pigment dispersion when cAMP levels are elevated. Conversely, phosphatase activity has been shown to be necessary for pigment aggregation in melanophores (Rozdzial and Haimo, 1986*b*). In erythrophores, a third class of chromatophores, Ca^{2+} levels have been shown to control the direction of pigment movement in both live (Luby-Phelps and Porter, 1982) and lysed (McNiven and Ward, 1988) cells. It is not yet known if the Ca^{2+} signal in erythrophores is utilized to regulate a phosphorylation–dephosphorylation system.

Both cAMP-dependent protein phosphorylation and Ca^{2+} have been implicated in regulating ciliary and flagellar motility. Some axonemes can be reactivated only after first being phosphorylated via the cAMP-dependent protein kinase pathway (Brokaw, 1985; Lindemann, 1978; Murofushi et al., 1986; Tash et al., 1984, 1986), and Ca^{2+} has been shown

to regulate the wave form of various axonemes (Bessen et al., 1980; Brokaw et al., 1974; Brokaw and Nagayama, 1985; Hyams and Borisy, 1978).

Recently, it has been demonstrated that wave form can be affected by the Ca^{2+}/calmodulin (CaM)[1]-stimulated phosphatase, calcineurin (CN) (Tash et al., 1988). CN was originally isolated from brain (Klee et al., 1979), is identical to phosphatase 2B (Stewart et al., 1982; Ingebritsen and Cohen, 1983*a*,*b*), and has a wide phylogenetic and tissue distribution in eukaryotes (Tallant and Cheung, 1983; Klee et al., 1988). It is possible that the regulatory role of Ca^{2+} in axonemes is mediated via CN-induced protein dephosphorylation (Tash et al., 1988). Potentially, in organelle transport in melanophores, the cAMP-dependent phosphorylation events required for dispersion are reversed by Ca^{2+} activation of CN to generate protein dephosphorylation and, thus, aggregation.

As a first step in unraveling the regulatory events governing pigment aggregation in melanophores, we sought to determine the identity of the specific phosphatase responsible for mediating pigment aggregation. In the work presented here, we provide evidence to indicate that CN is a component of melanophores and that pigment granule aggregation is mediated by CN phosphatase activity.

Materials and Methods

Solutions and Materials

Goldfish Ringer's (Kagawa et al., 1984) without $CaCl_2$, glucose, and antibiotics was used for all incubations of live cells. For cell lysis and reactivation of pigment granule transport, lysis buffer consisting of 30 mM Hepes, 2 mM EGTA, 33 mM potassium acetate, 2.5% polyethylene glycol (20,000 mol wt), and 0.0016% digitonin was adjusted to 1 mM free Mg^{2+} ($MgSO_4$) using the algorithms of Brokaw (1986). The phosphatase rescue experiments described below were conducted in buffer with 2 μM free Ca^{2+}; all other experiments were performed without Ca^{2+} in the buffer. *Tilapia mossambica* were purchased from Pacific Aquafarms (Niland, CA). Vectastain reagents for immunoblotting, biotinylated anti-rabbit IgG, and avidin-RITC for immunofluorescence were purchased from Vector Laboratories, Inc. (Burlingame, CA). Rabbit anti-mouse IgG used as a control nonimmune antibody was purchased from Miles Scientific Div. (Naperville, IL). Acetoxymethyl ester of 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*',*N*'-tetraacetic acid (BAPTA/AM) was purchased from Molecular Probes Inc. (Junction City, OR). Taxol was the generous gift of Dr. M. Suffness, National Institutes of Health (NIH). Bovine brain CN and anti-CN (a rabbit polyclonal antibody used either as an ammonium sulfate-precipitated IgG fraction or as a CN affinity column-purified fraction) were the generous gift of Claude B. Klee, NIH; the anti-CN has been characterized previously (Tash et al., 1988). Bovine brain CN was also purchased from Sigma Chemical Co. (St. Louis, MO); all other chemicals were purchased from Sigma Chemical Co.

Cell Lysis and Reactivations

Scales were prepared as previously described (Rozdzial and Haimo, 1986*a*; Haimo and Rozdzial, 1989).

Reactivations. Dermal melanophores were lysed by agitating scale pieces, with adherent dermis, in 1 ml of lysis buffer containing 10^{-5} M epinephrine in a 1.5-ml microfuge tube for 2–2.5 min. Cells were lysed in the presence of epinephrine to induce pigment granule aggregation so that all experiments were initiated with pigment in the aggregated state. Reactivation of pigment granule dispersion was induced by placing or agitating the lysed scale pieces in lysis buffer containing 0.5 mM ATP and 1 mM cAMP. Since cAMP will only induce dispersion in lysed melanophores (Rozdzial and Haimo, 1986*a*), this experimental design includes a simple, rapid visual assay (pigment dispersion) to ensure that cells were indeed lysed before further experimental manipulations were undertaken. Reactivation of pigment granule aggregation was carried out by placing scale pieces in lysis buffer containing 0.5 mM ATP.

Gentle vs. Extensive Lysis. The extent of lysis and ability of cells to undergo reactivation of pigment transport is directly correlated with the total amount of agitation in the lysis and reactivation steps. Normal lysis consisted of a 30-s total agitation during the lysis step with no further agitation during reactivation. Extensive lysis consisted of a 60-s agitation during the lysis step and an additional 30-s agitation in the dispersion step. The terms gentle and extensive, respectively, will be used throughout to describe these two procedures.

Okadaic Acid Inhibition of Aggregation

Live cells with dispersed pigment were incubated for 30 min in Ringer's containing various concentrations (0–10 μM) of okadaic acid (OA), and then challenged with epinephrine to aggregate pigment. Gently lysed melanophores were induced to disperse pigment in cAMP and ATP, incubated with OA (0–5 μM) for 10 min, and subsequently challenged to aggregate pigment by addition of ATP. Live melanophores with aggregated pigment were incubated in 10 μM OA for 30 min and then challenged to disperse pigment by addition of 25 μM iso-butyl-methylxanthine.

BAPTA Loading and Aggregation

Live melanophores were incubated for 30 min with 1 μM BAPTA/AM in Ringer's. Cells were then washed in fresh Ringer's for 1 min and challenged to aggregate pigment by incubating them with epinephrine or methoxamine.

