Endothelins Disperse Light-Scattering Organelles in Leucophores of the Medaka, Oryzias latipes

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ABSTRACT—Mammalian endothelins (ETs: ET-1, -2 and -3) effectively disperse the light-scattering organelles (leucosomes) in leucophores of the medaka, *Oryzias latipes*, in a dose-dependent manner. They were almost equally effective, their minimal effective concentrations being less than 100 pM, with EC_{50} value about 8.3–8.4 nM. Endothelins act directly on the leucophores, since denervated cells responded to the peptides quite similarly. Phentolamine, an α-adrenergic blocker, propranolol, a β-adrenergic blocker, and BQ-123, an inhibitor of mammalian ET_A receptor, did not interfere with the action of ETs. By contrast, BQ-788, an inhibitor of the mammalian ET_B receptor, potently blocked the action of ETs. Sarafotoxin S6c and IRL 1620, both mammalian ET_B receptor-selective agonists, were also found to disperse leucosomes effectively, mimicking the effect of ETs. Thus, ETs may act through the mediation of specific receptors existing in the leucophores. Along with their recently disclosed actions on light-absorbing chromatophores, ETs may play an important role in the delicate and exquisite control of integumentary hues and patterns.

INTRODUCTION

Endothelin (ET), an extremely potent vasoconstrictive polypeptide, was first found in medium in which porcine vascular endothelial cells had been cultured (Yanagisawa *et al.*, 1988). In mammals, ETs consist of a family of three isopeptides, designated ET-1, ET-2 and ET-3 (Inoue *et al.*, 1989), and they have pleiotropic effects on a variety of tissues (Sakurai *et al.*, 1992), in addition to their ability to constrict vessels (Masaki *et al.*, 1991). By contrast, few studies have reported the presence of ETs or their functional roles in poikilothermic vertebrates. Among them, Uemura *et al.* (1991) detected immunoreactive ET in the plasma of several lower vertebrates including two teleost species. The same group further showed the presence of the immunoreactive ET in the central nervous system of the medaka, *Oryzias latipes* (Kasuya *et al.*, 1991). Poder *et al.* (1991) demonstrated that ET-1 induced constriction of blood vessels of several 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 mammalian ET-1 induces contraction of cardiovascular tissues. It is therefore quite possible that ETs take part in the vascular physiology of lower vertebrates.

Working on the pigmentary effector system of fish, we recently demonstrated that cells other than those of the vascular tissues were also potently influenced by mammalian ET-1 (Fujii *et al.*, 1993). Melanophores in the skin, as a representative kind of chromatophore, play a primary role in the integumentary colorations and their changes. In almost all species of fish examined, ET-1 effectively aggregated the pigmentary organelles, the melanosomes. In addition, our more recent work showed that the other isopeptides of mammalian ET family, namely, ET-2 and ET-3, were also effective in aggregating pigment granules in teleostean melanophores (Hayashi *et al.*, 1996). Our preliminary observations, furthermore, revealed that other light-absorbing chromatophores in addition to melanophores, namely, xanthophores and erythrophores, were also responsive to ETs in a similar manner (Murata and Fujii, 1995).

In the skin of teleosts, light-scattering and/or reflecting chromatophores exist in addition to the light-absorbing chromatophores mentioned above, known as leucophores and iridophores, respectively (Fujii, 1993a, b). Having different optical properties, these chromatophores are generally regulated rather differently from the light-absorbing chromatophores (Fujii, 1993a; Fujii and Oshima, 1986, 1994). In this study, we now report the effects of mammalian endothelin isopeptides on the light-scattering chromatophores, the leucophores.

MATERIALS AND METHODS

Materials

Adult specimens of the medaka, *Oryzias latipes* (wild type and orange-red variety) were employed in this study. They were obtained from local dealers in Tokyo and in the Chiba Prefecture, and reared in freshwater aquariums in our facilities for at least a week for acclimatization. Usually, those reared under constant illumination on a

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white background for more than a week were used, because under such conditions, the number of leucophores, as well as the number of light-scattering leucosomes in each leucophore, were increased as a result of the so-called "morphological color change" (Sugimoto, 1993). Such circumstances allow us to measure the responses of the cells more easily and reliably. In addition, the leucophore responsiveness to various stimuli becomes more remarkable than for the cells from fish reared under normal conditions. In most experiments, fish of the orange-red variety were used, because they could be obtained more easily. There were no detectable differences in the physiological characteristics of leucophores obtained from these two groups of fish, namely, the wild type and the orange-red variety.

Scales on the dorso-lateral part of the trunk were isolated 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). In order to facilitate the penetration of stimulant molecules through the dermis in which the chromatophores were present, the epidermal layer overlying the dermis was removed in the following manner. The scales were stored at 4°C for 30 - 60 min in Ca^{2+}; Mg^{2+}-free saline, which had the following composition (in mM): NaCl, 130.7; KCl, 2.7; D-(+)-glucose, 5.6; ethylenediamine tetraacetic acid (trisodium salt; Dojindo Lab., Kumamoto, Japan), 0.5; Tris-HCl buffer, 5.0 (pH 7.3). The epidermis was then carefully removed with a finely pointed forceps under a binocular dissecting microscope.

Occasionally, the responses of melanophores and/or xanthophores on the same scale were also observed for the sake of comparison with those of the leucophores. In order to confirm that the leucophore to be examined retained normal motile responsiveness, a K^+-rich saline, in which the concentration of K^+ ions was raised to 50.0 mM, was usually applied to the scale prior to examination of the effects of ETs. In this solution, the concentration of Na^+ was compensatorily decreased to 78.0 mM in order to keep the osmolarity of the medium equal to normal saline. This type of chemical stimulation for testing cellular responsiveness was adopted since the induced dispersion of leucosomes was rapidly reversible, and the aftereffects of the stimulation were minimal (Miyoshi, 1952; Iga, 1978). Such a sudden increase in the concentration of K^+ ions is known to liberate neurotransmitter from presynaptic elements of nerves that control chromatophores, thus acting as a sympathetic stimulus (Fujii, 1959; Iga, 1978). Positive responsiveness to heightened K^+ concentration therefore indicates that the cell is normally innervated, in addition to the normal reponsiveness of the cell itself.

Method of denervation

In some experiments, the responsiveness of chemically sympathectomized leucophores was examined. Such leucophores were obtained by treating fish with 6-hydroxydopamine (6-OHDA; hydrobromide salt, Sigma Chemical, St. Louis, MO) (Iga and Takabatake, 1982). The fish were anesthetized by immersion in a 0.05% solution of 3-aminobenzoic acid ethyl ester (MS-222; methanesulfonate salt; Nacalai Tesque, Kyoto) and were intraperitoneally injected with 6-OHDA at a dose of 80 μg per gram body weight. The leucophores on scales excised from fish left overnight in a small aquarium were first tested to determine whether they had been denervated. If the leucophores were found to be refractory to an elevation in K^+ concentration, they were regarded as having been denervated. The K^+-rich saline described above was employed for this purpose.

