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Pigment-Aggregating Action of Endothelins on Medaka Xanthophores

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ABSTRACT—Mammalian endothelins (ETs) -1, -2 and -3 effectively aggregated pigmentary organelles in xanthophores of the medaka, *Oryzias latipes*. IRL 1620 and sarafotoxin S6c, both being selective agonists of the mammalian ET_B receptor, were also found to aggregate xanthosomes in those cells. Quantitative studies on the action of ET-1, ET-3 and IRL 1620 on xanthophores indicated that the responses were concentration-dependent, and that they may act directly on xanthophores, because denervated cells responded to the peptides quite similarly. Blockers of some receptors known to mediate motile activity of chromatophores were ineffective in inhibiting the action of ETs. In addition, other blockers of mammalian ET_A receptors (BQ-123 and TTA-386) or ET_B receptors (BQ-788) were also disclosed to be ineffective in interfering with the action of ET. Therefore, ETs may act through the mediation of ET receptors on the medaka xanthophores that do not resemble mammalian ET receptors pharmacologically. Thus, along with their effects on other kinds of chromatophores, ETs may take part in the delicate and exquisite control of integumentary hues and patterns.

INTRODUCTION

As an extremely potent vasoconstrictive principle, endothelin (ET) was first found in medium in which porcine vascular endothelial cells had been cultured (Yanagisawa *et al*., 1988). Since then, various action of ETs on mammalian tissues have been demonstrated. Following the detection by Uemura *et al*. (1991) of immunoreactive ET in the plasma of several poikilothermic vertebrates, a considerable number of studies have also reported the presence of ETs as well as their functional roles in poikilothermal vertebrates. For example, Poder *et al*. (1991) described that ET-1 induced constriction of blood vessels of a few poikilothermal vertebrates, including mesenteric arteries in a species of catfish *Ictalurus* (=*Ameiurus*) *melas*. Working on the rainbow trout *Salmo gairdneri* (=*Oncorhynchus mykiss*), Olson *et al*. (1991) reported that ET-1 induces contraction of cardiovascular tissues. Arad *et al*. (1992) showed that ET-1 acts on the heart and brain of two fish species (tilapia and torpedo) and a reptile (agama) via specific binding sites on the cells. By means of immunohistochemical tests, Goniakousca-Witalinska *et al*. (1995) have recently detected the presence of ETs in neuroendocrine cells of gills of the bowfin, *Amia calva*. It is therefore quite possible that ETs play diverse physiological roles in poikilothermal vertebrates as well as higher vertebrates.

Meanwhile, ETs have been found to influence integumentary chromatophores of various species of teleostean fish: they aggregated melanosomes in dermal melanophores (Fujii *et al*., 1993; Hayashi *et al*., 1996), but dispersed light-scattering organelles in leucophores of the medaka, *Oryzias latipes* (Fujita and Fujii, 1997). Having different optical properties, such reciprocal responses to various stimuli between melanophores and leucophores exist rather normally (Fujii and Oshima, 1986; Fujii, 1993a).

In addition to melanophores and leucophores, chromatophores exist in the skin of teleosts that contain yellowish, reddish and sometimes bluish chromatosomes. These are called xanthophores, erythrophores, and cyanophores, respectively, and they are of course responsible for the yellowish, reddish and bluish components of the integumentary tones (Fujii, 1993a, 2000). Incidentally, cyanophores are novel blue chromatophores that have recently been found in the skin of callionymid fish (Goda and Fujii, 1995). Among them, xanthophores of an oryziatid fish, the medaka *Oryzias latipes* (Iga, 1969), and erythrophores of the holocentrid squirrelfish *Holocentrus ascensionis* (Luby-Phelps and Porter, 1982) are known to be rather peculiar chromatophores, in that entry of extracellular Ca^{2+} ions into the cytosol is required for triggering pigment aggregation. Namely, their membranes may be more excitable to depolarizing stimuli, and the physiological characteristics of these brightly-colored chromatophores are

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probably considerably different from those of other chromatophores elucidated to date. It was therefore interesting to know the influence of ETs on these cells. Since holocentrid squirrelfish are rather difficult to obtain here in the central part of Japan, we decided to examine the effects of ETs on xanthophores of an easily available species.

MATERIALS AND METHODS

Materials

Individuals of the orange-red variety of the medaka, *Oryzias latipes* (Oryziatidae; Cyprinodontiformes) were used in this study. Adult specimens irrespective of sex were purchased from dealers in Tokyo.

Scales plucked from the dorso-lateral part of the trunk were used. They were excised in a physiological saline solution for teleosts, which had the following composition (in mM): NaCl 125.3, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.8, D-(+)-glucose 5.6, Tris-HCl buffer 5.0 (pH 7.3). They were then kept at 4°C for 10 min in a refrigerator, while being immersed in a Ca^{2+}: Mg^{2+}-free saline that had the following composition (in mM): NaCl 130.7, KCl 2.7, D-(+)-glucose 5.6, ethylenediamine tetraacetic acid (EDTA, trisodium salt; Dojindo Lab., Kumamoto, Japan) 0.2, Tris-HCl buffer 5.0 (pH 7.3). After subsequent transfer into standard saline again, they were held individually with a finely pointed forceps, and the epidermal layer overlying the dermis was removed by gentle shaking in the medium.

Recording of motile responses

For recording motile responses of xanthophores, a microscopic photoelectric system was employed. The system was essentially the same as that described previously (Oshima and Fujii, 1984), but with an improved electronic processing part for easier operation and increased stability (Fujii *et al.*, 2000). The new system turned out to be quite suitable for the present use, because the measurements usually required a longer period of time, sometimes extending to 5 hr or more. For increasing the contrast of the chromatophore images against the background in the microscopic field, a violet bandpass filter was employed. In the initial stage of this work, a glass filter which had a spectral transmittance peak of 440 nm (V-44; Toshiba Glass, Tokyo) was employed. Later, we adopted a triacetate plastic one (BPB-45, Fuji Photo Film, Tokyo), which had a peak wavelength of 450 nm. When we used the glass filter, it was placed between the light source and the condenser of the microscope. When the plastic filter was used, it was trimmed to a 12 mm square which was installed inside the eyepiece for photosensing within the photographic column of the microscope (Fujii *et al.*, 2000). Using the plastic filter allowed the state of chromatophores to be monitored in natural color.

In the present study, a 40X objective lens was exclusively employed. In order to restrict the area within the microscopic field, a circular aperture of 3.0 mm in diameter was placed at the plane of the rear real image of the objective (Fujii *et al.*, 2000). Thus, the diameter of the circular area of the skin to be measured for the light transmittance was 75 μm. *Oryzias* xanthophores are a little smaller than other dendritic chromatophores of most fishes, including melanophores and leucophores of the same species. Therefore, motile responses of a few xanthophores were recorded integratively.

Prior to examining the effects of ET, a K^+-rich saline, in which the concentration of K^+ ions was raised to 50 mM, was usually applied. This was to confirm that the chromatophores to be examined retained normal motile responsiveness. In this solution, the concentration of Na^+ was compensatorily decreased to 78 mM in order to keep the osmolarity identical to primary saline.