CaM Antagonists

Live melanophores were incubated in Ringer's containing 24 μM taxol for 30 min before treatment with CaM antagonists. Cells were then incubated with CaM antagonists, trifluoperazine (TFP), calmidizolium (CZM), *N*-(6-aminohexyl)-1-naphthalenesulfonamide (W-7), or Compound 48/80, and taxol for 30 min and subsequently challenged with epinephrine to aggregate pigment.

Phosphatase Rescue Experiments

The relative ability of alkaline phosphatase (AP) vs. CN to rescue aggregation in extensively lysed melanophores was compared in lysis buffer containing 2 μM free Ca^{2+} and 0.5 μM CaM to stimulate CN activity. Two concentrations of AP, having phosphatase activity equivalent to 10× and 50× the activity of 20 μg/ml CN in this buffer, were tested. Phosphatase activity was assayed using *p*-nitro-phenylphosphate as the substrate (Pallen and Wang, 1983).

CN Antibody Inhibition

The ability of anti-CN IgG to inhibit pigment transport was examined in gently lysed cells. To facilitate entry of the IgGs, melanophores were lysed in buffer containing elevated (0.002%) digitonin. All reactivation steps were performed in the presence or absence of rabbit polyclonal anti-CN IgG, which had been affinity purified by passing the IgG fraction over a CN column. Affinity-purified anti-CN (1×10^{-7} M) was preincubated with 0, 5×10^{-8}, or 1×10^{-7} M CN for 10 min before use. Lysed, dispersed melanophores were then incubated in anti-CN or anti-CN preincubated with CN, as indicated, for 25 min and subsequently challenged to aggregate pigment by addition of ATP.

Immunoblotting

To determine if CN was present in the dermis on scales, dermis was prepared as described (Haimo and Rozdzial, 1989). The dermis was homogenized in 2 ml of lysis buffer with protease inhibitors (1 mM PMSF, 0.5 mM DTT, 10 μg/ml of leupeptin, Nα-*p*-tosyl-L-arginine methyl ester, and aprotinin), centrifuged at 10,000 *g* for 1 h, and the supernatant collected and concentrated with a Centricon filter (Amicon, Inc., Danvers, MA). A crude extract of fish brain was prepared by homogenizing the tissue in buffer (50

1. *Abbreviations used in this paper*: AP, alkaline phosphatase; APV, average pixel value; BAPTA/AM, acetoxymethyl ester of 1,2-bis(2-aminophenoxy) ethane-*N*,*N*,*N*',*N*'-tetraacetic acid, CaM, calmodulin; CN, calcineurin; CZM, calmidizolium; IC_{50}, inhibitory concentration resulting in 50% inhibition; OA, okadaic acid; TFP, trifluoperazine; W-7, *N*-(6-aminohexyl)-1-naphthalenesulforamide.

mM Tris, pH 7.8, 3 mM $MgSO_4$, 1 mM EGTA, 0.5 mM DTT, 1 mM PMSF). The homogenate was centrifuged at 10,000 *g* and concentrated with a Centricon filter. The samples were electrophoresed on a 10% SDS-polyacrylamide gel, blotted onto nitrocellulose according to the method of Towbin et al. (1979), incubated with an ammonium sulfate-purified IgG fraction (7.3 μg/ml) containing anti-CN, and stained according to the Vectastain procedure (Vector Laboratories, Inc.).

Immunofluorescence

Scale pieces were fixed with 3.7% formaldehyde in Ringer's for 20 min, permeabilized in acetone for 5 s, and washed in PBS (50 mM K_2HPO_4, pH 7.4, 150 mM NaCl). All subsequent washes were in PBS-BSA (1 mg/ml BSA). Scale pieces were incubated with anti-CN IgG (ammonium sulfate-precipitated IgG fraction) in PBS-BSA for 24 h at 4°C, washed, incubated in biotinylated anti-rabbit IgG for 12 h, washed, incubated in avidin-RITC for 1 h, and finally washed. Controls were treated identically except that anti-CN IgG was omitted. To reduce photobleaching, scales were mounted in *p*-phenylenediamine (1 mg/ml) in 90% glycerol (Johnson and Nogueira-Araujo, 1981).

Image Analysis and Photography

The rate and extent of aggregation were quantitated by video image analysis, unless otherwise indicated. Scale pieces were viewed with a Zeiss Neofluor 6.3× lens on a Nikon Ophiphot microscope and imaged with a Newvicon video camera (Dage Series 67M; Dage-MTI, Inc., Wabash, IN) into the Image-1 (Universal Imaging Corporation, Westchester, PA) video processor. The pigment movements after experimental treatments were quantitated using the area brightness function of Image-1 which calculates the average pixel value (APV) on the monitor in the area of interest. Pixel values of 0 (black) to 255 (white) are assigned to each of the 255 gray levels that a pixel may hold. The change in APV is thus directly correlated with the area covered by the pigment in the melanophores, an increase in APV reflecting a decrease in area covered by pigment. APV readings were taken at intervals of 30 s (live cell experiments), or 1 min (lysed cell experiments). Data were normalized by setting the final extent of control aggregation at 100% change in APV. Several replicates (3–6) were conducted for each experiment. Each replicate consisted of 1 scale piece (50–100 melanophores) per treatment.

Additionally, images of the melanophores in the measured area were recorded before and after treatment. These images were photographed from the video monitor with Panatomic-X film, developed in Microdol-X, and printed on Kodak paper.

For immunofluorescence, melanophores were photographed with Kodak Technical Pan film on a Nikon Optiphot microscope with a 40× Neofluor objective using epifluorescence illumination. Micrographs are from negatives exposed for the same length of time and treated identically throughout processing.

Statistical Analysis

In experiments that were quantitated by video image analysis, data were analyzed using an analysis of variance for multiple comparisons (Duncan's multiple range test or Tukey's honest significant difference) on SAS (SAS Institute, Cary, NC). All experiments were analyzed for significant differences at the $\alpha = 0.05$ level. In the text, "significant" is used exclusively to refer to statistical significance as determined by these analyses.