Recording of responses of individual leucophores

Physiological and pharmacological methods employed were fundamentally the same as those described in previous reports (Fujii and Miyashita, 1975; Oshima anf Fujii, 1984). The motile responses of individual leucophore were photoelectrically recorded as recently described by us elsewhere (Fujii et al., 1997). In this method, the amount of light scattered from the peripheral dendritic area and that

from the cell body were transduced separately and concurrently into voltage changes. The changes due to the dispersion of leucosomes into the dendritic processes were then added electronically to the negative value of changes due to the evacuation of leucosomes from the cell body. The integrated voltage changes were recorded as a single trace on a chart recorder (EPR-231A, Toa Electronics, Tokyo).

At the end of each series of measurements, a sufficiently strong solution of norepinephrine hydrochloride (NE; racemic modification; Sankyo, Tokyo) in physiological saline was applied for a few min to bring about full dispersion of leucosomes for reference. Usually, a 2.5 μM solution of NE is strong enough for this purpose, the concentration being expressed in terms of the active L-(–)-isomer. In some experiments, melatonin (Sigma Chemical) was employed to aggregate leucosomes in leucophores (Obika, 1976). In all cases, the magnitude of the leucosome-dispersion response is expressed as a percentage of the maximal response observed during the course of measurements, with the fully aggregated state taken as zero.

Drugs used

The amino acid sequences of teleostean ETs have not yet been determined. Among the currently known three isopeptide groups of mammalian ETs, therefore, we selected one from each group. They were ET-1 (human, porcine, canine, rat, mouse, bovine), ET-2 (human, canine) and ET-3 (human, porcine, rat, rabbit), available as synthetic polypeptides (Sigma Chemical). These were selected since these molecular species were reported to produce significant contraction of vascular smooth muscles in many animal species (Sakurai et al., 1992), and to affect melanophores of various teleosts (Fujii et al., 1993; Hayashi et al., 1996).

Selective agonists of the ET_B receptor employed included sarafotoxin S6c (Williams et al., 1991; Sigma Chemical) and IRL 1620 (Takai et al., 1992; Peptide Institute, Osaka, Japan). Sarafotoxin S6c is a snake venom; its chemical structure and physiological action are similar to the ETs (Kloog and Sokolovsky, 1989). IRL 1620, a ligand for the ET_B receptor, is a synthetic product (Takai et al.,1992). BQ-123, an antagonist for mammalian ET receptors of the ET_A type (Na salt; Ihara et al., 1991) is a product of Research Biochemicals International (Natick, MA). As a selective antagonist for the ET_B receptor, we used BQ-788 (Na salt; Ishikawa et al., 1994), a gift from Banyu Pharmaceutical (Tokyo). Other drugs employed included a β-adrenolytic agent, propranolol hydrochloride (Sigma Chemical) and an α-adrenergic agent, phentolamine mesylate (Ciba-Geigy, Basel). Stock solutions of these drugs were diluted with the physiological saline immediately before use.

All physiological and pharmacological measurements were made at room temperature (20–25°C).

RESULTS

Leucosome-dispersing effects of ETs

When we equilibrated isolated scales in physiological saline, the light-scattering organelles (the leucosomes) in leucophores aggregated. Therefore, we first examined whether the three isopeptides of endothelin (ET) could elicit the dispersion of leucosomes within the cells.

A typical series of photomicrographs showing the responses of leucophores is shown in Fig. 1, where ET-3 was tested for its action. In this particular series, a scale from a wild-type individual was employed to examine the responses of melanophores as well as leucophores. Equilibration in physiological saline brought about the complete dispersion of pigmentary granules (melanosomes) in the melanophores (Fig. 1A, C). By contrast, leucosomes in the leucophores were completely aggregated in the perikarya (Fig. 1B, C). When K^+-rich
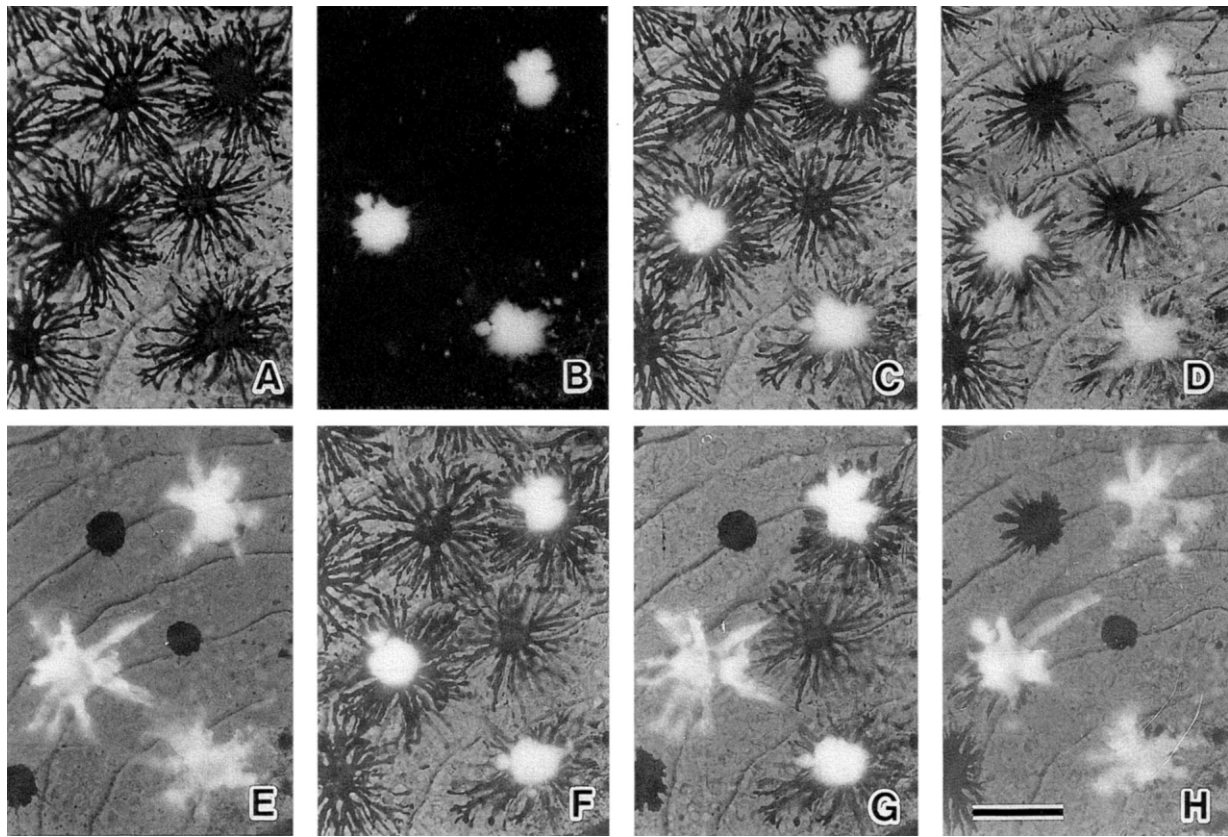
Fig. 1. Serial photomicrographs of the same field showing motile responses of leucophores and melanophores on an isolated scale of a wild-type medaka, *Oryzias latipes*, viewed from the dermal side. (**A-C**) Equilibrated in physiological saline. (**A**) Ordinary transmission optics; melanophores with fully dispersed melanosomes are visible. (**B**) Epi-illumination optics; The three bright light-scattering masses are the leucosome aggregates in the perikarya of leucophores. (**C-H**) Transmission and epi-illuminations are employed to observe melanophores and leucophores at the same time. (**D**) One min after application of K$^+$-rich saline (K$^+$: 50 mM); melanosomes in melanophores are becoming aggregated into the perikarya, while leucosomes in leucophores have just begun to disperse into the dendrites. (**E**) Two min after the application of K$^+$-rich saline; melanosomes are completely aggregated, and leucosomes are almost totally dispersed. (**F**) Equilibrated again in physiological saline for dispersal of melanosomes and aggregation of leucosomes. (**G**) Three min after the application of 100 nM ET-1; melanosomes in melanophores and leucosomes in the leucophore on the left are almost completely aggregated and dispersed, respectively, while the chromatophores on the right have just begun to respond. (**H**) Five min after the application of ET-1; reversal of the responses have already begun among the chromatophores on the left, while maximal responses have just been attained among the cells on the right. Scale bar: 100 μm.