At the end of each series of measurements, a sufficiently strong solution of norepinephrine hydrochloride (NE, racemic modification; Sankyo, Tokyo) was applied to induce the maximal aggregation of chromatosomes for reference. Usually, the application of 2.5 μM NE for more than 20 min was sufficient for this purpose. Incidentally, concentrations of NE are expressed in terms of its biologically active L-(–)-isomer, namely, half of the net concentration of its synthetic racemic modification. In all cases, the magnitude of response is expressed as a percentage of the maximal xanthosome aggregation recorded during the course of measurements, with the fully dispersed state taken as zero. All recordings were carried out at room temperature between 20 and 25°C.

In most original photoelectric recordings, the breadth (abscissa: time) was too long in comparison with the length (ordinate: magnitude of response) because of the slow responses of the xanthophores. In order to show those responses more clearly therefore, the ratio of the length and the breadth of an original recording was converted to be about 1 : 5. To do that, the original recording was copied to have an appropriately reduced size, and by means of an image-scanner coupled with a photo-retouching software on a personal computer, it was transformed to have the above-mentioned length-breadth ratio. The plot was then printed and traced with India ink, and finally image-scanned again to be labeled for presentation.

Denervation of xanthophores

Scales excised and soaked in physiological saline solution overnight were employed as specimens for chromatophore denervation, because nervous elements in the skin are known to lose their function during that period of time (Iwata *et al.*, 1959). Before examining the effects of ETs on xanthophores, we always observed the responses to a K^+-rich solution of leucophores on the same scale. When the leucosomes did not disperse, we regarded that chromatophores on that scale had been denervated. The reason why we examined the responses of leucophores rather than xanthophores was that in this species of fish, xanthophores are still responsive to elevated K^+ concentrations, even after denervation (Iga, 1969; Oshima *et al.*, 1998). By contrast, denervated leucophores are known to be unresponsive to the K^+ increase (Iga, 1978; Fujita and Fujii, 1997). The above-mentioned 50 mM-K^+ saline was employed for this purpose.

Drugs used

The chemical structures of teleostean ETs have not yet been determined. Therefore, we employed commercially available mammalian ETs (from Peptide Institute, Osaka, Japan, and Sigma Chemical, St.Louis, MO), each of which belongs to one of the three groups of isopeptides, designated inclusively as ET-1, ET-2 and ET-3 (Inoue *et al.*, 1989). The ones used in this study comprised ET-1 (human, porcine, canine, rat, mouse, bovine), ET-2 (human, canine) and ET-3 (human, porcine, rat, rabbit). They were selected as representatives of the three isopeptide groups because those molecular species had been previously shown to be effective in inducing responses in melanophores and leucophores in fish (Fujii *et al.*, 1993; Hayashi *et al.*, 1996; Fujita and Fujii, 1997).

IRL 1620 (Peptide Institute; Takai *et al.*, 1992) and sarafotoxin S6c (SRTX S6c; Sigma Chemical; Williams *et al.*, 1991) were used as ET_B receptor-specific agonists. BQ-123 (Research Biochemicals International, Natick, MA; Ihara *et al.*, 1991) and TTA-386 (Wako Pure Chemical, Osaka, Japan; Kitada *et al.*, 1993) were employed as ET_A receptor-specific antagonists, while BQ-788 (Research Biochemicals International; Ishikawa *et al.*, 1994) was used to antagonize ET_B receptors. Stock concentrated solutions of those drugs were prepared in doubly distilled water, aliquotted in small plastic centrifuge tubes, and stored in a deep freezer at - 70°C; They were diluted with physiological saline just prior to use.

Statistical analysis

Statistical comparisons were performed by two factor factorial analysis of variance (ANOVA). A *P* value of less than 0.05 was considered to indicate a significant difference.

RESULTS

Effects of ETs on xanthophores

At the beginning of each series of measurements, we usually confirmed whether the xanthophores to be examined possessed normal responsiveness by applying a K^+-rich saline (K^+: 50 mM) solution for a short period of time. When rapid aggregation of xanthosomes took place, the cells were regarded as normal. After ascertaining the redispersion of chromatosomes in primary physiological saline, we examined the effects of ETs.

As a typical series of photomicrographic examinations on the effects of ETs, Figure 1 illustrates the responses of xanthophores on a scale to 10 nM ET-1. The peptide effectively induced the aggregation of xanthosomes (Panels B-E), with a response that was comparable to the maximal one induced by NE (Panel H). In this particular series of photomicrographs incidentally, we can also recognize the responses of an amelanotic melanophore to ET-1 and also to NE: Although the action of ET-1 seemed to be relatively weak, NE aggregated its organelles (amelanotic melanosomes) effectively (Sugimoto *et al*., 1985). Microscopic observations also showed that both ET-2 and ET-3 were similarly effective in inducing xanthosome aggregation in *Oryzias* xanthophores (not shown).

We then recorded the motile responses of xanthophores photoelectrically. Panels A, B and C of Figure 2 exhibit typical recordings showing the responses to ET-1, ET-2 and ET-3, respectively. The rate of the xanthophore response to ETs was generally low, when compared with that of the melanophores of several teleosts (Fujii *et al*., 1993; Hayashi *et al*., 1996) or with leucophores of the medaka (Fujita and Fujii, 1997). It should also be noted that, even during application of the ET solution, redispersion of xanthosomes took place. It is further noteworthy that once ETs induced the aggregation of pigment, the cells became almost completely irresponsive to the peptides. A slight aggregation of xanthosomes, however, was sometimes detectable in xanthophores, as exemplified in Figure 2C, where ET-3 was applied again after thorough washing of the initially applied peptide. Such a phenomenon may be due to the fact that the first stimulus was rather weak in bringing about the down regulation of receptor of the homologous desensitization type (Hayashi *et al*., 1996; Fujita and Fujii, 1997). Actually, no such responses were observable when stronger solutions were applied.

Quantitative studies on the actions of ET-1 and ET-3 were then made, and the relationships between the concentration of the agonists and the magnitude of xanthosome aggregation are exhibited in Figure 3. Before the application of ETs and the agonist to skin specimens, we always checked the motile responsiveness of chromatophores by treating them briefly with the K^+-rich saline (K^+: 50 mM) as detailed above. After subsequent washing of the skin specimens with normal physiological saline for more than 30 min, ETs at concentra-