Results

OA Inhibition of Aggregation

OA is a toxin produced by the black sponge, *Halichondria okadai* (Bialojan and Takai, 1988). It has been found to inhibit phosphatases 1, 2A, and 2B and has been used both in vitro (Bialojan and Takai, 1988) and in vivo (Haystead et al., 1989) since it is permeable to intact cells. OA inhibits phosphatase 2A at very low concentrations (IC_{50} = 1.2 nM), phosphatase 1 at intermediate concentrations (IC_{50} = 315 nM), and phosphatase 2B (CN) at higher concentrations (IC_{50} = 4.5 μM), while it has relatively little effect on acid and alkaline phosphatases and on phosphatase 2C at concentrations up to 10 μM (Bialojan and Takai, 1988). Thus, OA has the potential to provide information concerning the type of phosphatase responsible for mediating pigment aggregation.

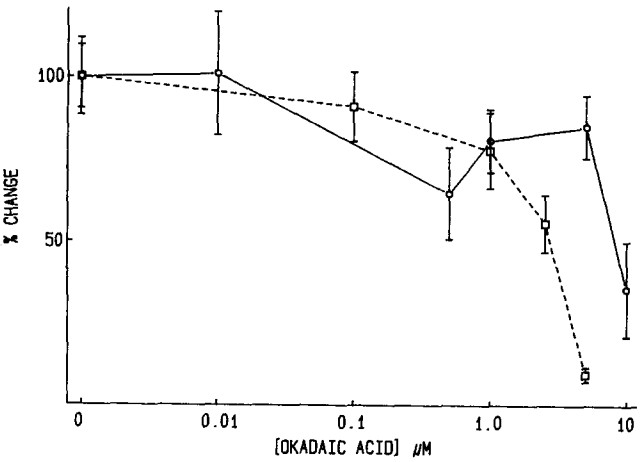

Figure 1. OA inhibition of pigment aggregation in live and lysed melanophores. The extent of pigment aggregation after treatment with OA is expressed as percent change in APV. The data have been normalized by setting the extent of aggregation in controls (0 OA) as 100% aggregation. In both live (○) and lysed (□) cell preparations there is no significant inhibition of aggregation at concentrations ≤ 1 μM compared with controls ($p < 0.05$). Live cells exhibit an IC_{50} of 7.8 μM. In lysed cells the IC_{50} is reduced to 3.1 μM, perhaps an indication of the accessibility of OA to the cells' interior. n = 4 for both live and lysed cell experiments; error bars are ± SEM.

Live melanophores with dispersed pigment were incubated in the presence or absence of OA and subsequently challenged with epinephrine to aggregate pigment. The extent of aggregation was quantitated and compared to controls (Fig. 1). Concentrations up to and including 1 μM OA had relatively little effect on pigment aggregation; the extent of aggregation at 1 μM OA was 81% that of control aggregation. Aggregation was inhibited by 50% (IC_{50}) at a concentration of 7.8 μM, and was almost completely inhibited at 10 μM, at which concentration melanophores aggregated only 35% the extent of controls.

Similar experiments were conducted using lysed cells. After gentle lysis, melanophores with dispersed pigment were incubated in the presence or absence of OA for 10 min and subsequently challenged to aggregate pigment. The results are analogous to those seen in live cells; concentrations of 1 μM or less had relatively little effect on the extent of pigment aggregation. Aggregation at 1 μM OA was 83.4% of control aggregation, while inhibition was virtually complete at 5 μM (Fig. 1). The IC_{50} for lysed cells was calculated to be 3.1 μM.

OA, at a concentration of 10 μM, had no effect on dispersion in live cells. Dispersion after OA treatment was 85% that of control, and was not significantly different ($P < 0.05$).

Ca^{2+} and CaM Involvement in Aggregation

The concentration of OA required for inhibition of aggregation is consistent with phosphatase 2B, CN, being the phos-

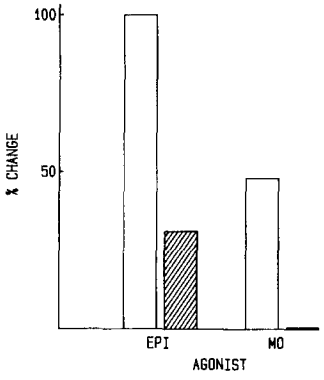

Figure 2. Inhibition of aggregation after BAPTA loading of live melanophores. Live melanophores were incubated in 1 μM BAPTA/AM for 30 min, washed in Ringer's, and challenged to aggregate pigment with 10^{-5} M epinephrine (*EPI*) or 10^{-5} M methoxamine (*MO*). The extent of aggregation is presented as percent change in average pixel value. Control aggregation in epinephrine has been normalized to 100%. Aggregation is severely inhibited in BAPTA loaded cells (▨) compared to untreated cells (□).

phatase responsible for the dephosphorylation required during aggregation. CN is activated by Ca^{2+} and CaM and, thus, these might also be required during aggregation. In fact, aggregation in live melanophores is induced by α-adrenergic agonists (Fujii and Novales, 1969), and stimulation of α_1 adrenergic receptors has been linked to a rise in intracellular free Ca^{2+} (Exton, 1985).

To test whether Ca^{2+} is involved in aggregation, live melanophores were loaded with the calcium chelator BAPTA, using the cell-permeant acetoxymethyl derivative (BAPTA/AM) (Tsien, 1981), and challenged to aggregate pigment. Response to adrenergic stimulation at very low free Ca^{2+} concentrations can thus be tested. After loading with BAPTA, melanophores were challenged to aggregate pigment with epinephrine, which binds to both α_1 and α_2 adrenergic receptors, or with methoxamine, which is an α_1-specific adrenergic agonist. In untreated cells, methoxamine does not induce as tightly aggregated a pigment mass as does epinephrine, although pigment does aggregate. After epinephrine stimulation of BAPTA-loaded cells, pigment transport was only 31% the extent of pigment movements in cells not exposed to BAPTA (Fig. 2). In BAPTA-loaded melanophores challenged to aggregate pigment with methoxamine, pigment migration was only 0.6% that of aggregation in control cells challenged with epinephrine, or 1.3% the extent of pigment transport in the methoxamine control (Fig. 2).

CaM involvement in aggregation was tested by incubating live, dispersed melanophores with CaM antagonists and, subsequently, challenging the cells to aggregate pigment. Three membrane-permeant antagonists—TFP, CZM, and W-7—were used. Each antagonist substantially inhibited aggregation compared with untreated controls (Table I). Compound 48/80, which is not membrane permeant, resulted in only a slight decrease in the extent of aggregation. In lysed cells, all four antagonists inhibited aggregation (data not shown).