saline was applied, melanosomes in the melanophores aggregated, and leucosomes in the leucophores dispersed (Fig. 1D, E). In order to reverse the distribution of pigment in the chromatophores, the scale was again equilibrated in the normal saline (Fig. 1F), after which 100 nM ET-3 was applied. A rapid aggregation of melanosomes in the melanophores and a slower but remarkable dispersion of leucosomes in the leucophores took place (Fig. 1G, H).

We also found that the other ET isopeptides, namely ET-1 and ET-2, effectively dispersed the leucosomes. A typical photoelectric recording of the responses to ET-1 (Fig. 2A) exhibits the responses of a leucophore from an orange-red individual. First, 50 mM K$^+$-saline was applied, and a rapid dispersion of leucosomes occurred, indicating that the cell under examination possessed normal motile activity. After re-equilibration in normal saline, 100 nM ET-1 was applied, which also dispersed the leucosomes quite effectively. If compared with the response to elevated K$^+$ concentration, however, the

reaction time was notably longer, and the rate of the response was rather slower. The level of leucosome dispersion was always significant, if ET at concentrations higher than 10 nM were applied.

It is noteworthy that, even during application of the ET solution, reaggregation of leucosomes took place, and this was not restricted to the action of ET-1. In almost all measurements of the leucosome-dispersing effect of ETs, we observed such a spontaneous reversal after peak response. Further, once a scale had been treated with ET-1, the leucophores became refractory to restimulation with the same isopeptide, as seen in Fig. 2. Such a phenomenon was observed even when ET isopeptides other than that applied originally were tested.

At the end of each measurement, a strong solution (2.5 μM) of norepinephrine (NE) was applied to induce a maximal degree of leucosome dispersion for reference. In most cases, the response levels to ETs were somewhat less than those
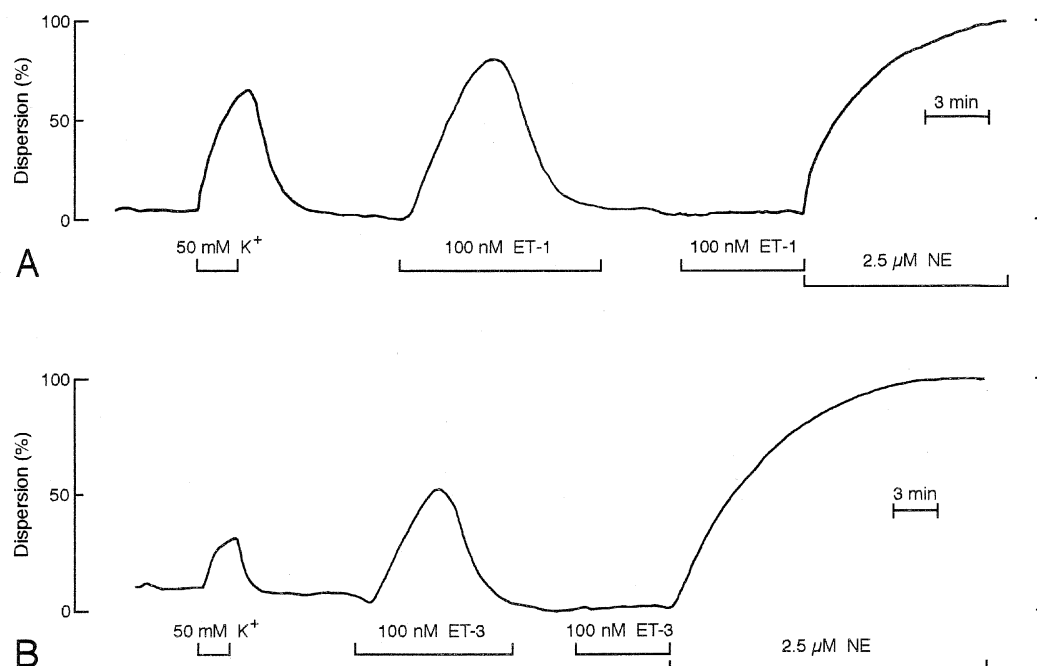
Fig. 2. Typical photoelectric recordings of response of individual leucophores of the medaka (orange-red variety) to two isopeptides of ET. In both recordings, K$^+$-rich saline (K$^+$: 50.0 mM) was first applied for 2 min to confirm the normal responsiveness of the cell, and a prompt dispersion of leucosomes took place. After equilibration of the scale again in normal saline, 100 nM ET-1 (**A**) or ET-3 (**B**) was applied. In both cases, a gradual but remarkable dispersion of leucosomes was elicited although the response was transient, since the leucosomes reaggregated even in the continued presence of ET. Finally, the maximal level of the response was attained by the addition of 2.5 μM norepinephrine (NE).

achieved by the strong solution of NE. Similar recordings of the responses have been performed when the effects of ET-2 or of ET-3 were tested (Fig. 2B illustrates a case in which 100 nM ET-3 was used). The refractory nature of the cell to the peptide after it has already been treated is also seen in this figure.