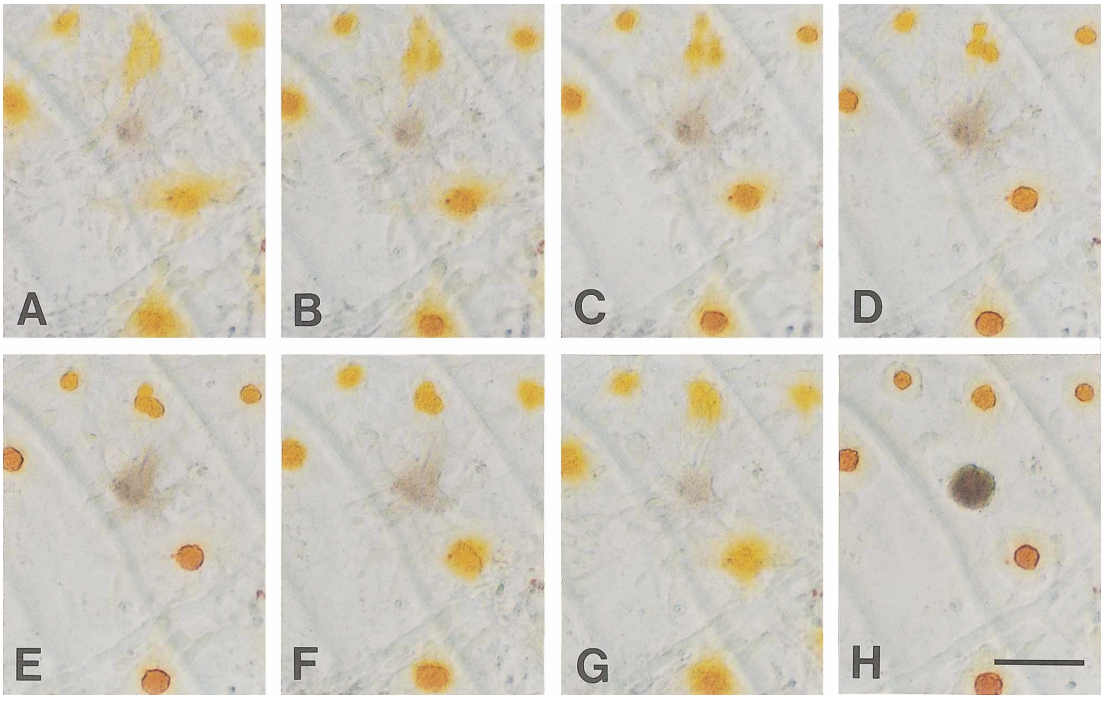

Fig. 1. Serial photomicrographs showing the effects of ET-1 on xanthophores in a scale of the medaka (*Oryzias latipes*, orange-red variety). (**A**) Equilibrated in physiological saline; xanthosomes are completely dispersed throughout the cytoplasm. (**B**–**G**) 1, 2 , 5, 10, 30 and 60 min after the application of 10 nM ET-1, respectively; ET-1 effectively aggregated pigment within the xanthophores. It may be noted however, that once the maximal level was reached (**E**), the effect was gradually reversed (**F**, **G**). (**H**) 10 min after the application of 2.5 μM NE. A large brownish grey figure observable slightly above the middle of each panel is an amelanotic melanophore, which responded to ET-1 and to NE by the aggregation of amelanotic melanosomes. The bar in panel **H** indicates 50 μm.

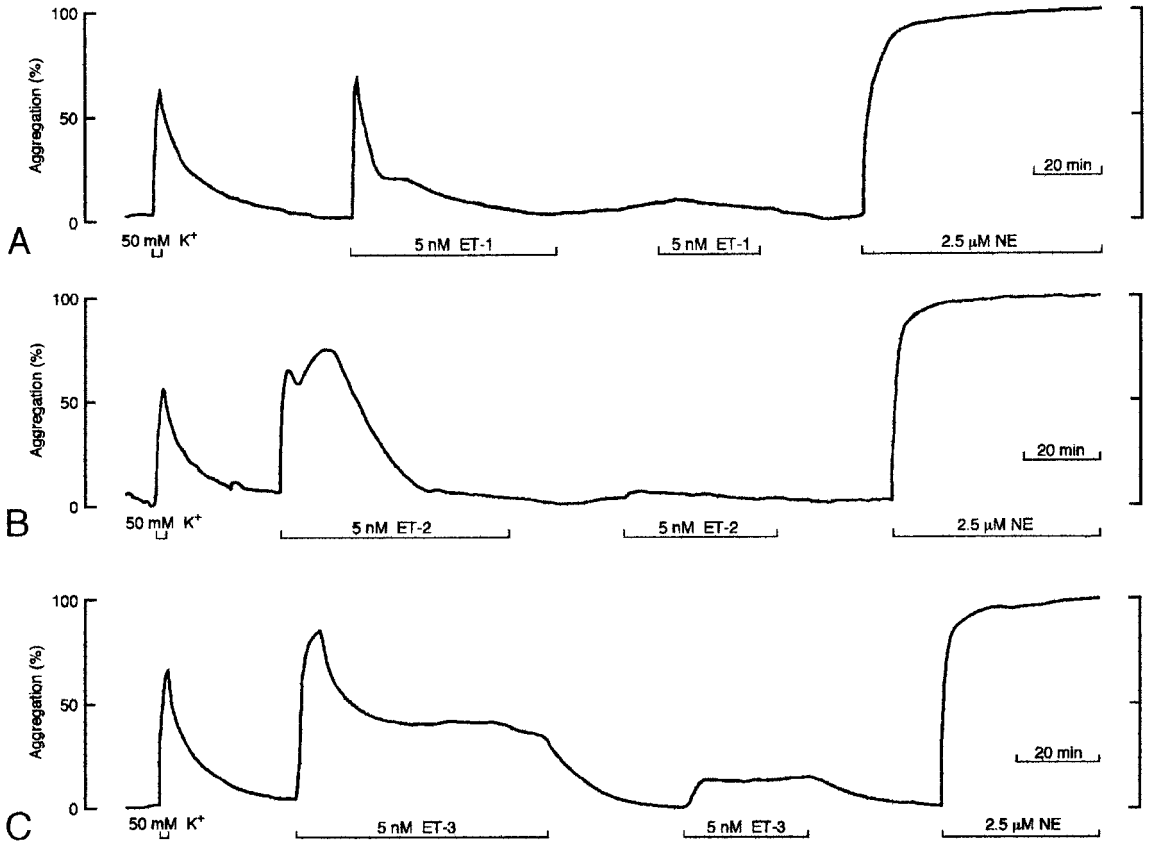

Fig. 2. Photoelectric recordings showing the typical effects of ETs on *Oryzias* xanthophores. In all measurements, K^+-rich saline (50 mM K^+) was applied first to confirm that the cells to be examined possessed normal responsiveness. Finally, 2.5 μM NE was applied to arouse the maximal response for reference. (**A**) 5 nM ET-1 was applied. (**B**) 5 nM ET-2 was applied. (**C**) 5 nM ET-3 was applied. In all recordings, remarkable aggregation of pigment, took place in response to ETs, but the responses were rather quickly reversed even in the presence of the peptides. It should also be noted that only slight aggregation of xanthosomes was induced by each peptide when it was applied a second time.

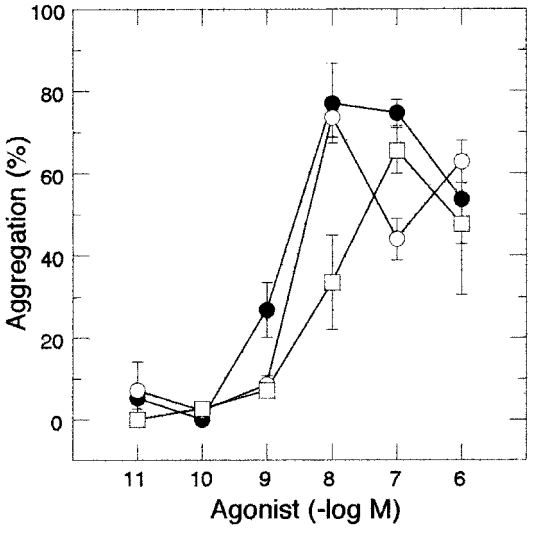

Fig. 3. Relationships of the concentration of ET-1 (solid circles), ET-3 (open circles) and IRL 1620 (open squares) to the magnitude of the pigment-aggregating response of *Oryzias* xanthophores. Abscissa, negative logarithm of the molar concentration. Ordinate, magnitude of the response as a percentage of the full response induced by 30 min application of 2.5 μM NE. Vertical lines indicate SE.

tions from 10 pM to 1 μM (in 10-fold increments) were applied for at least 30 min. The specimen was then washed again with the normal saline to disperse the xanthosomes. Finally, 2.5 μM NE was applied for 30 min to bring about the maximal aggregation of pigment for reference. The degree of xanthosome aggregation attained during the application of an ET agonist at a certain concentration was compared with that induced by 2.5 μM NE. Six measurements were done for each concentration of an ET agonist, and the data were processed to display the concentration-response relationships.

