The Zoological Society of Japan

ZOOLOGICAL SCIENCE 2: 317–322 (1985)

© 1985 Zoological Society of Japan

Mechanisms Controlling Motile Responses of Amelanotic Melanophores in the Medaka, *Oryzias latipes*

MASAZUMI SUGIMOTO, NORIKO OSHIMA and RYOZO FUJII[1]

Department of Biology, Faculty of Science, Toho University, Funabashi, Chiba 274, Japan

ABSTRACT—Using light-colored varieties of the medaka, *Oryzias latipes*, we have studied the mechanisms by which the aggregation and dispersion of premelanosomes within amelanotic melanophores are controlled. An alpha-adrenergic agonist (norepinephrine), melatonin, melanin-concentrating hormone (MCH), and K ions gave rise to the aggregation of the cellular inclusions, while a beta-agonist (isoxsuprine), adenosine, alpha-melanophore-stimulating hormone (alpha-MSH) and atropine were effective in dispersing them. Acetylcholine was without effects. Pharmacological analyses on the mode of action of these agents indicate that, like other usual motile chromatophores, the amelanotic melanophores are motile, and further that they are regulated in the same way as the chromatophores of common teleosts, comprizing the melanophores of the wild-type specimens of the present species, *Oryzias latipes*. Implication of the presence of the sound but purposeless controlling system for those colorless chromatophores was discussed.

INTRODUCTION

Hishida *et al.* [1] first demonstrated that the so-called "colorless" or "amelanotic" melanophores are present in the skin of lightly colored varieties of the medaka, *Oryzias latipes*. Later, Oikawa [2] showed that, like most colored chromatophores, these cells are motile, namely, the cellular inclusions can migrate centripetally into the perikarya or centrifugally into the dendritic processes in response to epinephrine or to atropine, respectively.

As for the regulatory mechanisms for colored pigment cells of vertebrates, much information has accumulated up to the present time, especially on those for the melanophores of teleosts [3, 4]: The melanophores are primarily controlled by the sympathetic system through the liberation of the neurotransmitter, norepinephrine, which acts to aggregate the pigmentary inclusions, the melanosomes [5, 6]. In addition, the cellular movements are influenced humorally by a pineal principle, melatonin [5, 7], and further by pituitary ones, i.e., melanophore-stimulating hormone (MSH) [3, 8] and possibly melanin-concentrating hormone (MCH) [9–11].

Until recently, other sorts of motile chromatophores, i.e., xanthophores, erythrophores and leucophores, have also become known to be similarly controlled by the sympathetics and/or the endocrines [3, 4, 12–15]. No trials, however, have ever been made to clarify the mechanisms by which the motility of the amelanotic melanophores are controlled. In the present study, therefore, we have tried to elucidate the problem.

MATERIALS AND METHODS

Two varieties of the medaka, *Oryzias latipes*, i.e., orange-red (*bR*) and white (*br*), of both sexes were used as the experimental materials. The adult specimens of the former variety were obtained from a commercial source, while those of the latter were given by Dr. S. Kikuchi of the Chiba University.

Scales on the dorsal trunk were isolated in a physiological saline solution, which had the following composition (mM): NaCl 128, KCl 2.7, $CaCl_2$

Accepted January 8, 1985
Received December 26, 1984

[1] To whom reprints should be requested.

NII-Electronic Library Service

The Zoological Society of Japan

1.8, $MgCl_2$ 1.8, D-glucose 5.6, Tris-HCl buffer 5.0 (pH 7.2). The perfusing system employed for observing and measuring the responses of pigment cells were essentially the same as that described elsewhere [16].

No darkly pigmented cells were found in the integumental tissues in either variety used. Instead, amelanotic melanophores were observable by carefully surveying the granular feature of their cellular inclusions, the premelanosomes, even through the ordinary light microscope. That those cells were indeed amelanotic melanophores was ascertained by the method described elsewhere [2].