The dose-dependent nature of responses to the three ET isopeptides to the extent of the response was quantified, and the results are summarized in Fig. 3. In this series of measurements, the scales were first equilibrated in physiological saline. After confirming the complete aggregation of leucosomes in the perikaryon of the targeted leucophore, a physiological saline solution containing one of the ETs at concentrations from 10 pM to 1 μM was applied for 10 min. As before, in order to determine the maximal level of leucosome-dispersion, 2.5 μM NE solution was applied at the end of each series of measurements. It is clearly seen that all three peptides acted to disperse the leucosomes in a concentration-dependent manner, and that the response curves assumed a typical sigmoid shape. Discernible dispersion of leucosomes was recognizable at concentrations as low as 100 pM, and the maximal level was attained at a concentration of 1 μM. The EC$_{50}$, namely, the concentration of agonist necessary to elicit half the maximal response, was calculated to be 8.3, 8.4 or 8.4 nM, for ET-1, ET-2 or ET-3, respectively.

Leucosome-aggregating effects of ETs

We then examined whether the ETs possess leucosome-
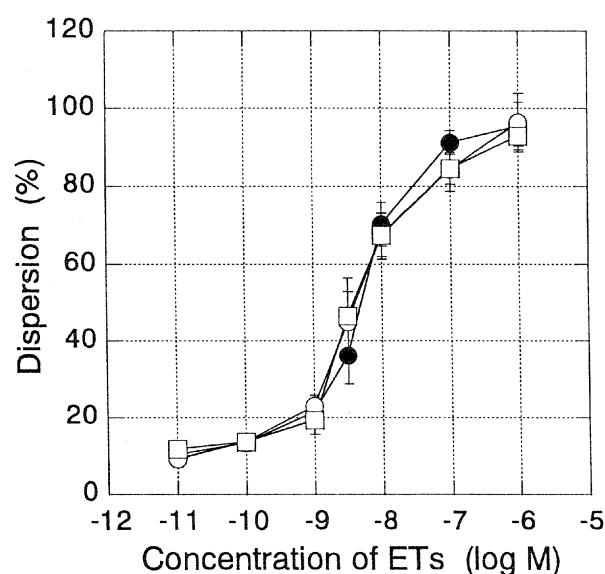
Fig. 3. Dose-response curves of ETs and leucosome dispersion in the medaka (orange-red variety). After equilibration of the scale in normal saline, a solution containing one of the ETs at concentrations ranging from 10 pM to 1 μM was applied for 10 min. At the end of each series of measurements, 2.5 μM norepinephrine solution was applied to obtain the maximal level of leucosome dispersion for reference. Curves with solid circles, open circles and open squares show the data for ET-1, ET-2 and ET-3, respectively. Each point is the mean of 7 measurements from different fish, and the vertical bars indicate SE.

aggregating action. In order to detect such an effect, the leucosomes had to be dispersed beforehand, and to this end, we employed a dilute solution of NE. Preliminary tests indicated that treatment of a scale with 250 nM NE for 10 min was sufficient to bring the leucosomes to an almost fully dispersed state. In the present study, 100 nM solutions of the ETs were tested. As a typical example of such a test, Fig. 4 exhibits a recording in which ET-3 was applied. Whether the cell was responsive either to NE or to melatonin was also tested, the latter being employed to show that the cell was responsive to leucosome-aggregating signals. After equilibration in normal saline, leucosomes were again dispersed by applying NE. After 10 min, 100 nM ET-3 was applied in the presence of NE, but leucosome aggregation persisted. Then, the NE was removed but ET-3, even in the absence of NE, failed to aggregate the leucosomes. Similar tests on the other ET species were performed, but in no case was aggregation of leucosomes due to the action of the peptides detected.

## Effects of ETs on denervated leucophores

The effects of ETs on the leucophores which had been chemically sympathectomized by 6-hydroxydopamine were examined. A typical series of photoelectric measurement is displayed in Fig. 5 in which ET-2 was employed. The lack of response of the leucophore to an increase in K^+ ions shows that the cell had successfully been denervated. When 10 nM ET-2 was applied, a marked dispersion of leucosomes comparable to that of normally innervated cells was elicited. ET-1 and ET-3 also effectively dispersed the leucosomes in denervated cells (data not shown).

Effects of adrenergic blocking agents

The neurally evoked dispersion of leucosomes is mediated by adrenoceptors of the β type (Obika, 1976; Iga et al., 1977), and thus, the effects of blocking these receptors on the leucophore were studied. As representative of β-adrenolytic agents, propranolol was tested for its possible inhibitory effects on the leucosome-dispersing action of ETs. Figure 6 shows that propranolol did not interfere with the action of ET-1, nor did it affect the leucosome-dispersing action of ET-2 or ET-3 (data not shown).

In addition to the β-adrenergic antagonist, an α-adrenergic antagonist was also tested for possible inhibitory effects on the dispersion of leucosomes elicited by ETs. Phentol-
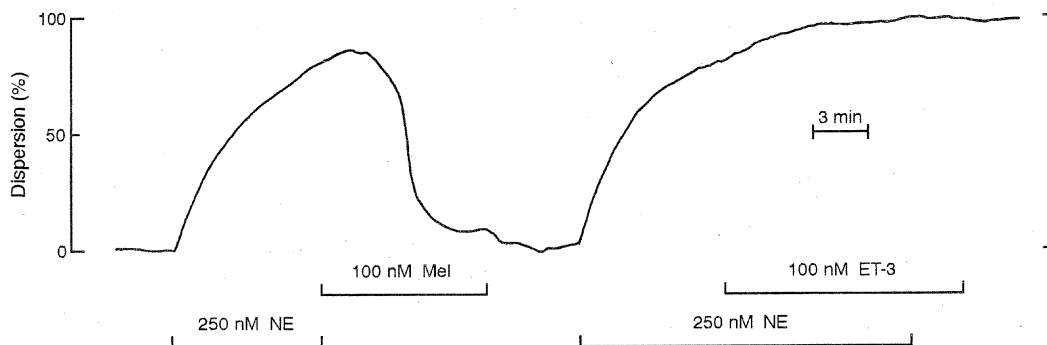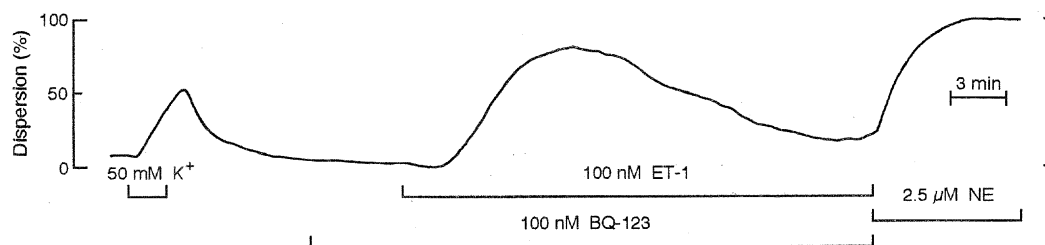

