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Pigment granule translocation in red ovarian chromatophores from the palaemonid shrimp *Macrobrachium olfersi* (Weigmann, 1836): Functional roles for the cytoskeleton and its molecular motors

Sarah Ribeiro Milograna [a], Márcia Regina Ribeiro [a], Munira Muhammad Abdel Baqui [b], John Campbell McNamara [a,*]

[a] Departamento de Biologia, FFCLRP, Universidade de São Paulo, Ribeirão Preto 14040-901, São Paulo, Brazil
[b] Departamento de Biologia Celular, Molecular e Bioagentes Patogênicos, FMRP, Universidade de São Paulo, Ribeirão Preto 14040-901, São Paulo, Brazil

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ABSTRACT

The binding of red pigment concentrating hormone (RPCH) to membrane receptors in crustacean chromatophores triggers Ca^{2+}/cGMP signaling cascades that activate cytoskeletal motors, driving pigment granule translocation. We investigate the distributions of microfilaments and microtubules and their associated molecular motors, myosin and dynein, by confocal and transmission electron microscopy, evaluating a functional role for the cytoskeleton in pigment translocation using inhibitors of polymer turnover and motor activity *in vitro*. Microtubules occupy the chromatophore cell extensions whether the pigment granules are aggregated or dispersed. The inhibition of microtubule turnover by taxol induces pigment aggregation and inhibits re-dispersion. Phalloidin-FITC actin labeling, together with tannic acid fixation and ultrastructural analysis, reveals that microfilaments form networks associated with the pigment granules. Actin polymerization induced by jasplaquinolide strongly inhibits RPCH-induced aggregation, causes spontaneous pigment dispersion, and inhibits pigment re-dispersion. Inhibition of actin polymerization by latrunculin-A completely impedes pigment aggregation and re-dispersion. Confocal immunocytochemistry shows that non-muscle myosin II (NMMII) co-localizes mainly with pigment granules while blebbistatin inhibition of NMMII strongly reduces the RPCH response, also inducing spontaneous pigment dispersion. Myosin II and dynein also co-localize with the pigment granules. Inhibition of dynein ATPase by erythro-9-(2-hydroxy-3-nonyl) adenine induces aggregation, inhibits RPCH-triggered aggregation, and inhibits re-dispersion. Granule aggregation and dispersion depend mainly on microfilament integrity although microtubules may be involved. Both cytoskeletal polymers are functional only when subunit turnover is active. Myosin and dynein may be the molecular motors that drive pigment aggregation. These mechanisms of granule translocation in crustacean chromatophores share various features with those of vertebrate pigment cells.

1. Introduction

Animal coloration has long provoked scientific curiosity, owing not only to esthetic appeal, but as an aspect of biological diversity that determines an individual's performance and ultimately, species' adaptation (Forsman et al., 2008). Color changes play important roles in mate signaling, warning to potential predators, thermoregulation, protection against UV radiation, and in camouflage. Among the Crustacea, camouflage strategies like aposematism, mimetism and disruption (Stevens and Merilaita, 2009) depend on a strong, light-mediated capability to imitate substrate chromatic characteristics mediated by pigment movements within pigment bearing cells or chromatophores (reviewed in McNamara and Milograna, in press).

Like most crustacean chromatophores (Bauer, 2004), those of palaemonid shrimps are highly asymmetrical and consist of a spherical cell body containing a single nucleus and the main pigment granule mass, and one or two cell extensions that ramify into fine dendrites through which granules migrate (McNamara, 1981). These single cells form assemblages of 10–15 structurally and functionally linked effectors known as chromatosomes (McNamara and Taylor, 1987). Ultrastructurally, palaemonid chromatophores are characterized by various types of pigment granules, microtubules, well-developed, regionally differentiated smooth endoplasmic reticulum, occasional semi-spherical mitochondria, rough endoplasmic reticulum cisternae, polyribosomes and infrequent Golgi bodies (McNamara, 1981).