CN Rescue of Aggregation

Lysed melanophores normally respond to cAMP and ATP by dispersing pigment throughout the cell (Rozdzial and Haimo, 1986*a*). Dispersion can be assayed visually and allows a rapid determination of cell lysis. All experiments were conducted on cells that had dispersed in vitro to ensure that data were only collected from cells that had indeed been lysed at the initiation of the experimental treatment.

Gently lysed melanophores are capable of a complete round of pigment transport; dispersion and subsequent reaggregation of pigment can be reactivated in vitro. When melanophores are gently lysed, almost all cells can reaggregate pigment completely after dispersion in vitro (Fig. 3, *a* and *b*). However, if melanophores are extensively lysed, their ability to reaggregate pigment after dispersion is substantially impaired (Fig. 3, *c* and *d*). Potentially, extensive lysis results in the loss from the cells of a soluble component required for aggregation. Phosphatase activity is necessary for this direction of transport (Rozdzial and Haimo, 1986*b*), and accordingly, the phosphatase CN was tested for its ability to rescue pigment aggregation. Addition of CN to extensively lysed cells resulted in the rescue of pigment aggregation (Fig. 3, *e* and *f*) which was indistinguishable ($p < 0.05$) from control aggregation after gentle lysis (Fig. 4).

To determine the specificity of pigment aggregation rescue by CN, AP was also tested for its ability to rescue pigment aggregation. Incubation of extensively lysed cells in AP (Fig. 3, *g* and *h*) resulted in partial restoration of aggregation. AP, however, was not able to restore complete aggregation of pigment, even when used at a concentration 50-fold the activity of the CN used. Although aggregation was significantly greater than the extensively lysed cells ($p < 0.05$), AP treatment did not result in aggregation comparable to controls (Fig. 4).

CN Is a Component of Melanophores

To determine if CN is present in melanophores, immunoblots and immunofluorescence of the dermis were performed using an ammonium sulfate–purified IgG fraction containing polyclonal rabbit IgG raised against bovine brain CN. This antibody recognizes both A (61-kD) and B (15–19-kD) subunits of CN (Klee et al., 1988). Using this antibody, immunoblots of purified CN from bovine brain revealed the presence of the A and B subunits as well as some proteolytic fragments of the A subunit (Fig. 5, lane *I*). Immunoblots of a soluble extract of *T. mossambica* brain demonstrated cross

Table I. Aggregation in Live Melanophores after Treatment with CaM Antagonists

CaM antagonists	Treatment	Extent of aggregation
	μM	%
Control		100
48/80	10	75.5
W-7	50	27.3
W-7	100	5.0
CZM	12	28.3
CZM	24	3.5
TFP	50	7.6
TFP	100	1.4

Live, dispersed melanophores were incubated with CaM antagonists, and then challenged to aggregate pigment. Aggregation was quantitated with video image analysis and data were normalized by setting the exent of control aggregation to 100%. The membrane-permeant antagonists—CZM, TFP, and W-7—all resulted in substantial inhibition of aggregation. The nonmembrane-permeant antagonists, compound 48/80, resulted in only a slight inhibition, which may be nonspecific.