Fig. 4. Typical photoelectric recording of a single leucophore of the medaka (orange-red variety), for detecting any leucosome-aggregating effect of ET-3. A weak norepinephrine solution (NE, 250 nM) was first applied to the scale to disperse the leucosomes in the cell, and gradual dispersion of leucosomes took place. In order to reverse the effect of NE quickly, 100 nM melatonin was then applied, and after confirming the reaggregation of leucosomes, 250 nM NE was added again. Ten min later, saline containing 100 nM ET-3 and 250 nM NE was applied. However, no aggregation of leucosomes could be detected, and the dispersion persisted for some time, even when NE was withdrawn.
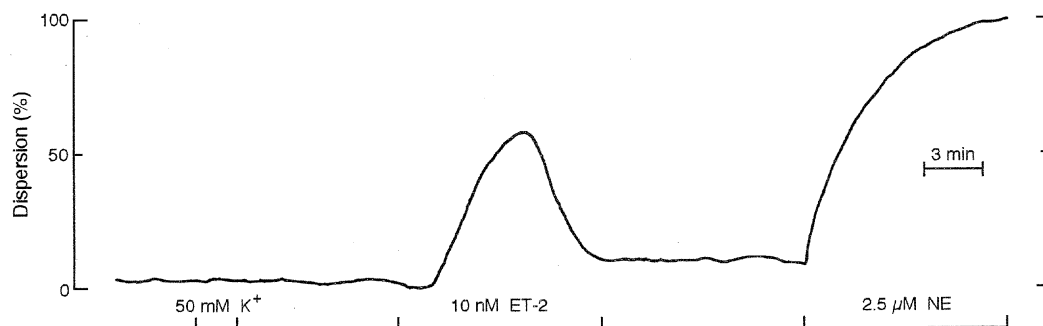
Fig. 5. Typical photoelectric recording of the response of a denervated leucophore of the medaka (orange-red variety), showing the effect of ET-2. First, the scale was perfused with 50 mM K^+ saline; the fact that the cell was refractory to the increase in K^+ concentration indicates that the cell had been denervated. In response to 10 nM ET-2, by contrast, a remarkable dispersion of leucosomes, comparable to that observable among normally innervated cells, took place. At the end of measurement, 2.5 μM norepinephrine (NE) solution was applied to bring about the full level of the response for reference.
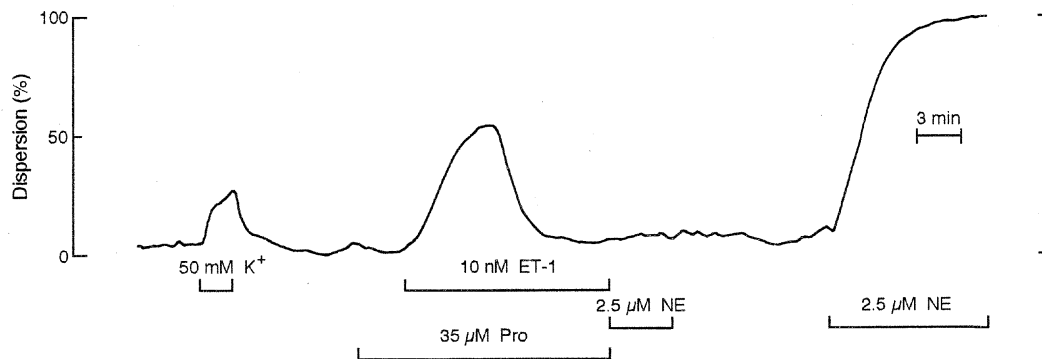
Fig. 6. Typical photoelectric recording of a single leucophore of the medaka (orange-red variety), showing the effect of the β-adrenergic blocking agent propranolol on the leucosome-dispersing action of ET-1. In order to confirm that the cell retained normal responsiveness, 50 mM K+ saline was first applied for 2 min. Following a 3 min treatment with 35 μM propranolol (Pro), 10 nM ET-1 in the presence of the blocker was applied. Propranolol did not interfere with the action of ET-1. NE: norepinephrine.

Fig. 7. Typical photoelectric recording of a single leucophore of the medaka (orange-red variety), showing the effect of BQ-123, a blocker for mammalian ET_A receptor, on the leucosome-dispersing action of ET-1. To confirm the normal responsiveness of the cell, 50 mM K+ saline was first applied for 2 min. The scale was then treated with 100 nM BQ-123 which was followed by perfusion with 100 nM ET-1 in the presence of BQ-123. ET-1 elicited the dispersion of leucosomes normally even in the presence of the blocker. NE: norepinephrine.
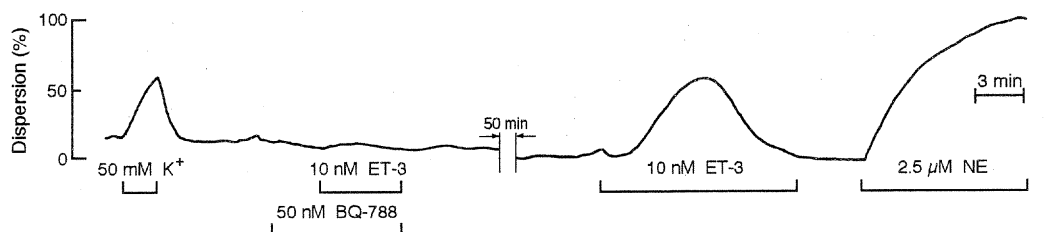

Fig. 8. Typical photoelectric recording of the response of a single leucophore of the medaka (orange-red variety), showing the effect of BQ-788, a blocker for the mammalian ET_B receptor, on the leucosome-dispersing action of ET-3. Treatment of a scale with 50 nM BQ-788 for 3 min was followed by perfusion with saline which contained 10 nM ET-3 in the presence of the blocker. No dispersion of leucosomes was observed. After a thorough washing of the scale with physiological saline, ET-3 induced leucosome dispersion normally. NE: norepinephrine.

amine, an effective blocker of sympathetic nervous signals to chromatophores was employed for this purpose (Fujii and Miyashita, 1975; Fujii and Oshima, 1994). As expected, phentolamine did not show any influence on the action of the three ET isopeptides (data not shown).