The aggregation into the perikarya of these organelles caused clearing of peripheral parts of the cells. Thus, the photoelectric method for recording chromatophore responses could be applied upon condition that a proper photosensor and appropriate electronic devices were employed [17]. In the present study, a photomultiplier tube (931A, Hamamatsu Photonics, Hamamatsu) was adopted as the transducer. By restricting the area of the skin to be measured of the light transmittance to be 150 μm in diameter, the response of a single amelanotic melanophore could usually be assessed. Care was taken in selecting the cell, around which no chromatophores of other sorts, i.e., leucophores and xanthophores, were present, especially when we used the scales of the orange-red variety, in order not to pick up the motile activities of xanthophores. The motile responses were registered on a paper-chart recorder (EPR-10B, or EPR-221A, Toa Electronics, Tokyo).

Distribution of the premelanosomes in live cells could be observed more clearly by employing the Nomarski differential interference optics of transmission type. In some experiments, therefore, the motile responses of amelanotic melanophores were observed and photographed with an Olympus (Tokyo) BHA-LB-N microscope.

Electrical nervous stimulation was done by putting the skin preparation in the A.C. (50 Hz) field [18]. In order to stimulate chemically the nervous elements around the chromatophores, an elevated K^+ concentration was used [19, 20]. For this purpose, the saline containing 50 mM K^+ was used, in which the concentration of Na^+ was compensatorily decreased to keep the tonicity of the solution constant.

In experiments in which the effects of chromatosome-dispersing agents were studied, we employed the scales taken from the fish which had been reared in reserpine (3×10^{-6}M; Nakarai Chemicals, Kyoto) solution made up in tap water for 12–24 hr [21].

Other drugs used in the present study included: norepinephrine hydrochloride (Sankyo, Tokyo), isoxsuprine hydrochloride (Daiichi Seiyaku, Tokyo), acetylcholine chloride (Daiichi Seiyaku), melatonin (Nakarai Chemicals), phentolamine mesylate (Ciba-Geigy, Basel), propranolol hydrochloride (ICI, St. Louis), adenosine (Kohjin, Tokyo) and synthetic alpha-melanophore-stimulating hormone (alpha-MSH, Sigma Chemical, St. Louis). Synthetic chum salmon melanin-concentrating hormone (MCH) was the gift from Dr. M. E. Hadley of the University of Arizona, which was synthesized by Dr. B. C. Wilkes [10], Department of Chemistry of the same University.

All measurements were performed at a room temperature between 17 and 25°C.

RESULTS

Carefully observing the dermal tissue in the scale, we could find amelanotic melanophores even through the ordinary light microscope. By employing the Nomarski optics, however, those cells were more clearly observable, and the motile responses to norepinephrine of a few chromatophore species including those amelanotic cells are demonstrated in Figure 1. The series of photomicrographs clearly indicate that in the amelanotic melanophores the premelanosomes migrated from the dendritic processes into the perikaryon just in the same manner as the melanosomes within the melanophore of many common teleost species, and further that the configuration of these cells resembled well that of the melanophores of the wild-type specimens of *Oryzias*.

A.C. field stimulation and K^+-rich saline, both being known to stimulate the chromatic nervous elements to liberate the neurotransmitter [5, 6, 19, 20], induced the aggregation of premelanosomes within the amelanotic melanophores. In the left

NII-Electronic Library Service

The Zoological Society of Japan

FIG. 1. Sequence of photomicrographs showing motile responses of chromatophores in the dermis of the medaka, *Oryzias latipes*. Nomarski optics was employed. In this field, two amelanotic melanophores (AM), two xanthophores (X) and a leucophore (L) are included. Among them, an amelanotic melanophore and a leucophore in the lower right part lie one upon another. a: Equilibrated in physiological saline. Premelanosomes in the amelanotic melanophores were in a dispersed state. b: 75 sec after the application of 5×10^{-6} M norepinephrine. Premelanosomes were on the way to aggregation. c: After 120 sec. Premelanosomes were maximally aggregated into the perikaryon. ×180.