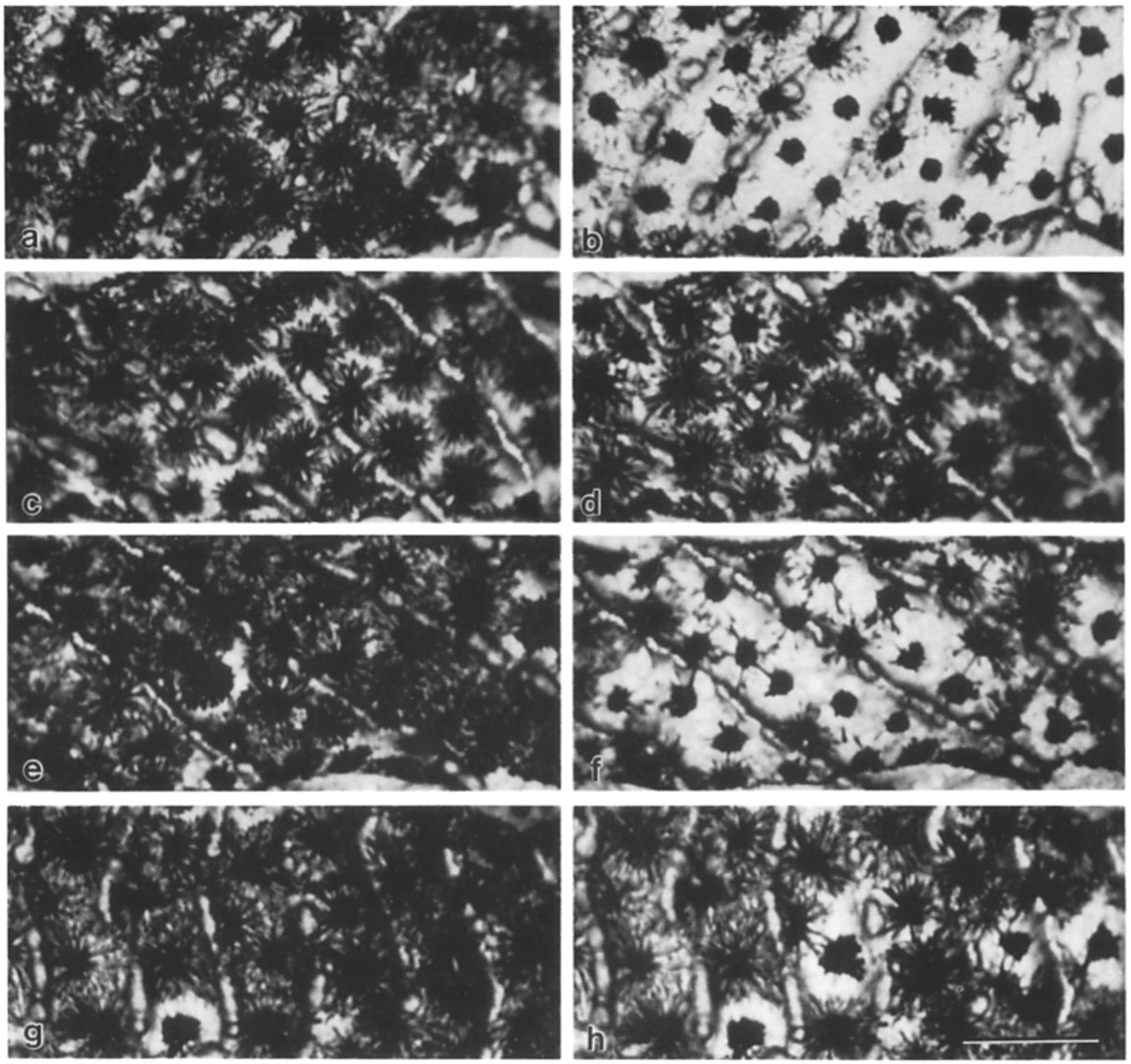

Figure 3. Phosphatase rescue of extensively lysed melanophores by CN and AP. Lysed melanophores with dispersed pigment (*a*, *c*, *e*, and *g*) were challenged to aggregate pigment (*b*, *d*, *f*, and *h*) under different conditions. After gentle lysis, melanophores with pigment dispersed (*a*) are capable of complete pigment aggregation (*b*). After extensive lysis, dispersed cells (*c*) exhibit only very limited pigment granule aggregation when challenged to transport pigment (*d*). Complete aggregation is rescued when extensively lysed, dispersed cells (*e*) are challenged to aggregate pigment in the presence of exogenous CN (*f*). Aggregation in extensively lysed melanophores treated with CN is not significantly different from control aggregations (see Fig. 4). Challenge of extensively lysed, dispersed cells (*g*) with AP results in partial recovery of aggregation (*h*). Ca^{2+} and CaM were present in all treatments. Bar, 200 μm.

reactivity with two polypeptides of ~60 and 19 kD (Fig. 5, lane *2*), indicating that the appropriate antigens in *T. mossambica* are recognized by the antibody.

Anti-CN also stained a 60-kD polypeptide in a soluble extract of the dermis (Fig. 5, lane *3*), indicating that CN is present in this tissue and that dermal CN is at least partially soluble, as has been previously reported for CN in other tissues (Ingebritsen, et al., 1983; Klee et al., 1988). Although a polypeptide corresponding to the B subunit could not be visualized in the whole extracts of dermis, the B subunit was apparent in soluble extracts that were partially purified by ammonium sulfate precipitation (data not shown).

Although melanophores are the major cell type of the dermis, there are a variety of other cell types present. Immunofluorescent localization of CN in intact dermal tissue was conducted to determine if CN were present in the melanophores themselves. CN was found to be specifically localized to melanophores, with diffuse fluorescence ob-

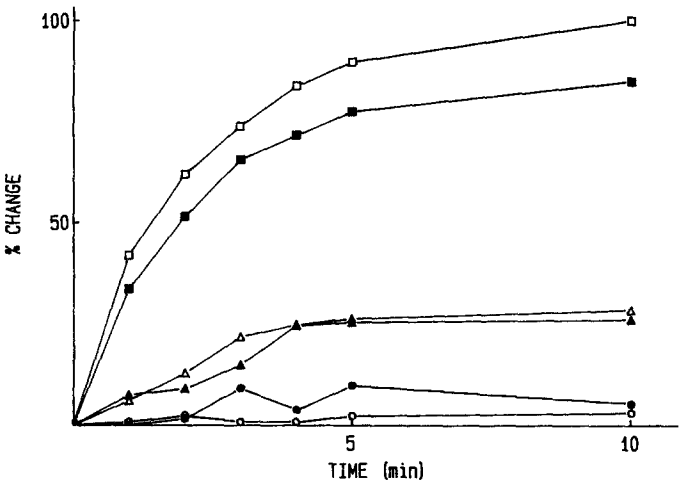

Figure 4. Rate and extent of aggregation mediated by phosphatase in extensively lysed melanophores. Aggregation is represented as percent change in APV vs. time. The data have been normalized by setting the final extent of aggregation in gently lysed control cells as 100% aggregation. Extensively lysed melanophores challenged to aggregate pigment with ATP in the presence (○) or absence (●) of Ca^{2+} and CaM are incapable of pigment aggregation. Extensively lysed cells incubated in AP (Ca^{2+} and CaM present) at either 10× (▲) or 50× (△) the phosphatase activity of the CN used show a significant ($p < 0.05$) increase in pigment transport. Extensively lysed melanophores incubated with CN in the presence of Ca^{2+} and CaM (■) show significantly more aggregation than with AP ($P < 0.05$), and are indistinguishable from controls (□). n = 4 for each treatment.

served throughout the cell body, as would be expected of a soluble protein (Fig. 6 *a*). Controls incubated without primary antibody showed only faint background staining with occasional bright spots that coincide with ridges formed by overlapping layers of the scale (Fig. 6 *b*). CN is also present in lysed cells (Fig. 6 *c*); however, in extensively lysed melanophores staining of CN is greatly reduced (Fig. 6 *d*). Since the samples were treated identically except for the lysis conditions, loss of CN staining qualitatively reflects a loss of the antigen as a consequence of the extensive lysis procedure.

Antibody Inhibition of Aggregation

Because OA inhibits aggregation in both live and lysed cells in a manner consistent with inhibition of CN phosphatase (see Fig. 1) and CN can rescue aggregation in extensively lysed cells (see Figs. 3 and 4), CN may be the phosphatase required for aggregation. If so, then specific inhibition of CN activity by anti-CN (Tash et al., 1988; Klee, C. B., personal communication) should result in loss of the melanophores' ability to aggregate pigment.

Gently lysed melanophores were dispersed and then incubated for 25 min in affinity-purified anti-CN (1×10^{-7} M). When challenged to aggregate pigment, the anti-CN treatment (Fig. 7, *c* and *d*) produced significant inhibition of pigment transport compared with control treatments without antibody addition (Fig. 7, *a* and *b*). When 1×10^{-7} M anti-CN was preabsorbed with CN (5×10^{-8} or 1×10^{-7} M), aggregation was partially restored (Fig. 7, *e* and *f*). Treatment with anti-CN alone significantly inhibited aggregation compared with control aggregation (Fig. 8). Aggregation in melanophores treated with anti-CN preabsorbed with CN resulted in significantly greater aggregation than in cells treated with anti-CN alone, but they were not equivalent ($p < 0.05$) to control aggregation (Fig. 8). Pigment dispersion was unaffected by the presence of the antibody ($p < 0.05$). The extent of dispersion in anti-CN was 98% of that in buffer alone.

