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Research Article

ROLE OF Ca^{2+} IN PIGMENT AGGREGATION AND DISPERSION IN THREE SPECIES OF FISH, *PUNTIUS*

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A R T I C L E I N F O

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A B S T R A C T

The roles of calcium in cell signaling consequent to chromatophoros in melanophores action and as an activator of mechanochemical transport proteins responsible for pigment granule translocation were investigated in the freshwater fish Puntius species In the present study, the pigment aggregating action induced by K^+ was completely inhibited in Ca^{2+}. The scales were first treated with CFR (calcium free ringer solution), the completely disperses state were found. When CFK (calcium free K^+ ringer) was added, fully aggregation stages were found. These aggregation dispersion stages were going on at about 2 hrs then after the process were stopped. Varapamil blocked the role of Ca^{2+}which produce their effect by binding to the α_1-subunit of the L-type Ca^{2+}channels and reducing Ca^{2+} flux through the channel. These data reveal an extracellular and an intracellular Ca^{2+}, and demonstrate that the centripetal or centrifugal direction of pigment movement, the translocation velocity, and the degree of pigment aggregation or dispersion attained are calcium-dependent properties of the granule translocation apparatus. The increase in Ca^{2+}may induce membrane depolarization of presynaptic nervous elements around the melanophores, which open the voltage-dependent Ca^{2+}channels. The liberation of adrenergic neurotransmitter follows, which induces the aggregation of pigment in melanophores

INTRODUCTION

Ca^{2+} is the major extra-cellular divalent cation. The normal adult man and women possess about 1300 and 1000 gm of Ca^{2+} respectively, of which more than 99 % is in bone. Ca^{2+} is present in small amount in extra-cellular fluids and to a minor extent within cells, where its ionized concentration under condition is about 0.1 цm. Ca^{2+} is essential for many important process including neural excitability, neurotransmitter release, muscular concentration, membrane integrity and blood coagulation. In addition, Ca^{2+} serve as second messenger function for the action of many hormones, an increased concentration of cytosolic Ca^{2+} caused increased concentration in cardiac and vascular smooth muscles cells. In addition to their primary role of revealing integumentary color, chromatophores of many species of animals including fish take dynamic part in the changes of these colors and pattern (Fujii, 1993). In fish, most chromatophores one dendritic cells and a number of processes emanate from their cell bodies parallel to the plane of the skin. Pigmentary organelles (termed chromatosomes) migrate centripetally into the perikarya (aggregation), or centrifugally (dispersion) in response to various signals, mostly brought about by sympathetic fibers or blood born hormones (Fujii, 1993; Fujii and Oshima, 1994).

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Voltage sensitive Ca^{2+} channel has been divided into at least three subtype based on the conductance and sensitivities to voltage (Tsien *et al.,* 1982). Large divalent cation such as $Ca^{2+}$and Mn^{2+} block a wider range of Ca^{2+} Channels. All approved Ca^{2+} bind to α1-subunit of L-type Ca^{2+} channel, which is main performing unit of the channel. Ca^{2+} antagonist also called Ca^{2+} entry blocker inhibits Ca^{2+} channel function. As reviewd by Blaustein (1985), the aspect of Ca^{2+} metabolism that enable thus cation to serve as on effective second messenger include: Ca^{2+} entry into the cell via voltage regulated or chemical transmitter activated channels through other pathway or "nerve mode" Na^{2+}-Ca^{2+} exchange.

The effect of changing ionic environment around the chromatophores have been infrequently investigated (Speath 1913; 1916; Yamamoto, 1933; Kamada and Kinoshita 1944; Fujii 1959; Iwata *et al.,* 1959). In relation to nervous system controlling fish chromatophores (Fujii, 1965) was reported that $Ca^{2+}$is required for catcholamine release from the sympathetic nerve terminals in the goby, *Chasmichthys gobius.* At up to the present time to sound explanation have been put forward about its effect or its mechanism of action, although the intra-cellular role of Ca^{2+} has some time been examined in some teleostean species (Luby-Phelps and Porter, 1982; Negieshi and Obika 1985; Oshima *et al*., 1988, Opsina *et al.*, 1998; Toyohara and Fujii, 1992; Kots and McNiven, 1994, Patil and Jain, 1993, Yamada and Fujii, 2002). In the present paper, we investigated

the role of Ca^{2+} in motile regulation of melanophores in fish *Puntius species*.