part of Figure 2, the neurally evoked response to an elevated K^+ concentration (50 mM) is exhibited. Further, norepinephrine of moderate strengths gave rise to a complete aggregation of the cellular inclusions. The action of these chromatosome-aggregating agents could easily be antagonized by the alpha adrenolytics, i.e., phentolamine and phenoxybenzamine. The inhibitory effect of phentolamine on the K^+ action is typically shown in Figure 2. On the other hand, acetylcholine, which is known to induce strongly the pigment aggregation in melanophores of some teleosts [22, 23], lacked the effect. It is therefore evident that the peripheral nerve-fibers controlling chromatosome aggregation in the amelanotic melanophores are quite orthodoxly adrenergic.

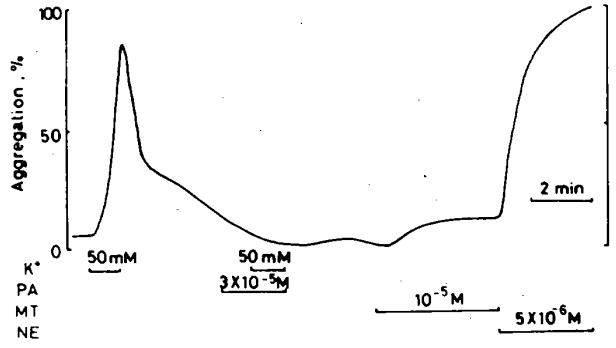

FIG. 2. Typical photoelectric recording showing the responses of an *Oryzias* amelanotic melanophore to K^+, melatonin (MT) and norepinephrine (NE). The effect of an alpha adrenolytic agent, phentolamine (PA), on the K^+ action is also exhibited in the central part of the figure.

Although not shown in the figure, melanin-concentrating hormone (MCH) was very effective in aggregating the premelanosomes within these cells. On the other hand, the premelanosome-aggregating action of melatonin was rather different from that of norepinephrine or of MCH. That is, the action was rather weak (cf. Fig. 2), and the effect was quite differential among the effector cells [7].

By making use of their moderate pigment-aggregating action, tolazoline and melatonin have been conveniently employed in the studies, in which various substances were tested for their possible melanosome-dispersing action [8, 21]. In the present materials, however, the former failed to induce the premelanosome aggregation within the cells, while the latter had only a weak action as stated above. So, the scales excised from the fish previously treated with reserpine was employed for this purpose [21]. It was clearly shown that a beta adrenergic agonist, isoxsuprine, was effective in inducing premelanosome dispersal (Fig. 3). As can be seen in the figure, the effect of the agonist could easily be antagonized by propranolol, a beta adrenergic blocking agent.

Adenosine, which has recently been shown to be the co-transmitter of the adrenergic postganglionic fibers to the melanophores of some teleosts [6, 24], was also effective in dispersing the premelanosomes (Fig. 4). Alpha-MSH, the intermediate lobe principle, also exhibited remarkable activity to disperse the cellular inclusions (Fig. 4). In addi-