Pigment aggregation is characterized by centripetal granule translocation into the chromatophore cell body from the cell extensions,

* Corresponding author at: Departamento de Biologia, FFCLRP, Universidade de São Paulo, Avenida dos Bandeirantes 3900, Monte Alegre, Ribeirão Preto 14040-901, São Paulo, Brazil. Tel.: +55 1636023687.
E-mail address: mcnamara@ffclrp.usp.br (J.C. McNamara).

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triggered by the release of red pigment concentrating hormone (RPCH) into the hemolymph *via* the sinus gland. RPCH is a small peptide neurosecretion or chromatophorotropin originating mainly in the eyestalk X-organ (Fingerman, 1985). Pigment dispersion, often mediated by pigment dispersing hormone (PDH), consists of centrifugal granule translocation, and the spread of granules into the cell extensions (Rao, 1985). The aggregation mechanism is triggered by RPCH binding to a putative 7-span G protein-linked membrane receptor (see Hammers et al., 2011; Anders, 2012; Milograna et al., unpublished data) whose activation increases intracellular second messengers like Ca^{2+} and cGMP that activate interdependent signaling cascades (Nery et al., 1998; Ribeiro and McNamara, 2009; Milograna et al., 2010). Ultimately, these result in interactions between actin microfilaments and myosin motors (McNamara and Ribeiro, 1999; Boyle and McNamara, 2006), and also between microtubule arrays and other motor molecules like kinesin and dynein (McNamara and Boyle, 2009), all collectively known as cytoskeletal, mechano-chemical, force-transducing proteins. However, the mechanisms of pigment granule translocation and the specific proteins that regulate such molecular motors in crustacean chromatophores remain obscure.

Various molecular motors may underpin granule translocation in crustacean pigmentary effectors since different pigments can migrate simultaneously in opposing directions in polychromatic chromatophores (McNamara, 1981). Further, ovarian and nerve cord red chromatophores contain two granule types with different surface characteristics: large (≈450 nm diameter) membrane limited granules; and small (≈140 nm diameter) carotenoid granules that lack a membrane (McNamara and Sesso, 1982, 1983). Pigment aggregation exhibits a biphasic velocity profile, displaying rapid and slow components putatively dependent on the activity of different motors (McNamara and Ribeiro, 1999). Actin-associated myosin II may drive pigment aggregation, while pigment dispersion may be powered by microtubule-associated kinesin (Boyle and McNamara, 2006).

Studies on vertebrate pigment cells show that kinesin I and II participate in mammalian (Hara et al., 2000) and amphibian melanosome transport (Gross et al., 2002). An actin–myosin system functions in granule transport in rat (Wu et al., 1998), fish (Rodionov et al., 2003) and amphibian (Rogers et al., 1999) melanophores. Non-muscle myosin II isoforms play a role in cell cleavage, spindle formation, contractile activity of lamellipodia (Betapudi, 2010), and in the tractional forces underlying cell migration and motility (Lo et al., 2004). This myosin is the one of the motors responsible for vesicle transport (DePina et al., 2007), and is often associated with organelle membranes (DeGiorgis et al., 2002). Non-muscle myosin II seems a likely candidate for pigment granule transport in chromatophores, both crustacean and vertebrate, and should be better investigated.

Granule translocation in many vertebrate pigment cells involves complex molecular interactions, such as mutual modulation among different motor classes (Even-Ram et al., 2007; Ali et al., 2008) that compete to move cargos towards their respective cytoskeletal polymers. Cargo-bearing motors may alternate from one cytoskeletal element to another during transport, and motors traditionally associated with microtubular transport, like kinesin for example, may participate in transport along actin microfilaments (Even-Ram et al., 2007). Microtubule- and microfilament-based motor proteins and pigment granules are functionally associated in fish and amphibian melanophores (Gross et al., 2002).

While such cellular machinery has been examined in vertebrate chromatophores, little is known of the mechanisms that underlie granule translocation in crustacean pigment cells. Here, we investigate the cytoskeletal polymers and respective molecular motors that drive pigment movements in the red ovarian chromatophores of a freshwater shrimp, *Macrobrachium olfersi*. We examine the locations and roles of actin microfilaments and microtubules, of non-muscle myosin II and other myosins, and of dynein in granule translocation. Understanding these mechanisms is fundamental to comprehending the functioning of pigment cells and color change mechanisms in invertebrates in general and their homologies with vertebrate pigment cells.