Discussion

The results of these studies indicate that CN mediates pigment aggregation in melanophores. CN, a Ca^{2+}/CaM-stimulated phosphatase, is a component of the melanophores as shown by immunofluorescent localization. OA inhibits pigment aggregation in both live and lysed melanophores at concentrations consistent with inhibition of CN phosphatase activity. CaM antagonists also inhibit aggregation and, additionally, chelation of intracellular Ca^{2+} blocks pigment transport to the cell center. Furthermore, exogenous bovine brain CN can rescue pigment aggregation in extensively lysed melanophores, while an anti-CN antibody specifically inhibits aggregation. Thus, CN activity appears to be required for pigment aggregation.

The high degree of specificity with which OA inhibits phosphatases has been used to implicate particular phosphatases in cellular processes (Haystead et al., 1989; Cohen et al., 1990). In live and lysed cells aggregation was inhibited

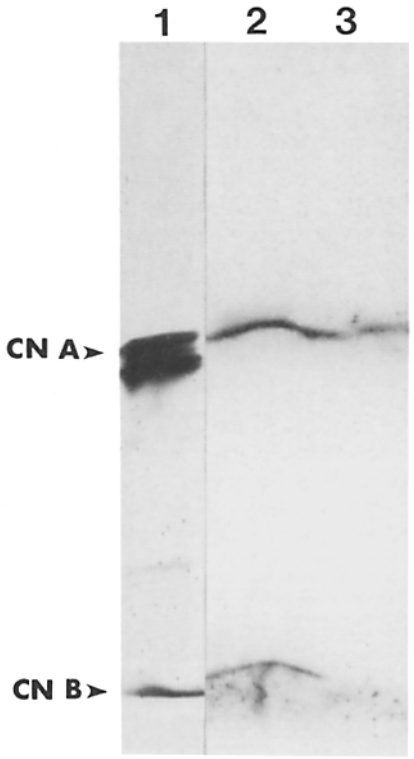

Figure 5. Immunoblots demonstrate that CN is a component of *T. mossambica* dermis. Anti-CN recognizes the A and B subunits of bovine brain CN as well as some proteolytic fragments of the A subunit (lane *1*) and of fish brain CN (lane *2*). A soluble extract of fish dermis (lane *3*) reveals the presence of the A subunit, although the B subunit was not detected in this sample.

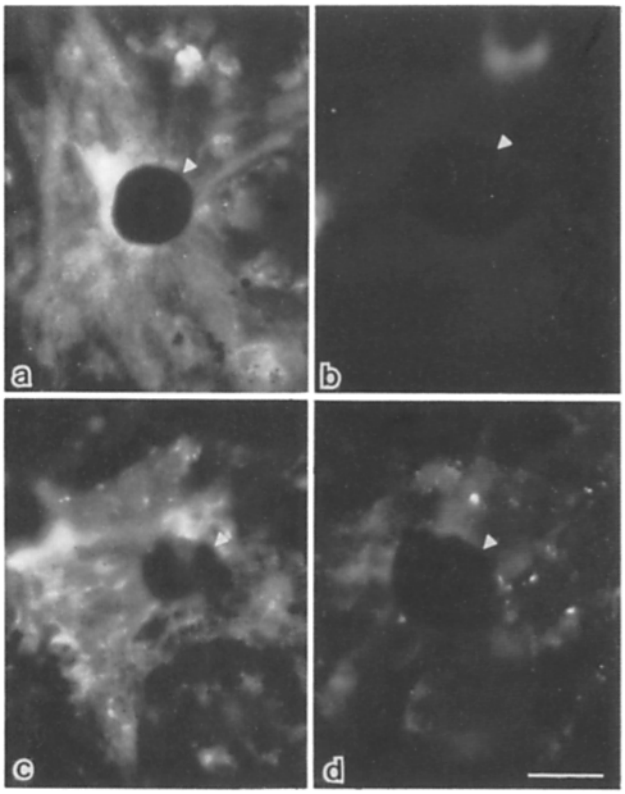

Figure 6. Immunofluorescent staining with anti-CN localizes CN in intact and lysed melanophores. The diffuse immunofluorescence throughout the cell body of an intact melanophore reflects the cytoplasmic distribution of CN (*a*). Control cell incubated with secondary antibody and avidin-RITC shows only background fluorescence which is intense at the scale ridges (*b*). A lysed melanophore also stains with anti-CN (*c*) at levels similar to live cells, but extensively lysed cells have greatly reduced staining (*d*). Arrowheads point out the aggregated pigment mass at the cell center. Bar, 50 μm.

at concentrations consistent with inhibition of CN. At 1 μM OA the extent of aggregation was 81–83% that of control aggregation, a difference that was not significant ($p < 0.05$). This lack of inhibition suggests that neither phosphatase 2A, which is inhibited at very low concentrations (nM), nor phosphatase 1, which occurs in cells at a concentration of ~1 μM (Cohen, 1989) and is inhibited maximally at 1 μM OA (Haystead et al., 1989), could be responsible for the phosphatase activity required for aggregation. If phosphatase 2C, acid phosphatase, or alkaline phosphatase were responsible, no inhibition would have occurred at the concentrations of OA tested. Instead, these results suggest that it is phosphatase 2B, CN, activity that is required for pigment aggregation.

It has been suggested that one of the roles of CN may be to activate phosphatase 1, by dephosphorylating, and thus deactivating, inhibitor-1 (Nimmo and Cohen, 1978), the endogenous negative modulator of phosphatase 1 (Cohen, 1989). If this were the case in pigment aggregation, OA should have inhibited aggregation at lower concentrations than were observed. It seems unlikely, therefore, that CN mediates aggregation through a cascade of phosphatase activation. Rather it may act directly to effect aggregation.

Ca^{2+} and CaM involvement would be predicted if CN is the phosphatase responsible for aggregation. Membrane-permeant CaM antagonists did indeed inhibit aggregation in live melanophores. In contrast, CaM antagonists failed to inhibit pigment aggregation in lysed erythrophores (McNiven and Ward, 1988), although the direction of pigment transport is Ca^{2+} regulated in these cells. This lack of inhibition may indicate that pigment movements are regulated differently in these two types of chromatophores.