Effects of ET_A receptor blockers

BQ-123, a recently developed blocker of mammalian ET_A receptors, was also examined for its possible effects. After a 5 min application of 100 nM BQ-123, the perfusing medium was changed to saline which contained 100 nM ETs in addition to 100 nM BQ-123 (Fig. 7). Even at such a high concen-

tration, BQ-123 did not inhibit the leucosome-dispersing action of ET isopeptides (ET-1 is shown in Fig. 7, but comparable results were found for ET-2 and ET-3).

Effects of ET_B receptor blockers

The effects on the action of ETs of a recently developed blocker of mammalian ET_B receptors, BQ-788, was examined next. After treating a scale with 50 nM BQ-788 for 5 min, the perfusing solution was changed to saline which contained 10 nM ETs as well as 50 nM BQ-788 (Fig. 8). No dispersion of leucosomes was observed. However, after washing the scale with physiological saline, ETs were able to induce dispersion
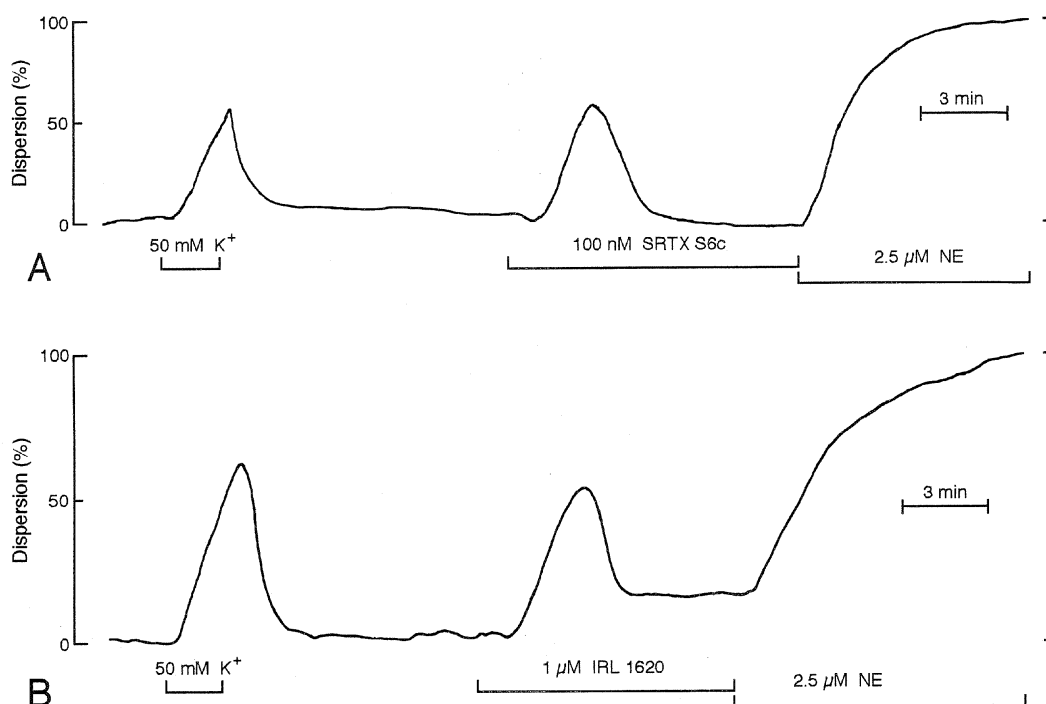
Fig. 9. Typical photoelectric recordings of single leucophores of the medaka (orange-red variety), showing the effect of two selective agonists for the mammalian ET_B receptor, namely, sarafotoxin S6c (SRTX S6c; **A**) and IRL 1620 (**B**). Both agonists induced the dispersion of leucosomes within the cells, and it should be noted that, even in the continued presence of the agonists, the leucosomes reaggregated comparable to the effects of the ETs. NE: norepinephrine.

quite normally. (ET-3 is shown in Fig. 8 but similar results were found for ET-1 and ET-2).

Effect of selective agonists for the ET_B receptor

Finally, we examined the effect of sarafotoxin S6c (SRTX S6c) and IRL 1620, both being selective agonists for mammalian ET_B receptors (Fig. 9). Either agonist effectively induced the dispersion of leucosomes within the leucophores.

DISCUSSION

The effects of ETs on the pigmentary system were first described by Yada et al. (1991), who reported the influences of these peptides on the proliferation and melanization of human melanocytes. As for their action on the motile responsiveness of pigment cells, our group has recently described that ETs efficiently aggregate pigment in melanophores of several teleostean species (Fujii et al., 1993; Hayashi et al., 1996). Our preliminary observations further showed that light-absorbing chromatophores other than melanophores, namely, erythrophores and xanthophores, also respond to ETs by pigment aggregation (Murata and Fujii, 1995).

A fairly large amount of information is now available about regulatory mechanisms for the motile activities of leucophores (Fujii, 1993a; Fujii and Oshima, 1986, 1994). Leucophores generally show motile responses opposite those of light-absorbing chromatophores in terms of the direction of pigmentary organelle displacement. For example, light-absorbing

chromatophores respond to sympathetic nervous stimulation, catecholamines and melanin-concentrating hormone (MCH) by aggregating chromatosomes. Leucophores, by contrast, respond to the same stimuli by dispersing organelles. When we think of the reciprocal optical properties of light-absorbing and light-scattering cells, such a difference may rather naturally be understood. In fact, organelles in melanophores and xanthophores aggregate, and those in the leucophores disperse, when medaka specimens are adapted to a white background, thus resulting in an effective blanching of the skin. The reverse movements occur when they are placed on a dark background, leading to effective darkening of the skin (Sugimoto, 1993). Such cooperative functions among different types of chromatophores may certainly favor effective dark-to-pale, or reverse, changes in the integumental coloration (Fujii, 1993a, b).