NII-Electronic Library Service

The Zoological Society of Japan

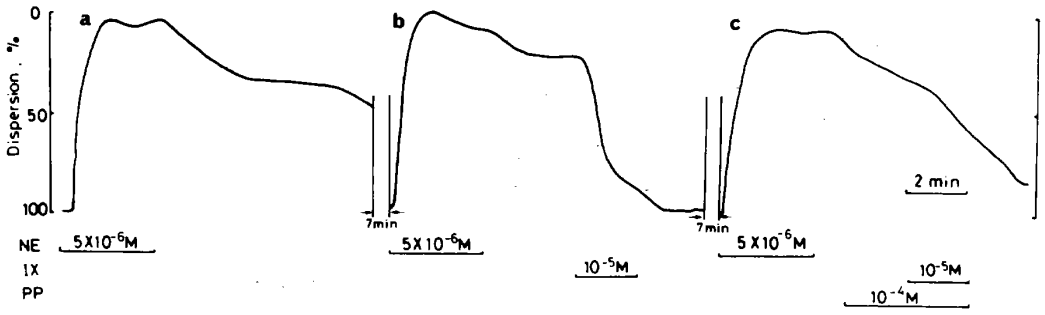

FIG. 3. Serial recordings obtained on an amelanotic melanophore to show the effect of a beta-adrenergic agonist, isoxsuprine (IX). a: Passive pigment dispersion in physiological saline following 3-min treatment with 5×10^{-6} M norepinephrine (NE). b: Premelanosome dispersion in response to 10^{-5} M isoxsuprine. c: Antagonistic effect of propranolol (PP), a beta blocker, on the action of isoxsuprine.

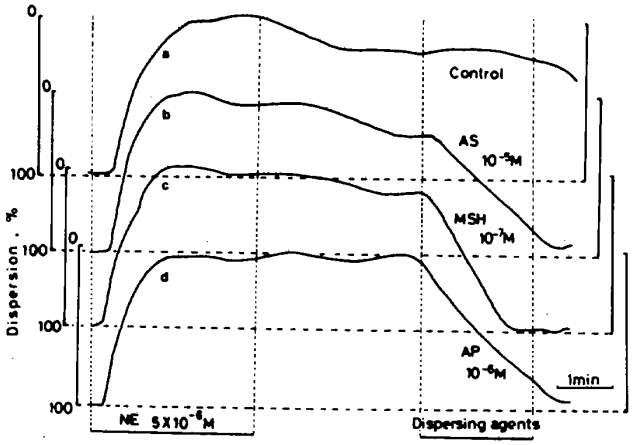

FIG. 4. Photoelectric recordings successively obtained on an amelanotic melanophore to show the premelanosome-dispersing action of some substances known to induce pigment dispersion in many chromatophore species. In each recording, the response to a test substance is preceded by a 3-min treatment with 5×10^{-6} M norepinephrine, which brought about the chromatosome aggregation almost completely. a: Passive gradual premelanosome dispersion in physiological saline. b: Premelanosome dispersion in 10^{-5} M adenosine (AS). c: Premelanosome dispersion in 10^{-7} M alpha-MSH. d: Premelanosome dispersion in 10^{-6} M atropine.

tion, it was ascertained that atropine, a cholinergic blocker of muscarinic type, strongly dispersed the premelanosomes (Fig. 4).

DISCUSSION

The present results have clearly shown that the amelanotic melanophores existing in pale varieties of the medaka are motile, and that they are controlled in the same way as the usual melanophores of the wild type or dark varieties of the same species of fish, and further as those in many other teleost species [3–8], notwithstanding the fact that the organelles of the present materials are practically colorless. That is, the sympathetic post-ganglionic fibers are certainly responsible for the rapid aggregation of the chromatosomes, like in chromatophores of usual sorts. It has also been shown that alpha adrenoceptors are involved in mediating the response. Further, possible involvement of adenosine or adenine nucleotides as the co-transmitter antagonizing the effect of the sympathetic true transmitter, norepinephrine [6, 24], has also been strongly suggested.

Positive effects of alpha-MSH, MCH and melatonin on those clear cells further indicate the possible participation of these hormonal substances in their motile responses, although the response rates to those endocrines should be much lower than those seen in the neural regulation.

That such colorless chromatophores are motile, and further regulated in the same manner as the usual pigmented chromatophores seems to be quite strange, if we come to think of it, because their motile activities must be practically meaningless in fulfilling part in the integumentary color change phenomena. Thus, we should consider these situations.