Melanophores aggregate pigment in response to adrenergic receptor stimulation (Fujii and Novales, 1969). The two subclasses of adrenergic receptors, α_1 and α_2, result in an increase of intracellular Ca^{2+} and a decrease of adenylate cyclase activity, respectively (Exton, 1985). Methoxamine, which binds only to α_1 receptors, was able to induce aggregation, suggesting that a rise in intracellular Ca^{2+} results in pigment aggregation. After BAPTA loading, methoxamine was no longer able to promote aggregation, confirming that methoxamine induces aggregation via an increase in Ca^{2+}. Epinephrine-induced aggregation of pigment was also severely inhibited after BAPTA loading. That some aggregation did occur may reflect binding of epinephrine to α_2 receptors. A decrease in cAMP, and therefore in cAMP-dependent kinase activity, after stimulation of α_2 receptors may allow some pigment transport due to the basal activity of CN. Conversely, when the cAMP concentration is elevated, an increase in Ca^{2+} may be necessary to stimulate CN activity to a level at which it can overcome the cAMP-dependent kinase activity and thereby result in pigment aggregation. These data suggest that both the levels of Ca^{2+} and cAMP are important in determining the pigment distribution in these cells.

Previous studies have shown that CN can be found in both the particulate and soluble fractions of brain tissue (Klee et al., 1988). In the present studies, extensive lysis significantly reduced the extent of pigment transport in melanophores while addition of CN rescued these movements. These results suggest that at least some of the CN in melanophores is soluble, that it is extracted during the extensive lysis, and that this loss results in the inability of the cells to undergo aggregation. That CN alone can rescue aggregation implies that all other components necessary for this direction of transport are retained in the cell during extensive lysis.

Interestingly, AP was not able to rescue pigment transport with the same efficiency as CN, even when used at an activity level 50-fold that of the CN used. These data suggest that the melanophore substrates are more effectively dephosphorylated by CN than by AP. In comparison to other phosphatases, CN has rather narrow substrate specificity in vitro (Klee et al., 1988; Cohen, 1989), and thus, its ability to rescue pigment transport may reflect the fact that CN is the endogenous enzyme.

Aggregation was specifically inhibited by addition of anti-CN to lysed cells; dispersion was unaffected. Inhibition of aggregation is unlikely to be due to a nonspecific interference by the antibody since preabsorption of the antibody with CN blocked this inhibition. Because anti-CN inhibits the phosphatase activity of CN (Tash et al., 1988), these data indicate that CN phosphatase activity is required for aggregation, but not dispersion, of pigment in melanophores and imply that phosphate turnover (continual dephosphorylation and subsequent rephosphorylation of phosphorylated proteins) is not required for dispersion to occur.

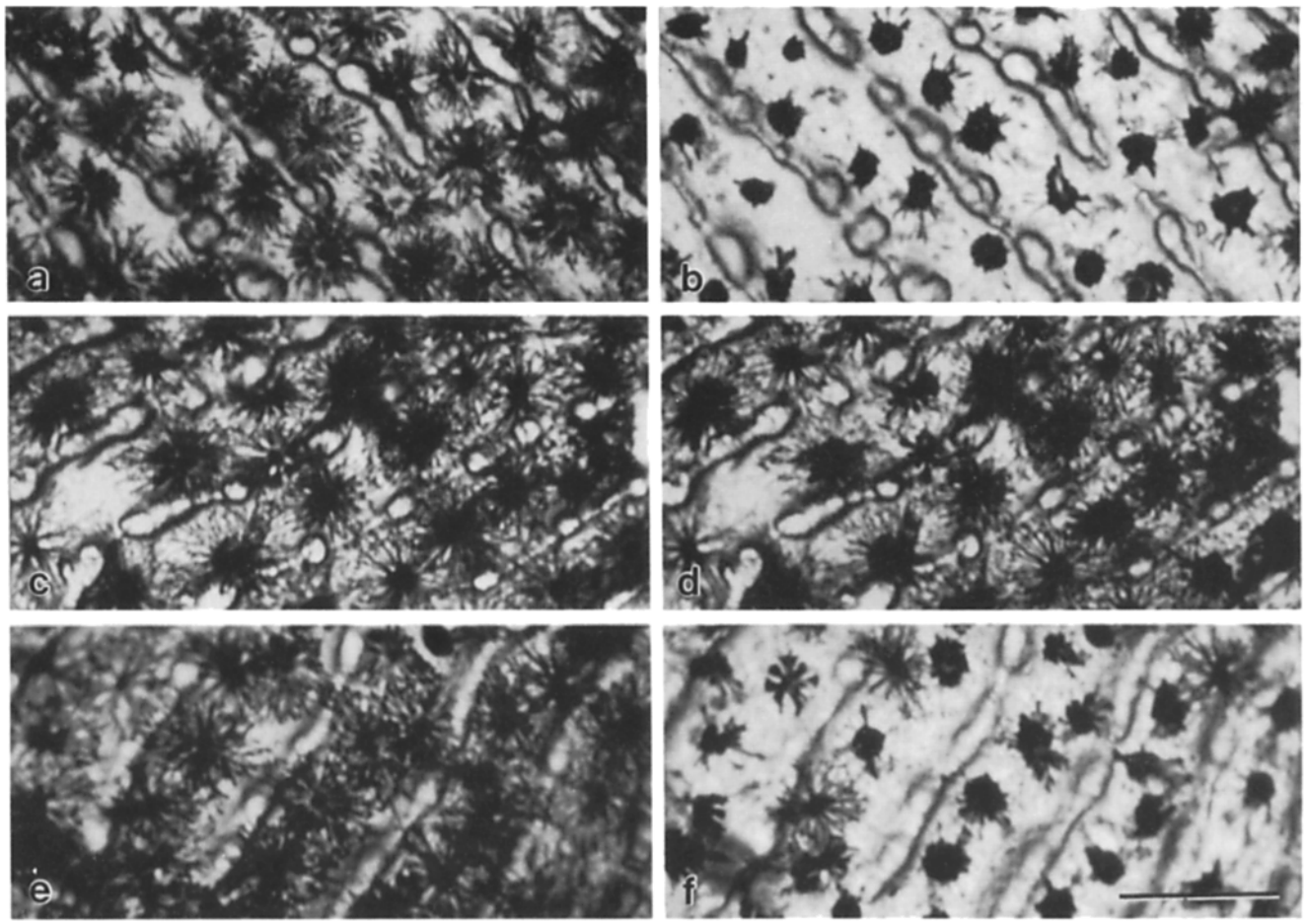

Figure 7. Anti-CN inhibits pigment aggregation in lysed melanophores. Lysed, dispersed melanophores (*a*, *c*, and *e*) were incubated in the presence or absence of antibody for 25 min and subsequently challenged to aggregate pigment (*b*, *d*, and *f*). In the absence of antibody, control cells (*a*) are able to aggregate pigment after the 25-min incubation (*b*). Melanophores incubated in the presence of 10^{-7} M anti-CN (*c*) exhibit only partial pigment aggregation with most cells undergoing little or no change in the distribution of pigment (*d*). Melanophores incubated in the presence of anti-CN preabsorbed with CN (1:1, mol/mol) (*e*) are capable of some aggregation (*f*). These experiments were conducted without addition of Ca^{2+} and CaM. Bar, 200 μm.