Both ET-1 and ET-3 induced the aggregation of xanthosomes in a concentration-dependent manner, and their concentration-response curves resemble each other. Discernible aggregation of *Oryzias* xanthosomes by these peptides could be observed at concentrations as low as 1 nM, while the maximal level of response was reached at about 10 nM. The concentrations of agonists that induced 50% of the maximal aggregation of pigment (EC_{50}) were 2.90 and 4.31 nM for ET-1 and ET-3, respectively, but there was no statistically significant difference between them. It was also shown that the maximal level attained became lower when ETs at higher concentrations were applied.

As shown in Figure 4, the xanthosome-aggregating re-

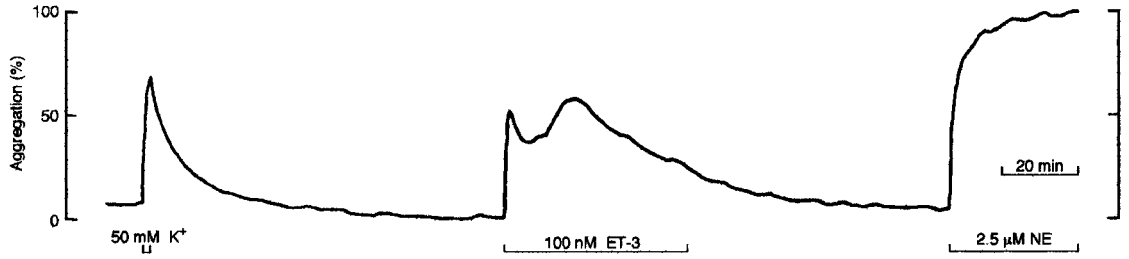

Fig. 4. Recording showing a peculiar but rather frequently occurring pattern of xanthophore response to ETs. In this particular recording, 100 nM ET-3 was used. ET-3 induced a rapid but transient aggregation of xanthosomes. Following the brief phase of xanthosome dispersion, they slowly aggregated again, and this was again reversed gradually. Similar phenomena were also observed rather frequently when ET-1 or ET-2 was applied at higher concentrations. As a result of such phenomena, the maximal level of pigment aggregation to ETs became smaller.

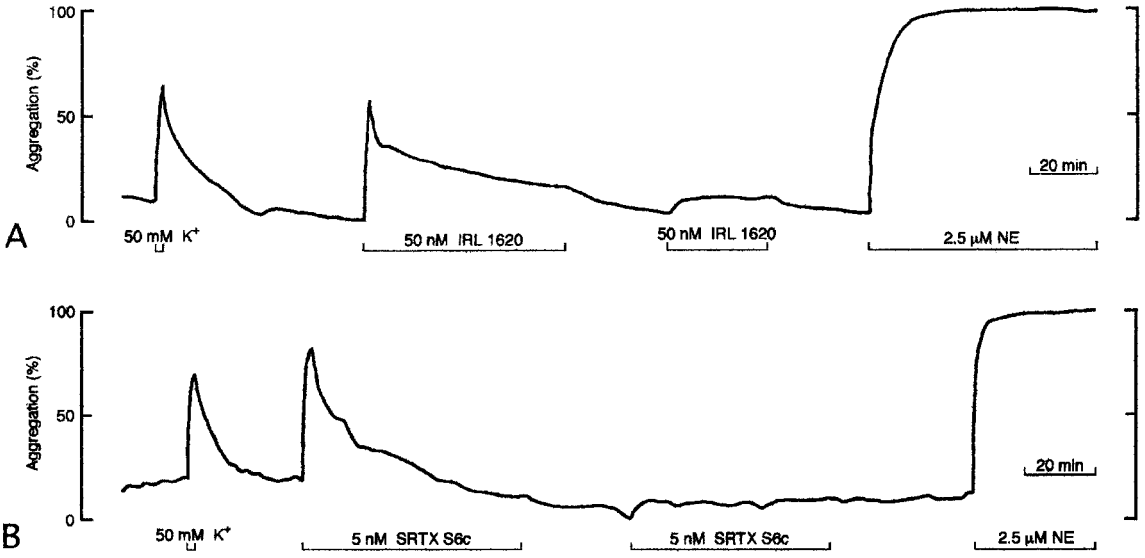

Fig. 5. Typical recordings showing the effects on xanthophores of two ET_B-selective agonists, IRL 1620 and sarafotoxin S6c (SRTX S6c). (A) 50 nM IRL 1620 was applied. (B) 5 nM SRTX S6c was applied. In each recording, the peptide induced aggregation of xanthosomes, but the response was gradually reversed even in the presence of the peptide. When it was applied again, only slight aggregation was induced.

sponse typically proceeded in two steps. An initial rapid aggregation of xanthosomes was quickly followed by their gradual dispersion, and then, a more slower aggregation took place. The pigment dispersing phase may be due to the down regulation of ET receptors mentioned above. Such a phenomenon may be at least partly responsible for lowering the maximal level of response.

Effects of ET_B receptor-specific agonists

The effects on xanthophores of two selective agonists for mammalian ET_B receptors, i.e. IRL 1620 and sarafotoxin S6c, were then examined. Both peptides potently induced the aggregation of xanthosomes, which was followed by their dispersion even in their continued presence (Fig. 5A, B). After removal of the peptides by thorough washing with normal saline, the same peptides were applied again. They sometimes induced a slight aggregation of pigment, but aggregation always failed to occur when it was applied a third time (data not shown). We then examined the concentration-response relationship with IRL 1620 which had an EC_{50} of 32.7 nM. As shown in Figure 3, its effect seems to be weaker than that of ETs, but the differences among the effects of IRL 1620, ET-1 and ET-3 were not significant statistically.

Effects of ETs on denervated chromatophores

Responses of denervated xanthophores to ET-1 were examined. By observing that leucophores on the same scale were not responsive to K^+-rich saline (50 mM K^+), we first confirmed that the xanthophores had been successfully denervated. Figure 6 shows a typical recording in which 10 nM ET-1 was applied, and a profound aggregation of xanthosomes ensued.