In the amelanotic melanophores, the process of melanization is believed to be blocked by the inhibition of tyrosinase activity [1, 2]. As a result, the vehicles for the black pigment, melanin, are vacant, being in contrast with mature melanized ones, the melanosomes, present in normal melano-

NII-Electronic Library Service

The Zoological Society of Japan

phores. As mentioned above, we have found in the present study that the motor systems for those unpigmented melanophores are functioning quite normally as those for the pigmented cells of the dark specimens. We presume here that the systems themselves may have been remaining unchanged ever since the time of the mutational change or the genetic transformation causing the blockade of tyrosinase activity, occurred many years ago in the wild-type individual(s). The presence of the sound, but meaningless regulatory systems may thus be smoothly understood. If the enzyme action is restored spontaneously or artificially by some means, therefore, the melanized cells can easily be supposed to be controlled quite normally like the melanophores found in wild forms or those of the recent dark varieties.

The present results may also deny the possibility that the presence of the innervation naturally leads to the induction of pigmentation or melanization in the teleostean chromatic effector cells: Even in the presence of the normal neural controlling system, the pigmentation has not developed at all in those cells. The reason why we want to discuss such a problem here is that nowadays dermatologists assume that human vitiligo, i.e., local depigmentation frequently seen in skin, might be caused by the defects in nervous supply [25]. By way of the side remark, the chromatic innervation mentioned above is of course the adrenergic motor one in this species of fish, but we like to consider it to include a putative "trophic" innervation, since the nervous supply of the latter sort has frequently been claimed to be deeply concerned with maintaining effector cells healthy and motile, and further responsive to regulatory agencies. In any case, further studies are naturally needed for understanding the chromatic as well as achromatic phenomena seen among those less investigated effector systems.

ACKNOWLEDGMENTS

We thank Dr. S. Kikuchi, Department of Biology, Faculty of Science, Chiba University, for providing us with white-variety specimens of the medaka, and Dr. M. E. Hadley, Department of Anatomy, University of Arizona, for the gift of synthetic salmon MCH. This work was aided by grants from the Ministry of Education, Science and Culture of Japan, and also by a grant from the Ito Foundation for the Promotion of Ichthyological Research.

REFERENCES

1 Hishida, T., Tomita, H. and Yamamoto, T. (1961) Melanin formation in color varieties of the medaka (*Oryzias latipes*). Embryologia, **5**: 335–346.

2 Oikawa, T. (1971) Histochemical and physiological study of chromaffin cells in the skin of the medaka, *Oryzias latipes*. Dev. Growth Differ., **13**: 125–130.

3 Pickford, G. E. and Atz, J. W. (1957) The Physiology of the Pituitary Gland of Fishes, New York Zool. Soc., New York.

4 Fujii, R. (1969) Chromatophores and pigments. In "Fish Physiology". Ed. by W. S. Hoar and D. J. Randall, Vol. 3, Academic Press, New York, pp. 307–353.

5 Fujii, R. (1961) Demonstration of the adrenergic nature of transmission at the junction between melanophore-concentrating nerve and melanophore in bony fish. J. Fac. Sci. Univ. Tokyo, Sect. IV, **9**: 171–196.

6 Kumazawa, T. and Fujii, R. (1984) Concurrent releases of norepinephrine and purines by potassium from adrenergic melanosome-aggregating nerve in tilapia. Comp. Biochem. Physiol., **78C**: 263–266.

7 Fujii, R. and Miyashita, Y. (1978) Receptor mechanisms in fish chromatophores—IV. Effects of melatonin and related substances on dermal and epidermal melanophores of the siluroid, *Parasilurus asotus*. Comp. Biochem. Physiol., **59C**: 59–63.

8 Fujii, R. and Miyashita, Y. (1982) Receptor mechanisms in fish chromatophores—V. MSH disperses melanosomes in both dermal and epidermal melanophores of a catfish (*Parasilurus asotus*). Comp. Biochem. Physiol., **71C**: 1–6.

9 Kawauchi, H., Kawazoe, I., Tsubokawa, M., Kishida, M. and Baker, B. I. (1983) Characterization of melanin-concentrating hormone in chum salmon pituitaries. Nature, **305**: 321–323.