2. Material and methods

2.1. Ethical procedures

Collection of animals, their maintenance in the laboratory and use were authorized by the relevant Brazilian federal agency, the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA/DIREN) under permits #070/2004 and #18559-1 to JCM.

Female freshwater shrimp, *Macrobrachium olfersi* (Weigmann 1836), possessing immature ovaries with red chromatophores visible in the fibrous ovarian capsule, were collected by sieving the marginal vegetation of the Paúba River (23° 47′ 51.30″ S; 45° 32′ 33.61″ W), São Paulo State, Brazil. This species is neither endangered nor protected. The shrimps were transported to the laboratory in 30-L carboys containing aerated water from the collection site where they were maintained at room temperature (23 °C) in 80-L tanks furnished with aerated Paúba River or local spring water. The shrimps were fed diced beef, carrot and beetroot on alternate days.

2.2. Immunocytochemistry: epifluorescence and confocal microscopy

For preparations requiring maximum red pigment dispersion within the chromatophores, the shrimps were held on a black background for 2 h before dissection. The intact ovaries were dissected and fixed/permeabilized in sodium biphosphate buffer (PBS; 100 mmol L^{-1} NaH_2PO_4 and 100 mmol L^{-1} Na_2HPO_4, pH 7.4), containing 4% *p*-formaldehyde (Sigma-Aldrich, USA) and 0.3% Triton X-100 (Sigma-Aldrich, USA) for 15 min at room temperature (23 °C). The ovarian preparations were rinsed in 100 mmol L^{-1} glycine in PBS for 5 min, then incubated at room temperature for 1 h in PBS containing 0.1% Triton X-100, 2% bovine serum albumin and 5% goat serum (Sigma-Aldrich, USA) to block non-specific antibody binding.

The whole ovaries were then incubated with their respective primary antibodies in PBS [mouse monoclonal anti-β-tubulin, diluted 1:100 (Sigma-Aldrich, USA); rabbit polyclonal anti-skeletal and smooth muscle myosin, 1:50 (Sigma-Aldrich, USA); polyclonal anti-non-muscle myosin II, 1:100 (a gift from Prof. Roy Larson, FMRP); and monoclonal anti-dynein heavy chains, 1:100 (Sigma-Aldrich, USA)] for 48 h at 4 °C, rinsed in PBS and incubated for 3 h with their respective Alexa Fluor 488-conjugated secondary antibodies (goat anti-rabbit IgG or goat anti-mouse IgG, diluted 1:400, Life Technologies, USA, gifts from Prof. Roy Larson, FMRP) at room temperature. They were then re-fixed in 4% *p*-formaldehyde in PBS for 15 min when necessary. The whole ovaries were carefully bisected in PBS with ophthalmic scissors, and the fine ovarian capsules containing the red chromatophores were separated from the oocytes, mounted on glass slides with Vectashield Mounting Medium and covered with 0.25 mm Knítel Glaser cover slips. Slides were stored in the dark at 4 °C.

Negative control preparations lacking primary antibodies were prepared using ovarian capsules containing chromatosomes. These capsules were incubated with either goat anti-mouse Alexa Fluor 488- or goat anti-rabbit Alexa Fluor 488-conjugated secondary antibodies. Such preparations showed no significant labeling (Supplementary Fig. 1). These red pigments absorb light at wavelengths between 400 and 600 nm, with peak absorbance at 500 nm and peak emission at 568 nm (Boyle, 2005).

Phalloidin-fluorescein isothiocyanate (phalloidin-FITC) (495/520 nm, Sigma-Aldrich, USA) (Wehland et al., 1977) was used to label actin microfilaments in the chromatophores. After fixing and permeabilizing as above, whole ovaries were also incubated for 3 h in 300 nmol L^{-1} phalloidin-FITC in PBS at room temperature. Their ovarian capsules were then dissected and mounted as described above.