Motile responses to some signals, however, result in similar directions of pigmentary movements in light-absorbing chromatophores and leucophores. For example, melatonin aggregates the cellular inclusions in both kinds of chromatophores, while α-melanophore-stimulating hormone (α-MSH) and adenylyl compounds disperse them (Fujii, 1993a; Fujii and Oshima, 1986, 1994). Such complexities in the chromatic system probably are indispensable features for producing various hues and patterns in animals that are necessary for adaptation to various environmental and ethological conditions (Fujii, 1993b). It was therefore a great challenge to characterize the effects of ETs on leucophores.

In contrast to their action on melanophores and other light-absorbing chromatophores, ETs dispersed pigment in leucophores, a result in accordance with the concept that light-absorbing and light-scattering chromatophores normally act synergistically in lightening or darkening the skin, as discussed above. It was found, moreover, that ETs induced dispersion of leucosomes at very low concentrations, which may indicate that the action of ETs is not merely a pharmacological one, but is relevant to the physiological control of cellular responses. Therefore, at least some molecular form(s) of ET family may be functioning to augment the whiteness of the skin, modifying the effects of sympathetic postganglionic nerves and of hormonal factors.

It has also become clear that ETs act directly on leucophores, but not via the stimulation of presynaptic nervous elements to liberate neurotransmitter, since denervated leucophores responded to them similarly to normally innervated cells. We have also found that sympatholytic agents of both α and β types, and a specific blocker of the mammalian ET_A receptor, did not interfere with the action of ETs on leucophores. By contrast, ET_B receptor-selective agonists effectively dispersed leucosomes comparably to the ETs, whereas an inhibitor of the mammalian ET_B receptor, potently blocked the action of ETs. These results strongly suggest that the action of ETs on leucophores is not through stimulation of receptors mediating first messenger signals, but rather is via stimulation of a receptor resembling the mammalian ET_B receptor.

Previous reports about the role of ETs have generally been concerned with their action on vertebrate musculatures that are exclusively of mesodermal origin. Categorized as "paraneurons", chromatophores of vertebrates are known to be of ectodermal origin (Bagnara and Hadley, 1973; Fujii, 1993a). Thus, we have added another example of a cell type that originates in germ layers other than the mesoderm, in addition to the melanophore (Fujii et al., 1993; Hayashi et al., 1996), the xanthophores and the erythrophores (Murata and Fujii, 1995). We hope that our studies on teleostean chromatophores will stimulate investigations on the roles of ETs in a variety of other animal tissues.

As to whether ETs act on chromatophores as neurological agents or as hormonal principles, we have discussed this in an earlier article dealing with the action of ET-1 on melanophores (Fujii et al., 1993). A similar discussion may also be applicable to the case of leucophores. In brief, we presume at this stage that ETs influence leucophores as hormones, the primary reason for this being that they were effective at very low concentrations. Results by Uemura et al. (1991) who detected ET-like immunoreactivity in the blood of teleostean species seems to be in accordance with this concept. In poikilothermal vertebrates, however, tissues or organs that secrete peptides of the ET family have not yet been determined. Based on their observations that higher immunoreactivity of ET-like substances was detectable in the neurohypophysis and urophysis of the medaka, Oryzias latipes, Kasuya et al. (1991) suggested that these organs might be the possible sites of secretion. On the other hand, we have suggested the possibility that dermal chromaffin cells might also be a source of secretion (Fujii et al., 1993). Incidentally, the possible role of dermal chromaffin cells in the control of chromatophores has been put forward by Miyashita and Fujii (1975), who assumed those cells to be the source of epinephrine which stimulates β-adrenoceptors of melanophores and induces the dispersion of melanosomes. At the present time, however, it is rather natural to assume the origin of the ETs to be from the endothelial cells, because, at least in mammals, they have generally been thought to originate from these cells.

Endothelial cells of capillaries or of small vessels and possibly the dermal chromaffin cells, are widely distributed in the dermis as are the chromatophores. If endothelial cells were the source of the ETs, one might assume that they would liberate peptides not from their luminal side but from the outer surface into the dermal connective tissue. In the case of dermal chromaffin cells, we can suppose more easily the sequence of hormonal action on the chromatophores since both types of cells may be closely located. In either case, their influence may be restricted within the neighborhood of secretion, and thus, the ETs may properly be categorized as local hormones.

If we restrict our discussion to pigmentary phenomena of the skin, we can now assume that ETs are involved in localized changes of color; i.e. the modification of color patterns. We now know that ETs aggregate chromatosomes in light-absorbing chromatophores, whereas they disperse organelles in leucophores. When they are secreted within a region of the skin, therefore, an effective blanching would take place there. If they are secreted within paler areas, we might expect an increased contrast of the pattern in that region of the skin. By contrast, if they affect chromatophores within darker parts, the pigmentation patterns may become less conspicuous. In this way, ETs might take part in the subtle and delicate control of integumentary hues, especially in the formation or the disappearance of pigmentary patterns.

We still have no information about the amino acid sequence of piscine ETs, and thus, we have had to employ commercially available mammalian ETs. As described in this paper, all these ETs were very effective in inducing dispersion of leucosomes in medaka leucophores. They were also very potent in eliciting aggregation of pigment in teleostean melanophores (Fujii et al., 1993; Hayashi et al., 1996), suggesting that ETs that actually regulate chromatophores have molecular structures not so different from those of mammals. The presumed conservative nature of peptides during phylogeny of vertebrates may inversely suggest that they should be of the crucial importance throughout the vertebrate classes. Characterization of the sequence of peptides belonging to the ET family in lower vertebrates are thus eagerly awaited.

Both α-melanophore-stimulating hormone (α-MSH) (Negishi and Obika, 1980; Oshima and Fujii, 1985) and melanin-concentrating hormone (MCH) (Oshima et al., 1986) are known to induce the dispersion of leucosomes in leucophores. We can now exclude the possibility that the action of ETs is

via secretion of these principles, since they are known to be secreted from the pituitary, and these experiments have been performed exclusively on isolated medaka scales.

The sympathetic nervous system is responsible for the dispersion of light-scattering organelles in leucophores (Fujii and Miyashita, 1979; Iwata et al., 1981; Iga, 1983). The neurotransmitter from the postganglionic fibers is catecholaminergic, and naturally, catecholamines give rise to the dispersion of leucosomes. The response was shown to be mediated by β-adrenoceptors, since propranolol, a β-adrenolytic agent, effectively blocked the response (Obika, 1976; Iga et al., 1977). In this study, however, propranolol did not interfere with the action of ETs, and thus, the action of ETs must not be via liberation of catecholamines either from the sympathetic postganglionics or from other secretory glandular cells, such as adrenal chromaffin cells.