10 Wilkes, B. C., Hruby, V. J., Castrucci, A. M. L., Sherbrooke, W. C. and Hadley, M. E. (1984) Synthesis of a cyclic melanotropic peptide exhibiting both melanin-concentrating and -dispersing activities. Science, **224**: 1111–1113.

11 Oshima, N., Kasukawa, H., Fujii, R., Wilkes, B. C., Hruby, V. J., Castrucci, A. M. L. and Hadley, M. E. (1985) Melanin concentrating hormone (MCH) effects on teleost (*Chrysiptera cyanea*) melanophores. J. Exp. Zool., in press.

12 Matsumoto, J., Watanabe, Y., Obika, M. and Hadley, M. E. (1978) Mechanisms controlling pigment movements within swordtail (*Xiphophorus helleri*)

NII-Electronic Library Service

The Zoological Society of Japan

erythrophores in primary culture. Comp. Biochem. Physiol., **61A**: 509–517.

13 Iga, T., Yamada, K. and Iwakiri, M. (1977) Adrenergic receptors mediating pigment dispersion in leucophores of a teleost, *Oryzias latipes*. Mem. Fac. Lit. Sci. Shimane Univ. Nat. Sci., **11**: 63–72.

14 Yamada, K. (1982) Sulfhydryl requirement for action of melanophore stimulating hormone on fish leucophores. J. Sci. Hiroshima Univ., Ser. B, Div. 1, **30**: 201–211.

15 Oshima, N. and Fujii, R. (1985) Calcium requirement for MSH action on non-melanophoral chromatophores of some teleosts. Zool. Sci., **2**: 127–129.

16 Fujii, R. and Miyashita, Y. (1975) Receptor mechanisms in fish chromatophores—I. Alpha nature of adrenoceptors mediating melanosome aggregation in guppy melanophores. Comp. Biochem. Physiol., **51C**: 171–178.

17 Oshima, N. and Fujii, R. (1984) A precision photoelectric method for recording chromatophore responses *in vitro*. Zool. Sci., **1**: 545–552.

18 Fujii, R. and Novales, R. R. (1968) Tetrodotoxin: effects on fish and frog melanophores. Science, **160**: 1123–1124.

19 Fujii, R. (1959) Mechanism of ionic action in the melanophore system of fish—I. Melanophore-concentrating action of potassium and some other ions. Annot. Zool. Japon., **32**: 47–58.

20 Iwata, K. S., Watanabe, M. and Nagao, K. (1959) The mode of action of pigment concentrating agents on the melanophores in an isolated fish scale. Biol. J. Okayama Univ., **5**: 195–206.

21 Miyashita, Y. and Fujii, R. (1975) Receptor mechanisms in fish chromatophores—II. Evidence for beta adrenoceptors mediating melanosome dispersion in guppy melanophores. Comp. Biochem. Physiol., **51C**: 179–187.

22 Fujii, R. and Miyashita, Y. (1976) Receptor mechanisms in fish chromatophores—III. Neurally controlled melanosome aggregation in a siluroid (*Parasilus asotus*) is strangely mediated by cholinoceptors. Comp. Biochem. Physiol., **55C**: 43–49.

23 Fujii, R., Miyashita, Y. and Fujii, Y. (1982) Muscarinic cholinoceptors mediate neurally evoked pigment aggregation in glass catfish melanophores. J. Neural Transmission, **54**: 29–39.

24 Kumazawa, T., Oshima, N., Fujii, R. and Miyashita, Y. (1984) Release of ATP from adrenergic nerves controlling pigment aggregation in tilapia melanophores. Comp. Biochem. Physiol., **78C**: 1–4.

25 Lerner, A. B. (1971) Neural control of pigment cells. In "Biology of Normal and Abnormal Melanocytes". Ed. by T. Kawamura, T. B. Fitzpatrick and M. Seiji, Univ. Tokyo Press, Tokyo, pp. 3–16.

NII-Electronic Library Service