MATERIAL AND METHOD

The *puntius species* (Pun*tius sophore, Puntius conchonius, Puntius ticto*) were used as the experimental material. The fish were collected from "Tighra reservoir" 23 km. from Gwalior (M.P). They were stocked in transparent glass aquarium containing fresh aerated water. Prior to use they were maintained in a fresh water aquarium for at least one weak for acclimatization. The scale slips were gently plucked by mean of fine forceps from the dorsal trunk surface of the animal. The isolated scale were immediately immersed in a physiological saline solution which had the following composition in mm (NaCl; 128.3, KCl; 2.8, Glucose; 5.6; $CaCl_2$; 1.8, 0.5 M Hepes-NaoH with pH value 7.4).

The K^+ rich saline was also used to prepare it an equimolar solution of KCl was substituted for NaCl in the PS. Ca^{2+} free ringer was prepare by withdrawing $CaCl_2$ and adding EDTA 0.2 mm to the recipe of the PS. Similarly Ca^{2+} free K^+ (CFK^+) was prepared by withdrawing $CaCl_2$ and adding EDTA 0.2 mm to the recipe of the K^+ rich saline. The effect of drug on the response of certain groups of melanophores were studied with light microscope and were evaluated according to Hogben and slome (1931) in amphibian melanophores where 1, representing the maximum aggregation and 5, representing maximum dispersion and 2, 3, 4 as inter-mediate stage of aggregation/ dispersion (Fig-1).

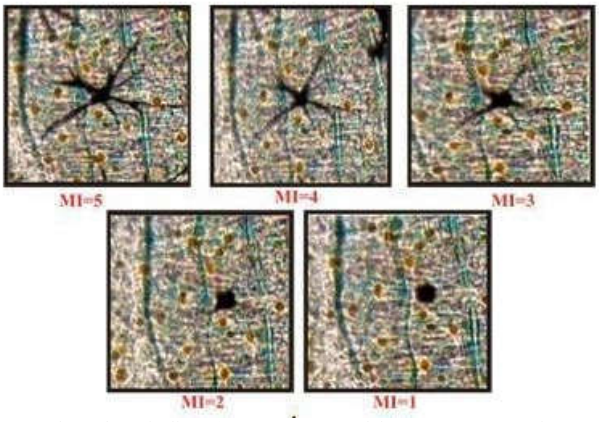
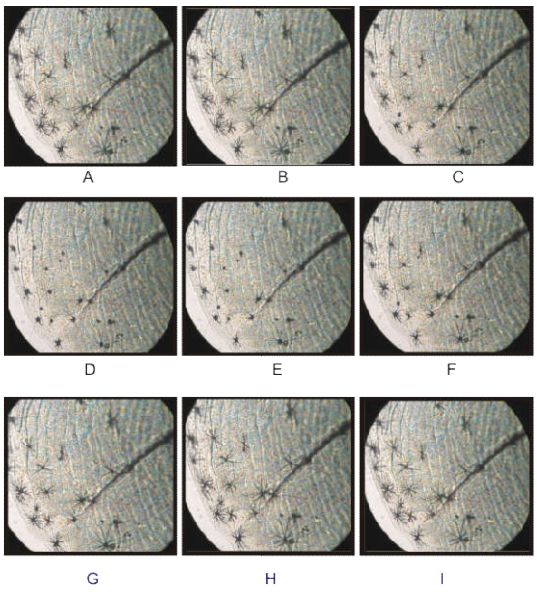


Fig 1 Melanophore indices (5-1) as were used for measurement of melanophore responses in the study

OBSERVATION AND RESULT

As in many other teleostean species, equilibrataion in normal physiological saline of a scale from the Puntius species brought about the dispersion of melanophores within the melanophores.When the skin preparation was perfused with Ca^{2+}-free Ringer solution (CFR), the melanophores remained in complete dispersion stage. When solution was substituted with CFK, melanosomes fully aggregated in melanophores within 5 min and the re-dispersion of pigment can be distinctly seen while the scale was still in the Ca^{2+} free saline. Such a typical response is seen in serial photomicrograph show in Fig-2 where a scale from a *Puntius species* was employed.

Fig-2 Serial photomicrographs (A-F) of the same field showing motile responses of melanophores on an isolated scale of the fish, *Puntius conchonius*, viewed from the dermal side. (A) Equilibrated in PS, melanophores with dispersed melanosomes are visible; (B) 3 min after the application of CFK^+, melanosomes are almost completely aggregated; (C-E) 5,10 min after the application of CFR respectively the melanosomes in all the melanophores are gradually dispersing. At 10 min stage, melanosomes are completely dispersed in the cells (F).