BQ-123, a selective antagonist developed for blocking ET_A receptors in mammals, was ineffective in inhibiting the action of ETs on leucophores. Such an outcome was not surprising to us, since the drug had already been shown to have no effect on melanophores (Fujii et al., 1993; Hayashi et al., 1996). These results may possibly be due to the fact that the ET receptors of fish take a rather different conformation from mammalian ET_A receptors. Incidentally, Karne et al. (1993) recently cloned the ET receptors of the clawed toad, Xenopus laevis, and reported that the homology in their amino acid sequence to mammalian ET receptors was about 70%. We naturally suppose the homology between mammalian and piscine ET receptors to be considerably lower than that. Future studies will be necessary to determine the molecular structures of ETs and their receptors in fish in order to understand their interactions in greater detail.

It should be noted here that, as in the case of melanophores, the effect of ETs gradually diminished, although the direction of organelle translocation in leucophores was reverse that in melanophores. This phenomenon might be ascribed to the desensitization of receptors involved or to the so-called "down regulation". Further, once a scale had been treated with an ET, the leucophores then became refractory to the same or to other ET isopeptides. Analogous phenomena have also been described recently by Hayashi et al. (1996) on the pigment-aggregating response of teleostean melanophores to ETs, and although they have briefly discussed their issues, further studies will be needed to understand these processes.

As for the process of signal transduction following ET treatment of chromatophores, we can now profitably employ information obtained from vascular smooth muscle cells of mammals. Various changes associated with the action of ETs on smooth muscle cells include the production of inositol 1,4,5-trisphosphate (IP_3) by activated phospholipase C, elevation of the intracellular concentration of Ca^{2+} ions, activation of protein kinase C, and so on (Little et al., 1992; Sakurai et al., 1992). In chromatophores of several teleost species, the aggregation of pigment has been shown to be associated with an increase in the intracellular concentration of Ca^{2+} (Luby-Phelps and Porter, 1982; Negishi and Obika, 1985; Oshima

et al., 1988; Fujii, 1993a). In addition, we recently reported that, in tilapia melanophores, the aggregation of pigment mediated by α-adrenoceptors (α_1 type) is preceded by the production of IP_3 as the second messenger (Fujii et al., 1991). ET-1 (Fujii et al., 1993) and ET-2 and ET-3 (Hayashi et al., 1996) effectively aggregated pigment in melanophores and other light-absorbing chromatophores (Murata and Fujii, 1995). Therefore, it seems quite possible that a mechanism similar to that involved in the action of ETs on mammalian smooth muscles is operating in motile responses of light-absorbing fish chromatophores. Along with increases in the levels of IP_3 and Ca^{2+} incidentally, the decrease in the intracellular level of cyclic AMP has long been known to be involved with pigment aggregation in many dendritic chromatophores (Fujii, 1993a). Both adenylyl cyclase and phosphatidyl inositol (PI) systems may be operating in concert during signal transduction of these cells to stimulate their motile response (Fujii et al., 1991; cf. Fujii, 1993a).

In contrast to the light-absorbing chromatophores, ETs disperse pigmentary inclusions. As in melanophores and other light-absorbing chromatophores, the dispersion of the organelles in response to catecholamines or to sympathetic nervous stimulation is mediated by β-adrenoceptors (Obika, 1976; Iga et al., 1977; Fujii, 1993a). Naturally, increases in cyclic AMP levels have been regarded as the main secondary messenger involved in the dispersion of the organelles (Yamada and Iwakiri, 1982; Obika, 1988; Fujii 1993a). Although the dynamics of Ca^{2+} and IP_3 levels in leucophores have not yet been clarified, the available evidence now strongly favors the view that decreases in their concentration in the cytosol are also associated with the aggregation of leucosomes.

ETs dispersed the leucosomes, the direction of the response being opposite that observed in melanophores (Fujii et al., 1993; Hayashi et al., 1996). In both leucophores and melanophores, however, α-adrenoceptors mediate pigment aggregation, while β-adrenoceptors mediate pigment dispersion. Receptors for MSH, adenosine and related substances mediate the dispersion of pigment in both kinds of chromatophores (cf. Fujii, 1993a; Fujii and Oshima, 1986, 1994). Thus, signal transduction systems existing in chromatophores may be rather common, suggesting that in leucophores the action of ETs may be mediated by receptors whose characteristics are quite different from those possessed by melanophores. Probably, receptors of the leucophores function to decrease intracellular levels of IP_3 and of Ca^{2+}. Yamada and Iwakiri (1982) showed that an increase in the cytosolic level of another second messenger, namely cyclic AMP, is correlated with the dispersion of leucosomes. Thus, the effect of ETs might also be transduced by an increase in the level of nucleotide.

Such reciprocal transduction of signals by first messengers may not be so strange amomg visceral effector cells. Adrenoceptors, for example, are commonly categorized into α and β subtypes. It is widely known that reverse effects are brought about when α or β receptors are differentially stimu-

lated is widely known. The circumstances may probably be similar to the adrenergic control of chromatophores in poikilothermic vertebrates (Bagnara and Hadley, 1973; Fujii 1993a; Fujii and Oshima, 1986, 1994). Furthermore, Nishi and Fujii (1992) recently showed that, in addition to known melatonin receptors responsible for the aggregation of pigment, novel receptors exist that mediate the dispersal of melanosomes in some melanophores of the common pencil fish, *Nannostomus*; they proposed naming these melatonin receptors to be of the α and β subtypes, respectively. In accordance with this, we would like to tentatively designate the receptors described in this study as "β-ET receptor". Accordingly, receptors mediating the aggregation of chromatosomes may be named "α-ET receptor".

Working on the melanophores of an amphibian species, the African clawed toad *Xenopus laevis*, Lerner and associates (Karne *et al.*, 1993) recently reported that ET-3 dispersed melanosomes mediated by ET$_C$ receptors. They further showed that ET-1 and ET-2 were also melanosome-dispersing, although their potencies were lower than that of ET-3. The direction of the pigmentary movements in response to ETs was identical to that examined in medaka leucophores in this study, but opposite to that observed in melanophores examined in many other fishes (Fujii *et al.*, 1993; Hayashi *et al.*, 1996). Lerner's group (Graminski *et al.*, 1993), also reported that inositol 1,4,5-trisphosphate (IP$_3$) is involved in pigment dispersion in *Xenopus* melanophores, which in terms of the direction of melanosome displacement, was quite opposite to that reported by us in fish (Fujii *et al.*, 1991). For a better comprehension of such confusing and complicated processes which exist among chromatophores of poikilothermal vertebrates, further comparative studies of the mechanisms involved in signaling either outside or inside the cells are definitely needed, in addition to determining the physiological and/or the ethological significance of the ET system in these lovely animals.

ACKNOWLEDGMENTS

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