Effects of some receptor antagonists

Blockers for some known pigment-aggregating stimuli were then tested for their possible effects. These included an α-adrenolytic, phentolamine, a β-adrenolytic, propranolol, and a cholinolytic agent, atropine. All of them were shown to be ineffective in inhibiting the xanthosome-aggregating action of ETs. As a typical example, Figure 7 exhibits a recording where

Fig. 6. Typical recordings showing the responses to ET-1 of the denervated xanthophores. Before examining the effects of ETs on xanthophores, we confirmed that leucosomes in leucophores on the same scale did not respond to the elevation of the K^+ concentration in the medium (cf. Materials and Methods section). In this recording, 10 nM ET-1 was then applied and it effectively aggregated xanthosomes in the denervated xanthophores. Analogous recordings were obtained when ET-2 and ET-3 were employed.

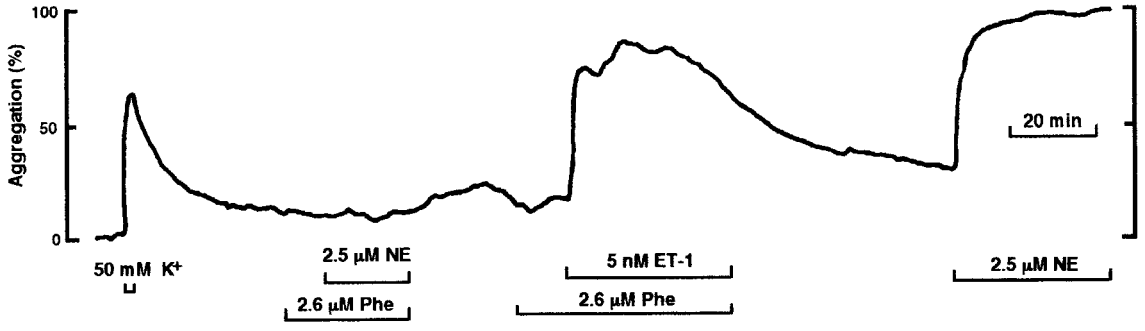

Fig. 7. Typical recording showing the effects of phentolamine, an α-adrenolytic agent, on the responses of xanthophores to ET-1. Phentolamine (Phe) effectively blocked the xanthosome aggregating action of NE, but it had no effect on the response to ET-1.

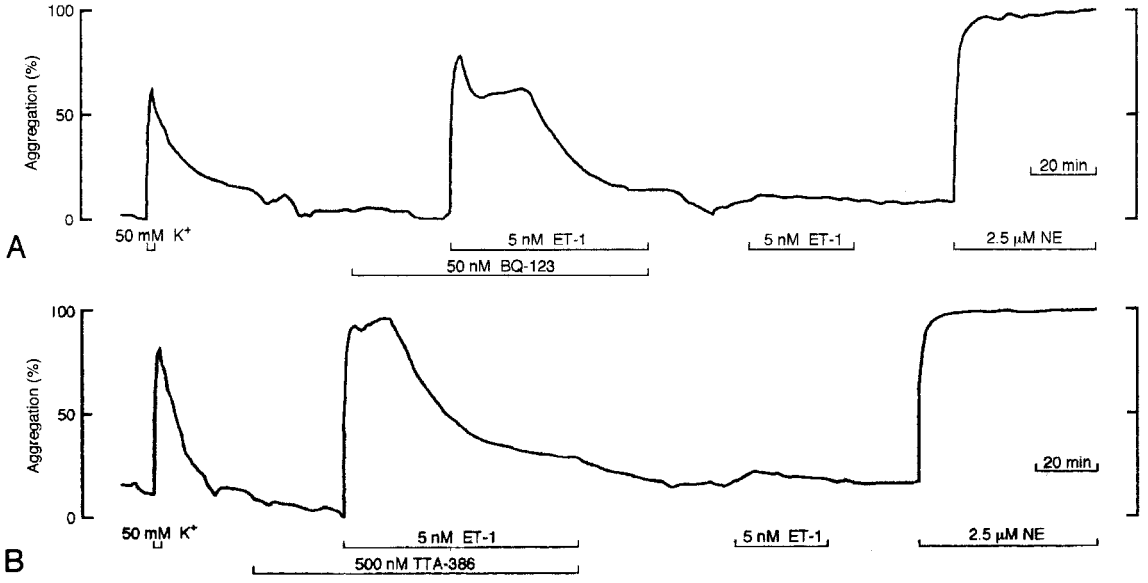

Fig. 8. Typical recordings showing the effects of two mammalian ET_A receptor specific antagonists, BQ-123 and TTA-386, on the responses of xanthophores to ET-1. (**A**) Effect of 50 nM BQ-123; pretreatment with BQ-123 for 30 min was followed by the application of ET-1 in the presence of BQ-123. (**B**) Effect of 500 nM TTA-386; after 30 min pretreatment with TTA-386, ET-1 was applied in the presence of TTA-386. In either case, the inhibition was not recognizable despite the fact that a very strong solution of the antagonist was applied.

phentolamine was tested for its effects on the action of ET-1. Phentolamine did not interfere with the action of ET-1, although it effectively blocked the action of NE.

Effects of ET_A receptor-selective antagonists

The effects of two antagonists specific for mammalian ET_A receptors, i.e. BQ-123 and TTA-386, on the xanthosome-aggregation action of ETs were examined. Panels A and B of

Fig. 9. Typical recording showing the effects of BQ-788, an antagonist specific for the mammalian ET_B receptor, on the responses of xanthophores to ET-1. Following treatment with 500 nM BQ-788 for 30 min, 5 nM ET-1 was applied in the presence of BQ-788. BQ-788 effectively interfered with the pigment-aggregating action of ET-1.

Figure 8 illustrate typical recordings showing the effects of BQ-123 and TTA-386 on the action of ET-1, respectively. After treating a scale with either of those blockers for 30 min, ET-1 was applied during the continued presence of the blocker at the same concentration. Even in the presence of sufficiently high concentrations of the blockers, the pigment-aggregating action of ET-1 was not inhibited. The actions of ET-2, ET-3, IRL 1620 and SRTX S6c were also unaffected.

Effects of an ET_B receptor-selective antagonist

The effects of BQ-788, an antagonist specific for mammalian ET_B receptors, on the action of ETs were finally examined. Figure 9 illustrates a typical recording obtained when ET-1 was used as the stimulant. After treating a scale with 500 nM BQ-788 for 30 min, 5 nM ET-1 was applied in the presence of BQ-788 at the same concentration, but no inhibition was evident.

After removal of the blocker by washing with the primary saline, ET-1 was again applied. Distinct from the case of blockade by ET_A antagonists, ET-1 aroused a fairly large response. It has generally been observed that once a response took place, either in the presence or the absence of an antagonist, ETs or agonists usually failed to induce the response again when they were applied for the second time (Figs. 2, 5 and 8). Such desensitization has already been observed for melanophores (Fujii *et al*., 1993, Hayashi *et al*, 1996) and also for leucophores (Fujita and Fujii, 1997), and has been thought to be due to the down regulation of the receptors. Presumably, BQ-788 binds to ET receptors more weakly, and has some inhibitory influence on that down regulation. Fundamentally similar results were obtained on ET-2 and ET-3.