When the skin preparation was perfused with Ca^{2+}-free Ringer solution (CFR), the melanophores remained in complete dispersion stage. When solution was substituted with CFK, melanosomes fully aggregated in melanophores within 5 min. These processes *i.e.,* melanophore aggregation by CFK^+ and redispersion by CFR were repeatable upto 50-55 min in all the studied fish. Upto this time there was no sign of fatigue on this stimulation (Fig.-3). Thereafter, the magnitude of response declined and then melanophores were refractory to K^+ stimulation.

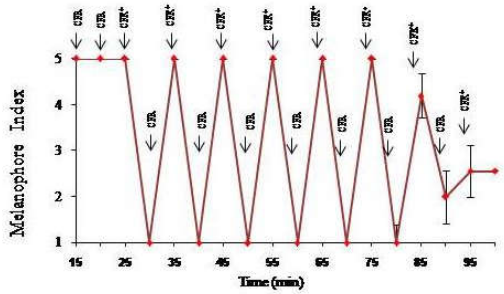


Fig 3 Effect of CFR and CFK^+ in the PS equilibrated isolated scale melanophores of the fish, *Puntius*. The vertical lines show the standard deviation.

Verapamil is a calcium channel blocking agent (also termed Ca^{2+} antagonist) which belongs to the chemical class of phenylalkylamines. The inhibitory action of verapamil depended on its concentrations (10^{-6} to 10^{-4}M), for 60 min incubation in the perfusing medium. The inhibitory effect was partial at 10^{-6}M while melanosome aggregation induced by CFK^+ was completely inhibited by the drug at 10^{-4}M (Fig-4).

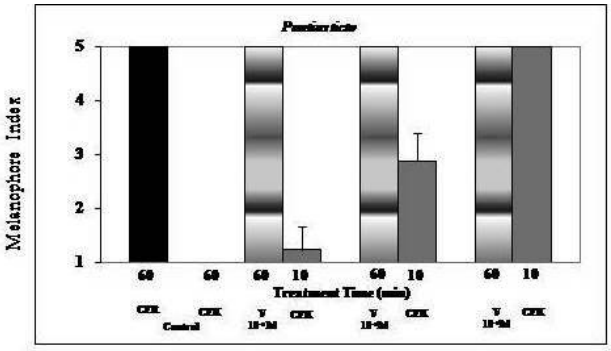


Fig 4 The effect of different concentrations (10^{-6} to 10^{-4}M) of Ca^{2+} antagonist, verapamil on the action of CFK^{+}. Central represents the response of CFR equilibrated melanophores to CFK^{+}. Each point is the mean of 25 measurments (5 animals). Perpendiculars drawn indicate S.D.

DISCUSSION

The entry of extra-cellular Ca^{2+} is more important in initiating the concentration of cardiac myocytes (Ca^{2+} induced Ca^{2+} release). The release of Ca^{2+} from intracellular storage sites also contributes to concentration of vascular smooth muscles particularly in some vascular beds. Cytosolic Ca^{2+} concentration may hormones and neurohormones increase Ca^{2+} influx through so called receptor- operated channels, whereas high external concentration of K^{+} and depolarizing electrical stimuli increase Ca^{2+} influx through voltage sensitive or potential operated channels. The Ca^{2+} channel antagonists produce their effect by binding to the α_1-subunit of the L-type Ca^{2+}channels and reducing Ca^{2+} flux through the channel. According to Schwartz, 1992 Ca^{2+} is a trigger for concentration, albeit by different mechanism and Ca^{2+} channels contain several other associated subunit (α, β, γ, δ). The melanosome-aggregating action of elevated concentrations of K^{+} or other cations had been thought to be due to their direct action on the melanophores. Indeed, it was rather natural to think so when one recollects the "potassium contracture" that is ubiquitously observed among striated and smooth muscle tissues. It is now well known that the depolarization of the effectors cell membrane due to the effect of high $[K^{+}]o$ leads to the muscular contraction via the elevation of intracellular levels of Ca^{2+} ions. Primarily based on observations that denervated melanophores were unresponsive to pigment-aggregating ions, and that the liberation of the sympathetic neurotransmitter was certainly involved in the action on innervated cells, Fujii (1959) first showed that these ions do not act directly on melanophores but rather they act on presynaptic elements of sympathetic postganglionic fibers to release the neurotransmitter. The liberated transmitter then brings about the aggregation of pigment in the melanophore. Working on the crucian carp, *Carassius auratu*s, Iwata *et al*. (1959a) soon came to the same conclusion as had Fujii. 1993). Our goal of the present work was to elucidate whether the mechanism of pigment aggregation in response to an increase in extracellular $[Ca^{2+}]o$ is identical to that of the action of increasing $[K^{+}]o$.