CN is highly susceptible to proteolysis (Manalan and Klee, 1983; Tallant et al., 1988; Hubbard and Klee, 1989). In bovine CN, proteolysis leads to removal of inhibitory and CaM binding domains of the A subunit while the B subunit remains intact (Tallant et al., 1988; Hubbard and Klee, 1989). The proteolyzed enzyme is in an activated state, has lost its Ca^{2+}/CaM sensitivity, but can be further stimulated by Ca^{2+} via the intact B subunit (Manalan and Klee, 1983; Tallant et al., 1988; Hubbard and Klee, 1989). Proteolysis of endogenous CN in the lysed melanophores could explain why no Ca^{2+} dependence of aggregation was observed in these or earlier studies (Rozdzial and Haimo, 1986*a*); proteolyzed CN is active in the absence of Ca^{2+}. Removal of cAMP, required by lysed cells to maintain pigment in the dispersed state (Rozdzial and Haimo, 1986*a*), would thus result in net dephosphorylation via the activated CN, and hence, pigment aggregation. Samples of scales incubated exactly as they would be before lysis and then used for immunoblotting show that some CN is proteolyzed to a 40-kD fragment in the intact melanophores (data not shown). CN may normally become proteolyzed in these cells; specific proteolysis of CN during platelet activation, for example, has been reported (Wallace et al., 1987). Alternatively, CN may be attacked by proteases some time after scales are removed from the fish and prepared for experimentation. Whichever the case, some CN is proteolyzed in these cells, and we suggest that this may be the reason that Ca^{2+} dependence has not been readily observed in lysed melanophores.

Live melanophores are likely to regulate both directions of pigment transport, dispersion being favored when the cAMP concentration is high and aggregation being favored when the Ca^{2+} concentration is high. The extent to which pigment is dispersed or aggregated may reflect the relative levels of kinase and phosphatase activity and, hence, the balance between Ca^{2+} and cAMP levels. In vitro, CN effectively dephosphorylates several substrates that would attenuate cAMP signals, including R_{II} (the regulatory subunit of cAMP-dependent kinase) and Ca^{2+}/CaM-dependent cAMP phosphodiesterase (Cohen, 1989). Thus, Ca^{2+} could modulate cAMP signals in vivo by activation of CN (Cohen, 1989; Klee et al., 1988).

It will be most interesting to determine if CN plays a role in the regulation of motility in other microtubule-based systems in addition to axonemes and melanophores. Although

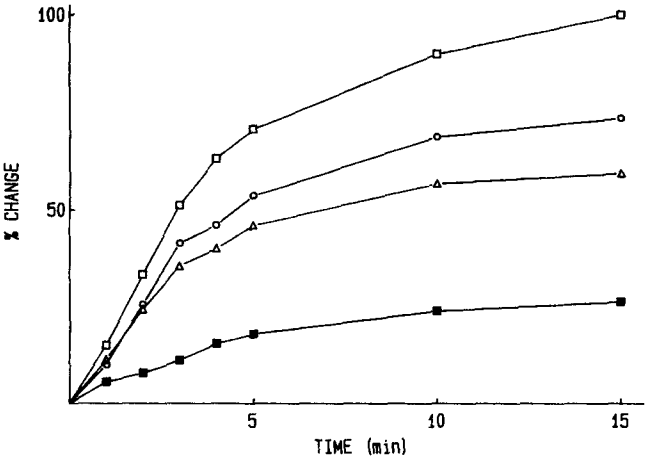

Figure 8. Anti-CN inhibition of pigment aggregation is blocked by preincubation of the antibody with CN. Pigment aggregation was followed by measuring change in APV vs. time. Control aggregation (□) of melanophores not exposed to anti-CN has been normalized to 100%. Melanophores incubated with 10^{-7} M anti-CN (■) aggregate only 28.9% the extent of controls. When anti-CN was preincubated with CN in molar ratios of 1:0.5 (△) or 1:1 (○) the inhibition is at least partially overcome since both these treatments demonstrate significantly greater extents of aggregation than the anti-CN treated cells ($p < 0.05$). These experiments were conducted without addition of Ca^{2+} and CaM. n=5 for each treatment.

CN was originally discovered in brain tissue, where it is present in relatively high concentration (Wallace et al., 1980), the processes for which this enzyme is required in the brain have not been elucidated. The B subunit of CN is myristylated, which may allow association with cellular membranes (Klee et al., 1988) including organelles. The subcellular localization of CN in melanophores has not been determined, but the possibility that it may be associated with the pigment granules is intriguing and should be investigated in future studies. CN is present in synaptosomes (Anthony et al., 1988); potentially, it could be involved in regulating the direction of vesicle transport in axons. CN might also be one of the accessory factors required for organelle transport in vitro (Schroer et al., 1988). The ability of exogenous CN from bovine brain to rescue motility in melanophores of a teleost fish may reflect the highly conserved mechanisms of organelle transport and regulation.

We are grateful to Dr. C. B. Klee for the generous gift of anti-CN antibody and bovine brain calcineurin and for helpful discussions; to Dr. J. S. Tash for invaluable advice and discussions; to Dr. P. Cohen for the generous gift of OA and for helpful advice; to Dr. M. Suffness (National Cancer Institute) for the gift of taxol; and to Dr. B. Hyman, R. Fenton, and M. Lacey (University of California, Riverside) for suggestions and critical review of this work.

This work was supported by National Science Foundation grant DCB-8710428, University of California Riverside Committee on Research, and Biomedical Research Support Grant.

Received for publication 30 April 1990 and in revised form 19 June 1990.

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