DISCUSSION

When colored organelles (chromatosomes) disperse throughout the cytoplasm of dendritic chromatophores, the skin naturally tends to strengthen the hue of these organelles, whereas the hue fades when they aggregate into the perikarya of the cells. When a fish is adapted to a white background, for example, chromatosomes in the light-absorbing chromatophores (melanophores, erythrophores and xanthophores) aggregate, and those in the light-scattering chromatophores (leucophores) disperse, hence resulting in an effective blanching of the skin. The direction of movement of those chromatosomes is reversed when the animal is adapted to a dark background which causes an effective darkening of the skin. Such cooperative responses observed among various types of chromatophores elicit effective dark-to-pale, or reverse changes of the integumental coloration.

Such reciprocal responses existing between light-absorbing chromatophores and leucophores are also commonly observable when examinations are made *in vitro*. For example, light-absorbing chromatophores respond by aggregating of chromatosomes in response to sympathetic nervous stimuli, norepinephrine (NE) and to melanin-concentrating hormone (MCH), while leucophores respond to the same stimuli by dispersing their light-scattering organelles, the leucosomes (Oshima *et al*., 1986; Fujii, 1993a, 2000). When we take account of their reciprocal optical properties, such a reciprocity may rather naturally be understood since such dark-to-pale or reverse changes of color may be of extraordinary importance for survival (Fujii, 1993b).

However, there are several exceptions in the nature of such responses that exist between chromatophores and these belong to two categories. For example, melatonin generally aggregates pigmentary organelles in both kinds of chromatophores, while α-melanophore-stimulating hormone (α-MSH) and adenylyl compounds always disperse them (Fujii and Oshima, 1986; Fujii, 1993a, 2000). Furthermore, some exceptions exist even when we restrict the subject to the light-absorbing chromatophores. Using a few species of teleosts for example, Oshima and her associates reported recently that prolactin dispersed chromatosomes in erythrophores and xanthophores of some teleostean fish, while it had practically no influence on chromatophores of other sorts (Kitta *et al*., 1993; Oshima *et al*., 1996). Nishi and Fujii (1992) and Masagaki and Fujii (1999) described that melanophores in certain regions of the integument of *Nannostomus* pencilfish respond to melatonin by dispersing their melanosomes which had been thought to aggregate pigment. Such complexity in the chromatic system of fish may not be a strange phenomenon, but must rather be an important feature for generating delicate hues and patterns that fish need to adapt to various environmental conditions and ethological encounters (Fujii, 1993b). Although categorized into light-absorbing chromatophores, xanthophores and erythrophores possess optical properties

that stand between those of melanophores and leucophores in a certain sense. Until the present, furthermore, no results have been reported on the effects of ETs on these brightly-colored chromatophores. It was therefore very challenging for us to examine whether ETs aggregate or disperse pigmentary organelles in these chromatophores. In the present study, we have chosen xanthophores of medakas as representative of brightly colored chromatophores.

We found that ETs possess remarkable xanthosome-aggregating action, and the direction of xanthosome displacement was similar to that observed in melanophores (Fujii *et al*., 1993; Hayashi *et al*., 1996). Thus, the present observations are in conformity with many past reports that melanophores, xanthophores and erythrophores generally respond to neural or hormonal signals in the same manner in terms of the direction of the pigmentary translocation (Fujii, 1993a, 2000; Fujii and Oshima, 1986, 1994; Matsumoto *et al*., 1978; Obika and Meyer-Rochow, 1990). On the other hand, we have already shown that ETs disperse light-scattering organelles in leucophores of medakas (Fujita and Fujii, 1997). Namely, ETs may function to enhance the contrast of chromatic patterns on the skin. As mentioned above, such chromatic responses must be helpful for animals, at least under ethological conditions where an increased contrast of chromatic patterns is advantageous.

ETs showed their effects even at very low concentrations, which may indicate that the action of ETs on the cells is not merely a pharmacological one. Namely, at least some molecular form(s) of ET isopeptides may function to augment the whiteness of the skin, modifying the action of sympathetic postganglionic fibers and/or of hormonal factors (Fujii, 1993a, 2000; Fujii and Oshima, 1986, 1994). Very recently however, Visconti *et al*. (1999) reported that ETs were ineffective in inducing skin lightening or darkening of a freshwater ray, *Potamotrygon reticulatus*. That is, the melanophores of that species may be unresponsive to ETs. Since these peptides are thought to have definite roles in regulating chromatophores in teleosts, further comparative studies are needed in lower fishes, including elasmobranchs.

Denervated xanthophores responded to ETs quite normally. It was thus concluded that ET isopeptide(s) act directly on xanthophores, not via the stimulation of presynaptic nervous elements to liberate sympathetic neurotransmitter. IRL 1620 and sarafotoxin S6c, both being ET agonists, potently aggregated xanthosomes. Adrenergic blocking agents of the α- and β-types (phentolamine and propranolol), and a cholinolytic type (atropine) were all ineffective. Furthermore, specific blockers of the mammalian ET_A receptor (BQ-123, TTA-386) or for the ET_B receptor (BQ-788) were not effective in blocking the action of ETs on xanthophores. These results indicate that the ET action on *Oryzias* xanthophores is mediated by ET receptors that do not resemble mammalian ET_A or ET_B.

We now know that the classes Osteichthyes and Mammalia branched off more than 400 million years ago. Since then, receptor proteins in these two groups should have diverged independently. Therefore, even though their roles to bind to ET agonists still resemble each other, molecular structures as well as pharmacological properties of receptors should have altered considerably. It is therefore inappropriate to categorize fish ET receptors into ET_A or ET_B types on the basis of effects of drugs developed for human use, and fish ET receptors will have to be classified more precisely in the future.

Like melanophores (Fujii *et al*., 1993; Hayashi *et al*., 1996), *Oryzias* xanthophores responded to ETs by pigment aggregation, but there are some differences between their characteristics. The first is the slow rate of xanthosome displacement, but that can be ascribed to the intrinsic nature of the cells themselves. In xanthophores, secondly, the level of maximal aggregation was decreased at very high concentrations of agonists (Fig. 3). It is conceivable that xanthophores possess β-ET receptors in addition to α-ET receptors. At higher concentrations of ETs, the xanthosome-dispersing effect via β-ET receptors might become apparent, counteracting the xanthosome aggregating action mediated by α-ET receptors.

We suggest that ET may function as a paracrine factor or a local hormone to modify the actions of nervous or hormonal principles (Fujii *et al*, 1993; Hayashi *et al*., 1996; Fujita and Fujii, 1997; cf. also Fujii, 2000). Recently, Yada *et al*. (1991) showed that in humans, keratinocytes are a source of ETs which can be strong mitogens as well as melanogens for human melanocytes. In fish, tissues or organs that secrete ETs to control chromatophores have not yet been identified, but very recently, the secretion of ET from goldfish epidermal cells in culture was demonstrated (Akimoto *et al*., 2000). Thus, the possible source of ET regulating chromatophore movement might be sought there. In fact, epidermal melanophores are thickly surrounded by epidermal cells, and these two cell types may easily constitute the paracrine coupling. In fish however, chromatophores are rather infrequently found in the epidermis. Having been thought to have only minor roles in pigmentation, these cells have not yet been studied for the effects of ETs. By contrast, dermal chromatophores are separated from the epidermis by the basal lamina that is thought to be relatively impermeable, and also by rather thick, subepidermal collagenous lamella that belongs to the compact connective tissue (Fujii, 1968). It is therefore unlikely that epidermal cell-dermal chromatophore coupling forms the paracrine link. At this moment therefore, we presume that endothelial cells of blood capillaries that run close to the chromatophores are the source of ET. Capillaries are richly distributed in the dermis, and ETs would be liberated not from the luminal side but rather from the outer side of endothelial cells to reach the chromatophores (Fujita and Fujii, 1997; Fujii, 2000).