Adding further evidence for this concept, Fujii (1961) then showed that the effect of K^{+} ions could be blocked by treating the skin pieces with an adrenergic blocking agent, dibenamine. The present understanding f the process is that the depolarization due to the heightened $[K^{+}]o$ of the presynaptic membrane opens the voltage-dependent Ca^{2+} channels there. The resultant increase in the cytosolic level of Ca^{2+} ions triggers the exocytotic release of the neurotransmitter which finally signals the aggregation of melanosomes in the effector cells (Fujii, 2000). It is now known that various pigment-aggregating or dispersing substances retain their effects even in K^{+}-rich saline, when melanophores have previously been denervated, or when the release of the transmitter was inhibited (Fujii and Taguchi, 1969; cf. also Fujii, 1993; Fujii and Oshima, 1994). In other words, the motile responses of melanophores seem to be quite independent of the electrical potential across the membrane or of electrical activities there, since under K+-rich conditions, the membrane should be almost completely depolarized. The conclusion supports the current view that the stimulation of pigment-motor receptors in the cell membrane is transduced via G-proteins, which lead to changes in the intracellular levels of second messengers involved. Ionic channels in the cell membrane, which are directly involved with changes in potential across the membrane, as well as those which are voltage-dependent, seem not to be involved in the process of signal transduction in melanophores (Fujii, 1993). Nevertheless, an increase in $[K^{+}]o$ induces the aggregation of pigment in melanophores. As already mentioned above, that action results from the membrane depolarization of presynaptic nervous elements due to the elevated level of $[K^{+}]o$ which gives rise to the entry of Ca^{2+} ions through the voltage-dependent Ca^{2+} channels there, which then results in the release of the sympathetic neurotransmitter. It is well known that changes in the level of intracellular Ca^{2+} ions are critically involved in the motile activities of many types of cells. Chromatophores may not be an exception (cf. Fujii, 1993; Fujii and Oshima, 1994). For example, Luby-Phelps and Porter (1982) showed that the aggregation of pigmentary organelles in erythrophores of the squirrelfish, *Holocentrus ascensionis*, depends on extracellular Ca^{2+} ions, and that their inflow triggers the centripetal displacement of the pigment by increasing the intracellular level of Ca^{2+} ions. Kotz and McNiven (1994), working on erythrophores of the same squirrelfish, showed recently that in addition to low $[Ca^{2+}]i$, high [cyclic AMP]i is necessary to induce the dispersion of erythrosomes in the cells. Working on melanophores of the medaka, *Oryzias latipes*, Negishi and Obika (1985) came to the same conclusion. Using calcium probes, Oshima *et al*. (1988) showed that Ca^{2+} may function to elicit the aggregation of melanosomes in melanophores of the tilapia in culture. Fujii *et al*. (1991) then showed that inositol 1,4,5-trisphosphate acts as a second messenger, in addition to cyclic AMP, which acts via the release of Ca^{2+} from elements of the smooth endoplasmic reticulum to aggregate melanosomes. Having developed a method to simultaneously record motile responses and intracellular changes of Ca^{2+} ions, Toyohara and Fujii (1992) recently succeeded in showing a correlation between the Ca^{2+} increase and the aggregation of melanosomes in tilapia melanophores in culture. Thus, it is quite certain that in many teleost chromatophores the increase in $[Ca^{2+}]$ is deeply involved in the aggregation of pigmentary organelles. In this way, current studies on the functions on Ca^{2+} ions have mostly been concerned with the intracellular involvement of Ca^{2+} ions during pigment aggregation in chromatophores. In contrast, the present study deals with the effects of Ca^{2+} in the media outside the cells: The elevation of $[Ca^{2+}]o$ gave rise to the aggregation of pigment in melanophores of all three species of teleosts examined, and denervated melanophores were refractory to that $[Ca^{2+}]o$ increase. The response was completely blocked by adrenergic α-adrenergic blockers, phentolamine, yohimbine and prazosin,

and by bretylium (an inhibitor of the release of the neurotransmitter from adrenergic fibers). It was further shown that Ca^{2+} blockers including Mn2+, verapamil and gallopamil, also blocked the response. These results led us to conclude that the effect of increasing $[Ca^{2+}]o$ is not on the melanophores themselves, but on the nervous elements that control the aggregation of melanosomes.

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