It should be noted here that, as in the case of melanophores (Fujii *et al*., 1993; Hayashi *et al*., 1996) or leucophores (Fujita and Fujii, 1997), the xanthosome-aggregating effect of ETs was transient, although the time course was longer than that observed on other types of chromatophores examined thus far. It was also found that once a scale had been treated with ETs, the xanthophores became refractory to them. Such

refractoriness has also been observed on melanophores and leucophores. As described before, these phenomena might be ascribed to desensitization of receptors involved, or to the so-called "down regulation" (Hayashi *et al*., 1996; Fujita and Fujii, 1997).

Recent studies on chromatophores showed that the increase or the decrease in cytosolic level of a certain second messenger species always induces reciprocal pigmentary movements, and that the direction of the movement is common disregarding the species of chromatophores (Fujii, 1993a; 2000; Fujii and Oshima, 1994; Nery and Castrucci, 1997). That is, the increase in cyclic AMP levels always mediates dispersion of chromatosomes, while, in cases where Ca^{2+} ions (Luby-Phelps and Porter, 1982; Negishi and Obika, 1985; Oshima *et al*., 1988; Oshima *et al*., 1998) and inositol 1,4,5-trisphosphate (Fujii *et al*., 1991) are working as second messengers, they always induce aggregation of chromatosomes.

On the other hand, we know that adrenergic stimuli, for example, aggregate or disperse chromatosomes via α-adrenoceptors or β-adrenoceptors, respectively. Melatonin (MT) generally aggregates melanosomes, but in some cases (e.g. pencilfish) it disperses pigment in melanophores by novel MT receptors. We named them β-MT receptors, leaving the conventional and common ones to be called α-MT receptors. Similarly, ET receptors may also be categorized tentatively into two groups, i.e. α-ET and β-ET receptors. In accordance with the above cases, the α-ET receptors are assigned for those mediating chromatosome aggregation, while β-ET receptors mediate their dispersion. To date, only leucophores are known to possess β-ET receptors.

In relation to the designation of ET-receptors, the signal transduction mechanism involved in the ET action on chromatophores should be mentioned. Until recently, many researchers working on fish chromatophores have been inclined to consider that cyclic adenosine 3',5'-monophosphate (cAMP) is the sole second messenger for motile activities of chromatophores. As for hormonal regulation, cAMP may play the major role. Meanwhile, an increase in intracellular levels of Ca^{2+} ions has sometimes been shown to be associated with the aggregation of chromatosomes. Among chromatophores employed in those studies, erythrophores of *Holocentrus* squirrelfish and xanthophores of medakas are rather peculiar cells, in that they are distinct from many other chromatophores reported hitherto, in that the aggregation of pigment in these brightly-colored chromatophores is triggered by the penetration of extracellular Ca^{2+} into cytosol (Luby-Phelps and Porter, 1982; McNiven and Ward, 1988; Oshima *et al*., 1998). Thus, these cells may better be treated separately from common chromatophores (Fujii, 1993a, 2000). Working on medaka melanophores however, Negishi and Obika (1985) reported that an increase in cytosolic Ca^{2+} levels is associated with the aggregation of melanosomes. Oshima *et al*. (1988) then showed that catecholamine stimulation is accompanied by an increase in intracellular Ca^{2+}, resulting in the aggregation of pigment in melanophores and erythrophores of the platyfish (*Xiphophorus maculatus*). Based on their results on xanthophores of medakas, Oshima *et al*. (1998) suggest that $Ca2^{+}$ depletion in the cytosol may lead to the inhibition of adenylyl cyclase, and may finally result in the aggregation of xanthosomes.

Working on melanophores of the Nile tilapia, *Oreochromis niloticus*, Fujii *et al*. (1991) further showed that inositol 1,4,5-trisphosphate (IP_3) may function as another second messenger for signaling adrenergic stimulation in fish chromatophores, indicating the involvement of α_1-adrenoceptors. Namely, both α_1- and α_2-adrenoceptors are present in melanophores being controlled adrenergically. In many tissues, IP_3 has been shown to induce the liberation of Ca^{2+} ions from intracellular storage compartments. In common chromatophores as well, IP_3 may act via the release of Ca^{2+} ions from such compartments within the cells, because vesicular elements of smooth endoplasmic reticulum are abundantly found in the cytoplasm (Fujii, 1966, 1993a).

It has generally been accepted that signal transduction through ET receptors mainly proceeds by activating phospholipase C, which produces IP_3 and diacylglycerol (DG). As for the role played by DG in the motile responses of chromatophores, we still have no information, although those effects, if any, may be related to morphological color changes. As in the case of adrenergic stimulation of melanophores (Fujii *et al*., 1991), IP_3 produced via the action of ETs may act to aggregate pigment also in xanthophores. It seems likely therefore that a mechanism similar to that involved in the action of ETs on mammalian tissues is also operating in xanthophores of fish. Adenylyl cyclase and phosphatidyl inositol systems are probably operating in concert in the signal transduction of chromatophores to realize their motile responses (Fujii *et al*., 1991; cf. Fujii, 1993a).

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REFERENCES

Akimoto K, Sorimachi K, Takaoka T, Niwa A (2000) Development of a simple culture method for the tissue contaminated with microorganisms and application to establishment of a fish cell line. Zool Sci 17: 61–63

Arad TZ, Bdolah A, Kochva E, Wollberg Z (1992) Activity of sarafotoxin-endothelin peptides in the heart and brain of lower vertebrates. Toxicon 30: 439–448

Fujii R (1966) Correlation between fine structure and activity in fish melanophore. In "Structure and Control of the Melanocyte" Ed by G Della Porta, O Mühlbock, Springer-Verlag, Berlin, pp 114–123

Fujii R (1968) Fine structure of the collagenous lamella underlying the epidermis of goby, *Chasmichthys gulosus*. Annot Zool Japon 41: 95–106

Fujii R (1993a) Cytophysiology of fish chromatophores. Int Rev Cytol 143: 191–255

Fujii R (1993b) Coloration and Chromatophores. In "The Physiology of Fishes" Ed by DH Evans, CRC Press, Boca Raton, pp 535–562

Fujii R (2000) The regulation of motile activity in fish chromatophores.

Pigment Cell Res 13: 300–319
Fujii R, Oshima N (1986) Control of chromatophore movements in teleost fishes. Zool Sci 3: 13–47
Fujii R, Oshima N (1994) Factors influencing motile activities of fish chromatophores. In "Advances in Comparative and Environmental Physiology Vol 20" Ed by R Gilles, Springer-Verlag, Berlin, pp 1–54
Fujii R, Wakatabi H, Oshima N (1991) Inositol 1,4,5-trisphosphate signals the motile response of fish chromatophore—I. Aggregation of pigment in the tilapia melanophore. J Exp Zool 259: 9–17
Fujii R, Tanaka Y, Hayashi H (1993) Endothelin-1 causes aggregation of pigment in teleostean melanophores. Zool Sci 10: 763–772
Fujii R, Yamada T, Oshima N (2000) Further improvements of the photoelectric method for measuring motile responses of chromatophores. Zool Sci 17: 33–45
Fujita T, Fujii R (1997) Endothelins disperse light-scattering organelles in leucophores of the medaka, *Oryzias latipes*. Zool Sci 14: 559–569
Goda M, Fujii R (1995) Blue chromatophores in two species of callionymid fish. Zool Sci 12: 811–813
Goniakousca-Witalinska L, Zaccone G, Fasulo S, Mauceri A, Licata A, Youson J (1995) Neuroendocrine cells in the gills of the bowfin *Amia calva*: an ultrastructural and immunocytochemical study. Folia Histochem Cytobiol 33: 171–177
Hayashi H, Nakamura A, Fujii R (1996) The endothelin receptors that mediate aggregation of pigment in fish melanophores. Comp Biochem Physiol 115B: 141–152
Iga T (1969) The action of potassium ions on the xanthophores of the teleost, *Oryzias latipes.* Mem Fac Lit Sci Shimane Univ, Nat Sci 2: 67–75
Iga T (1978) The mode of action of potassium ions on the leukophores of a freshwater teleost, *Oryzias latipes*. J Exp Zool 205: 413–422
Ihara M, Noguchi K, Saeki T, Fukuroda T, Tsuchida S, Kimura S, Fukami T, Ishikawa K, Nishikibe M, Yano M (1991) Biological profiles of highly potent novel endothelin antagonist selective for the ET_A receptor. Life Sci 50: 247–255
Inoue A, Yanagisawa M, Kimura S, Kasuya Y, Miyauchi T, Goto K, Masaki T (1989) The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. Proc Nat Acad Sci USA 86: 2863–2867
Ishikawa K, Ihara M, Noguchi K, Mase T, Mino N, Saeki T, Fukuroda T, Fukami T, Ozaki S, Nagase T, Nishikibe M. and Yano M. (1994) Biochemical and pharmacological profiles of a potent and selective endothelin B-receptor antagonist, BQ-788. Proc Nat Acad Sci USA 91: 4892–4896
Iwata KS, Watanabe M, Kurihara T (1959) Changes of state and response of the fish scale melanophore during continuous immersion in Ringer's solution. Biol J Okayama Univ 5: 185–194
Kitada C, Ohtaki T, Masuda Y, Masuo Y, Nomura H, Asami T, Matsumoto Y, Satou M, Fujino M. (1993) Design and synthesis of ET_A receptor antagonists and study of ET_A receptor distribution. J. Cardiovascular Pharmacol. 22(Suppl. 8): S128–S131
Kitta K, Makino M, Oshima N, Bern HA (1993) Effects of prolactins on the chromatophores of the tilapia, *Oreochromis niloticus*. Gen Comp Endocrinol 92: 355–365
Luby-Phelps K, Porter KR (1982) The control of pigment migration in isolated erythrophores of *Holocentrus ascensionis* (Osbeck) II. The role of calcium. Cell 29: 441–450
Masagaki A, Fujii R (1999) Differential actions of melatonin on melanophores of the threeline pencilfish, *Nannostomus trifasciatus*. Zool Sci 16: 35–42
Matsumoto J, Watanabe Y, Obika M, Hadley ME (1978) Mechanisms controlling pigment movements within swordtail (*Xiphophorus helleri*) erythrophores in primary cell culture. Comp Biochem Physiol 61A: 509–517
McNiven MA, Ward JB (1988) Calcium regulation of pigment transport in vitro. J Cell Biol 106: 111–125
Negishi S, Obika M (1985) The role of calcium and magnesium on pigment translocation in melanophores of *Oryzias latipes*. In "Pigment Cell 1985: Biological, Molecular and Clinical Aspects of Pigmentation" Ed by J Bagnara, SN Klaus, E Paul, M Schartl, Univ Tokyo Press, Tokyo, pp 233–239
Nery LEM, Castrucci AML (1997) Pigment cell signalling for color change. Comp Biochem Physiol 118A: 1135–1144
Nishi H, Fujii R (1992) Novel receptors for melatonin that mediate pigment dispersion are present in some melanophores of the pencil fish (*Nannostomus*). Comp Biochem Physiol 103C: 263–268
Obika M, Meyer-Rochow VB (1990) Dermal and epidermal chromatophores of the antarctic teleost *Trematomus bernacchii*. Pigment Cell Res 3: 33–37
Olson KR, Duff DW, Farrell AP, Keen J, Kellogg MD, Kullman D, Villa J (1991) Cardiovascular effects of endothelin in trout. Am J Physiol 260: H1214–1223
Oshima N, Fujii R (1984) A precision photoelectric method for recording chromatophore responses *in vitro*. Zool. Sci. 1: 545–552
Oshima N, Kasukawa H, Fujii R, Wilkes BC, Hruby VJ, Hadley ME (1986) Action of melanin-concentrating hormone (MCH) on teleost chromatophores. Gen Comp Endocrinol 64: 381–388
Oshima N, Suzuki M, Yamaji N, Fujii R (1988) Pigment aggregation is triggered by an increase in free calcium ions within fish chromatophores. Comp Biochem Physiol 91A: 27–32
Oshima N, Makino M, Iwamuro S, Bern HA (1996) Pigment dispersion by prolactin in cultured xanthophores and erythrophores of some fish species. J Exp Zool 275: 45–52
Oshima N, Sekine H, Tanooka M (1998) Involvement of Ca^{2+} in the direct effect of K^+ on xanthophores of the medaka, *Oryzias latipes*. Zool Sci 15: 645–650
Poder TC, Silberberg SD, Rampe D (1991) Contraction of reptile, amphibian, and fish blood vessels by endothelin-1. Can J Pharmacol 69: 215–217
Sugimoto M, Oshima N, Fujii R (1985) Mechanisms controlling motile responses of amelanotic melanophores in the medaka, *Oryzias latipes*. Zool Sci 2: 317–322
Takai M, Umemura I, Yamasaki K, Watakabe T, Fujitani Y, Oda K, Urade Y, Inui T, Yamamura T and Okada T (1992) A potent and specific agonist, Suc-[Glu^9,$Ala^{11,15}$]-endothelin-1 (8-21), IRL 1620, for the ET_B receptor. Biochem Biophys Res Commun 184: 953–959
Uemura H, Naruse M, Naruse K, Hirohama T, Demura H, Kasuya Y (1991) Immunoreactive endothelin in plasma of nonmammalian vertebrates. J Cardiovascular Pharmacol 17: S414–416
Visconti MA, Romanzini GC, Camargo CR, Castrucci AML (1999) Elasmobranch color change: A short review and novel data on hormone regulation. J Exp Zool 284: 485–491.
Williams DL Jr, Jones KL, Pettibone DJ, Lis EV, Clineschmidt BV (1991) Sarafotoxin S6c: an antagonist which distinguishes between endothelin receptor subtype. Biochem Biophys Res Commun 175: 556–561
Yada Y, Higuchi K, Imokawa G (1991) Effects of endothelins on signal transduction and proliferation in human melanocytes. J Biol Chem 266: 18352–18357
Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T (1988) A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332: 411–415

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